Impact of Sodium Reduction on the Microbiota of Ready-to-Eat Meats

by

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Abstract

The growth of *Listeria monocytogenes* and spoilage microorganisms is a concern in sodium-reduced ready-to-eat (RTE) meats. To assess the microbial risk of sodium-reduced RTE meats, the microbiota of retail RTE meats was profiled and the growth of *L. monocytogenes* and an autochthonous microbiota was examined under experimental conditions relevant to sodium reduction.

To determine the effect of sodium reduction on the growth of *L. monocytogenes*, ham was manufactured with different concentrations of sodium chloride. Results confirmed that *L. monocytogenes* had shorter lag phase on ham formulated with less NaCl when no autochthonous microbiota was present. The autochthonous microbiota effectively controlled the growth of *L. monocytogenes* through competition regardless of the concentration of sodium chloride.

The antilisterial effect of carnocyclin A (CCLA) was examined. Global gene expression, protein production and morphological changes were profiled in *L. monocytogenes* exposed to a sublethal dose of CCLA in a low sodium environment. CCLA exposure resulted in down-regulation of genes involved in cell division, cell wall synthesis and motility in *L. monocytogenes* after 4 h and 30 h of exposure. Absence of flagella from the cell surface was observed.

The filamentation of *L. monocytogenes* was examined following exposure of cells to stress including acid, salt and antimicrobial peptides. *Listeria* formed filaments in low concentrations of acids and salts but not with purified CCLA. *L.*

monocytogenes was able to form filaments both *in vitro* and *in situ*. Down-regulation of cell division gene *ftsX* could be involved in filamentation.

The effect of a mixture of sodium lactate and sodium diacetate (NaL/DA) on gene expression in *L. monocytogenes* was examined. Global gene expression was examined after *Listeria* cells were exposed to NaL/DA in broth. The genes encoding glycolytic enzymes were down-regulated. The DNA repair gene, cell division gene and cell structure synthesis genes were up-regulated. The addition of NaL/DA inhibited the growth of *Listeria* on low sodium ham products.

In conclusion, this work demonstrated that the use of preservatives (CCLA and NaL/DA) and the presence of an autochthonous microbiota can effectively inhibit the growth and down-regulate the expression of genes essential for cellular functions in *L. monocytogenes*.

Preface

This thesis is an original work by Xiaoji Liu; with the culture-dependent analysis conducted by Dr. Petr Miller from Department of Agricultural, Food and Nutritional Sciences and characterization of the molecular weight of exopolysaccharide by size-exclusion chromatography by Dr. Januana Teixeira from the same department. Chapters that have been submitted for publications prior to the release of this thesis are indicated.

Dedication

To my beloved parents:

Mr. Xinming Liu and Ms. Li Luo

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List of Abbreviations

APT	All-Purpose-Tween
a_w	Water activity
GABA	Gamma-aminobutyric acid
CCLA	Carnocyclin A
CDS	coding sequence
DE	Differential expression
DGGE	Denaturing Gradient Gel Electrophoresis
EPS	Exopolysaccharides
FDR	False discovery rate
FSC	Forward scatter
GAD	Glutamate decarboxylase
HHP	High hydrostatic pressure
LAB	Lactic acid bacteria
MRS	de Man, Rogosa and Sharpe
NaL/DA	Sodium lactate and sodium diacetate
PCA	Plate Count Agar
PTS	Phosphotransferase
RAPD	Random Amplified Polymorphic DNA
RIN	RNA integrity number
RPKM	Reads per kilobase per million
RTE	Ready-to-eat
STAA	Streptomycin Thallous Acetate Actidione agar
TEM	Transmission electron microscope
TSB	Tryptic Soy Broth
TVC	Total viable counts
UPLC-ESI-Q-TOF-MS	Ultra-performance liquid chromatography - quadrupole time-of-flight mass spectrometer
UTRs	Untranslated regions
VRBG	Violet Red Bile Agar with Glucose

1. General Introduction and Literature Review

Sodium is necessary for the electrolyte balance and biological functions in humans. However, excess consumption of sodium can lead to health problems, such as cardiovascular disease and high blood pressure (Kawasaki et al., 1978). As a result, consumers are demanding products that contain less sodium. Sodium-reduced ready-to-eat (RTE) meats contain less sodium, but other ingredients, such as acids or lactates/ diacetates, as well as antimicrobial peptides, can be present to control the growth of spoilage and pathogenic bacteria. It is important to understand the impact on these antimicrobials on the physiology of bacteria, and to determine if these ingredients can effectively reduce the growth of bacteria in a sodium-reduced environment.

Sodium reduction can lead to a series of food safety and quality issues in RTE meats. The most prominent issue is an increased growth of spoilage microorganisms, as well as *L. monocytogenes*. *L. monocytogenes* is the causative agent of listeriosis. Current interventions to control the growth of *L. monocytogenes* in food processing environments include the addition of preservatives, such as bacteriocins. Bacteriocins are effective in controlling the growth of *Listeria* in RTE meats; however, there is potential for an increase in bacteriocin resistance in a sodium-reduced environment. Thus, it is important to understand the molecular response of *L. monocytogenes* to bacteriocin challenge. In addition, sodium reduction could also lead to a change in behaviour (cell

morphology and enzymatic activity) of *L. monocytogenes*, as well as the autochthonous microbiota. Therefore investigation of the impact of the sodium concentration on the biological activities of spoilage microorganisms and *L. monocytogenes* is important.

The purpose of this research was to examine the effect of sodium reduction on the microorganisms in RTE meats. Specifically, how the reduction of Na/NaCl concentration, the presence of autochthonous microbiota and the addition of preservatives would influence the physiology (growth, morphology, gene expression) of *L. monocytogenes*.

1.1. Sodium reduction strategy

The sodium intake reduction strategy was initiated by Health Canada in 2010. According to Health Canada's sodium reduction strategy, the sodium content of food products should be gradually reduced to the targeted levels to achieve a mean sodium intake of 2,300 mg per person per day by the year 2016. The strategy also requested the food industry to voluntarily reduce the amount of added sodium salts in a wide range of food products, including processed meat and poultry.

To meet the standards for each category, the levels of sodium in meat products are accurately defined. Depending on the concentration of sodium, sodium-reduced products are classified into 6 categories: free of sodium, low in sodium or

salt, reduced in sodium, lower in sodium, no added sodium and lightly salted (Salt reduction guide for the food industry, 2009).

For RTE meat products, specifically, the sodium level target for fully cooked RTE meats is 800 mg per 100 g of product (sales weighted average; Health Canada, CA). Sodium-reduced products have 25-50% less sodium than a regular sodium RTE meat.

1.2. Role of sodium in RTE meat

The main contributions of sodium to RTE meats are preservation and flavor enhancement (Dotsch et al., 2009). Sodium is present in meat in different forms of salts, and the most abundant form of sodium present in meat is sodium chloride. Sodium chloride preserves meat by inhibiting the growth of foodborne pathogens and spoilage microorganisms (Chen and Shelef, 1992) by reducing the water activity (a_w) of the meats (Troller and Christian, 1978). Water activity is defined as the ratio of the vapour pressure of the food (or solution) to that of pure water at the same temperature, i.e., the available moisture for the growth of microorganisms. The a_w correlates with the growth of microorganisms (Brown, 1982; Wijtzes et al., 1992). The minimal a_w required for the growth of *L. monocytogenes* is 0.92 (Fontana, 2008; Nolan et al., 1992).

1.3. Meat spoilage microorganisms

As a result of sodium reduction, the growth of spoilage microorganisms on RTE meats could increase. The spoilage microorganisms on meats include

Lactobacillus spp. (Korkeala and Björkroth, 1997) and *Leuconostoc* spp. (Johansson et al., 2011). On vacuum-packaged RTE meats, the bacteria frequently isolated are lactic acid bacteria such as *Lactobacillus sake* and *Lactobacillus curvatus* (Korkeala and Björkroth, 1997) and *Brochothrix* spp. (Jay, 2010). These microorganisms can spoil meat products by metabolic activity that degrades myoglobin, which changes the colour of meat products (Cai et al., 1998), and through the production of off-odour metabolites, such as acetoin, acetate, formate (Casaburi et al., 2011) and 2, 3-butanediol/diacetyl (Blickstad and Molin, 1984). In addition, bacteria such as *Leuconostoc* spp. can spoil RTE meats by producing exopolysaccharides (EPS) (Lyhs et al., 2004; Johansson et al., 2011). EPS are polymers consisting of monosaccharide units such as glucose, fructose, galactose or rhamnose (Degeest and de Vuyst, 1999).

1.4. *Listeria monocytogenes*

In addition to spoilage, sodium reduction in RTE meats could also lead to changes in the growth of *L. monocytogenes* (Bohaychuk et al., 2006; Dave and Ghaly, 2011). *L. monocytogenes* is a foodborne pathogen that can contaminate RTE meats and is a concern for the food industry and the human health because it can survive food processing hygiene management, such as high pressure processing, and can be found in food products in the retail market (Bohaychuk et al., 2006; Uyttendaele et al., 1999). *L. monocytogenes* is especially dangerous for pregnant women because it can pass through the blood-placenta barrier and affect the fetus, which leads to still birth or spontaneous abortion (Beck et al., 1966). *L.*

monocytogenes has been responsible for numerous foodborne illness outbreaks (Hof, 2003). In 2008, one *Listeria* outbreak from a single manufacturer in Canada resulted in 22 deaths and 57 confirmed positive cases (Gilmour et al., 2010). In Canada, there are between 100 and 140 cases of listeriosis reported each year (Public Health Agency of Canada, 2010).

To control the growth of spoilage and pathogenic bacteria in sodium-reduced RTE meats, preservatives such as organic acids and salts can be added. Acids (acetic acid, lactic acid) and non-NaCl salts (sodium acetate, sodium diacetate, and sodium lactate) are approved for use (Health Canada, 2013). Acetic acid has long been used to control the growth of food spoilage microorganisms (Levine and Fellers, 1940). Lactic acid can effectively reduce the growth of *L. monocytogenes* on cooked ham (Hwang et al., 2012). Sodium or potassium salts of lactate and diacetate not only reduce the growth of *L. monocytogenes*, but also the spoilage microorganisms (Mellefont and Ross, 2007; Sallam et al., 2007).

In addition to acids and salts, antimicrobial peptides (bacteriocins) can also be used to improve both the safety and the quality of RTE meats (Castellano and Vignolo, 2006; Galvez et al., 2008). Bacteriocins are secreted by certain bacteria that are naturally occurring in meat and the bacteriocins allow them to compete with spoilage or pathogenic microorganisms. Bacteriocin-producing lactic acid bacteria outcompete the pathogenic microorganisms without producing spoilage metabolites and they are not hazardous to humans. Some of these strains and/or their bacteriocins have been commercialized for application to RTE meat products

to improve the microbial safety. The products include Nisaplin® (DuPont™ Danisco®, contains nisin), ALTA™ 2431-Quest (Cotter et al., 2005; contains pediocin PA-1/AcH) and Micocin® (Griffith Laboratories Inc.). Nisin (Moll et al., 1996) and pediocin PA-1 (Chikindas et al., 1993) target the cell membrane via formation of pores on the cytoplasmic membrane. Micocin® is a preparation of bacteriocins containing carnobacteriocin BM1, piscicolin 126 and carnocyclin A. Carnocyclin A (CCLA) forms ion channels in the membrane and results in dissipation of membrane potential, which leads to cell death (Gong et al., 2009).

1.4.1. Acid stress and tolerance

Acids cause membrane dysfunction, denaturation of proteins and inhibit many biochemical reactions inside the cell that require a neutral pH. Acetic acid and lactic acid, which are both used as preservatives, inhibit the growth of *L. monocytogenes* Scott A (Young and Foegeding, 1993). Both low pH (H^+) and the undissociated acids stress bacterial cells (Janssen et al., 2007).

L. monocytogenes can gradually adapt to acid stress and develop resistance through altered gene regulation and protein expression. *L. monocytogenes* responds to acid stress by maintaining intracellular pH homeostasis via the activity of H^+-ATPase. The activity of H^+-ATPase depends on the nutrients in the environment, particularly glucose. In the presence of glucose, the intracellular pH remains constant when external pH drops, while corresponding net H^+ flux across the bacterial cell membrane is increased (Shabala et al., 2002). The glucose-

dependent acid resistance requires the activity of alternative sigma factor RpoS (Lin et al., 1996).

In addition to H^+-ATPase activity, the glutamate decarboxylase (GAD) system is another principal system that is used by *L. monocytogenes* to cope with acid stress. GAD catalyzes the decarboxylation of glutamate to gamma-aminobutyric acid and CO_2 by consuming a proton. Microorganisms use this mechanism to remove excess protons inside the cells when under acidic conditions (Feehily and Karatzas, 2013; Feehily et al., 2013). This system protects *L. monocytogenes* from acid stress (Karatzas et al., 2012), and also protects *L. monocytogenes* on modified atmosphere-packaged food products (lettuce, coleslaw mix and minced beef; pH 4-6; Francis et al., 2007).

1.4.2. Sodium lactate and sodium diacetate

Salts (high osmolarity) inhibit the growth of *L. monocytogenes* by dehydration of the cell due to osmosis. A combination of 3% sodium lactate and 0.25% sodium diacetate [concentrations permitted by the U.S. Department of Agriculture Food Safety and Inspection Service] effectively inhibit the growth of *L. monocytogenes* in vacuum-packaged frankfurters during refrigerated storage (Bedie et al., 2001). Sodium lactate and sodium diacetate also inhibit the growth of *L. monocytogenes* on vacuum-packaged wieners and cooked bratwurst (Glass et al., 2002). Potassium lactate inhibits the growth of *L. monocytogenes* on modified atmosphere packaged sliced ham (Mellefont and Ross, 2007).

In vitro studies suggest that *L. monocytogenes* responds to stress from various salts differently. Exposure to NaCl leads to differential expression of salt stress proteins, including the heat shock protein (DnaK), an elongation factor (EF-Tu), a transporter of glycine betaine (GbuA) and the catabolite control protein (CcpA) (Duché et al., 2002; Bae et al., 2012). Compared to stress from NaCl, exposing *L. monocytogenes* to sodium lactate combined with sodium diacetate could specifically shut down the expression of DNA-binding proteins (Mbandi et al., 2007). *L. monocytogenes* can develop osmotolerance through accumulation of compatible solutes such as glycine betaine (N, N, N-trimethylglycine; Ko et al., 1994) and carnitine [3-hydroxy-4-(trimethylazaniumyl) butanoate; Verheul et al., 1997; Mendum and Smith, 2002] from the growth medium. The expression levels of two betaine transporter genes, *betL* and *gbuB*, and the carnitine transporter gene *opuCA* are increased in the presence of NaCl (Okada et al., 2006). Another gene essential for growth under stress conditions is *htrA* (also known as *degP*), which encodes a homolog to protease, and is required for the growth of *L. monocytogenes* 10403S (serotype 1/2a) in an environment with an elevated NaCl concentration (Wonderling et al., 2004). In addition, the genes *ykpA*, *lmo0668* (encoding a permease protein), *lmo0990*, *lmo0989* (encoding a transcriptional regulator, MarR family), as well as *ntpJ* (gene product mediates K^+ and Na^+ co-transport) participate in adaptation of *L. monocytogenes* to a high salt environment (Gardan et al., 2003).

In conclusion, numerous stress proteins are involved in the stress response and tolerance of *L. monocytogenes* to salts *in vitro*. To date, no research has been reported on the role of stress proteins or genes encoding these proteins in *L. monocytogenes* growing on sodium-reduced RTE meats.

1.4.3. Autochthonous microbiota and class II bacteriocins

Autochthonous microbiota can suppress the growth of *L. monocytogenes*, mostly through production of bacteriocins or bacteriocin-like substances. For example, *Carnobacterium divergens* V41 and *C. piscicola* V1 inhibit the growth of *L. monocytogenes* on cold-smoked salmon at 4°C (Duffes et al., 1999). Another strain of *Carnobaterium* spp., *C. maltaromaticum* L103, inhibits the growth of *L. monocytogenes* on vacuum-packaged meats at 4°C (Schöbitz et al., 1999). *Carnobacterium* spp., *B. campestris* ATCC 43754, which is a bacteriocin-producer, controls the growth of both the spoilage organism *B. thermosphacta* and *L. monocytogenes* (Greer and Dilts, 2006). Other species of autochthonous microbiota that compete with *L. monocytogenes* for growth include *Enterococcus* spp. (Dallas et al., 1991). Some strains of autochthonous microbiota have been used as protective cultures to control the growth of *L. monocytogenes* in RTE meats.

Bacteriocins are categorized into Class I, II and III bacteriocins (Cintas et al., 2001). Class II bacteriocins are small (usually 5-10 kDa) cationic peptides produced mostly by lactic acid bacteria, which generally do not undergo posttranslational modifications before secretion outside the cell. Class II

bacteriocins are further categorized into Class IIa to Class IIe bacteriocins (Cotter et al., 2013):

- Class IIa are heat-stable bacteriocins, such as pediocin. The mode of action is through formation of pores in the cell membrane (Chikindas et al., 1993);
- Class IIb are two-peptide bacteriocins and the antimicrobial activity requires the presence of both peptides. The mode of action is through disruption of the gradient of monovalent ions, such as Na^+ and K^+, across the cell membrane (Nissen-Meyer et al., 2010);
- Class IIc (grouped as Class IV by Heng and Tag, 2006) are cyclic peptides including enterocin AS-48 (Gálvez et al., 1989) and carnocyclin A (Martin-Visscher et al., 2008). The modes of action of Class IIc bacteriocins are suggested to be diverse and based on their three-dimensional arrangement; they could either directly lyse the membranes of target cells or serve as activators of lipid-degrading enzymes (Martin-Visscher et al., 2009);
- Class IId bacteriocins are one-component, linear peptides (e.g., aureocin A53; Netz et al., 2002a and 2002b) that interact with the lipid component of the target cell membrane;
- Class IIe bacteriocins are peptides that contain two or more components (e.g., aureocin A70; Netz et al., 2001).

Microorganisms can adapt to sublethal dose of bacteriocins. There are bacterial strains that are spontaneously resistant to bacteriocins (Macwana and Muriana, 2012). Possible mechanisms of bacteriocin resistance include: thickened cell wall (Crandall and Montville, 1998), cell efflux (Hoang et al., 2011) and modification of receptor binding sites for bacteriocins (Arber, 1993). It has been suggested that spontaneous resistance results from the alteration of the cell surface, mainly from an increase in short-acyl-chain and unsaturated phosphatidylglycerol (PG) species, which indicates greater fluidity and a positive charge on the cell membranes (Vadyvaloo et al., 2004).

Strains of *L. monocytogenes* that are resistant to Class IIa bacteriocins have been discovered (Kaur et al., 2011). For example, certain strains of *L. monocytogenes* are resistant to pediocin PA-1 (Crandall and Montville, 1998). The loss of the AB subunit of mannose-specific phosphotransferase system ($EIIAB^{Man}$ PTS, responsible for the uptake of mannose) and the up-regulation of two β-glucoside-specific PTS genes are linked to the resistance to bacteriocins (Dalet et al., 2001; Gravesen et al., 2002; Ramnath et al., 2000).

1.4.4. General stress response and cross-protection

The cross-protection of *L. monocytogenes* to stresses from acids, salts, and bacteriocins has been observed in many studies. For example, the adaption of *L. monocytogenes* to acid stress is strongly dependent on the simultaneous presence of a second stress factor (NaCl) (Bergholz et al., 2012; Koutsoumanis et al., 2003). The cross-protection is possible from the regulation of gene expression by

alternative sigma factors. There are five sigma factors found in *L. monocytogenes*, including σ^A, sigH, σ^{54} (rpoN or SigL), sigV (or σ^C, encoded by *lmo0423*) and σ^B (Glaser et al., 2001).

The sigma factor σ^B is found in various low GC-content gram-positive bacteria including *Listeria*. The σ^B mediates general stress response and is up-regulated in *L. monocytogenes* in the presence of various environmental stresses including salts, acids and bacteriocins (Abram et al., 2008; Chaturongakul and Boor, 2006; Sue et al., 2004; Palmer et al., 2009).

Another alternative sigma factor involved in stress-response of *L. monocytogenes* is σ^{54} (Robichon et al., 1997; Dalet et al., 2001, 2003), which is required for resistance of *L. monocytogenes* to Class IIa antilisterial bacteriocin mesentericin Y105 and resistance to Class I bacteriocin (nisin) (Palmer et al., 2009). The σ^{54} participates in resistance to bacteriocins by regulating the expression of sugar-specific PTSs (Gravesen et al., 2002). The σ^{54} is also involved in osmotolerance and acid resistance in *L. monocytogenes*. Deletion of *rpoN* (σ^{54}) results in greater uptake of a compatible solute, carnitine (Okada et al., 2006), and deletion of *sigL* (σ^{54}) results in a decreased growth of *L. monocytogenes* at 4°C in the presence of lactic acid (Raimann et al., 2009).

The alternative sigma factor rpoS also regulates the expression of proteins involved in tolerance to heat, osmotic pressure and acids in both Gram-positive

and Gram-negative bacteria, including *L. monocytogenes* (Ait-Ouazzou et al., 2012), *E. coli* and *Salmonella* (Hengge-Aronis, 1993).

RNA-binding proteins, such as CsrA, are also responsible for the regulation of stress response (Romeo, 1998). Although the role of CsrA in stress response to preservatives in *L. monocytogenes* has not been reported, mutation in *csrA* results in the inability of *E. coli* to grow on media with acetate (Wei et al., 2000). The regulation of *csrA* expression has been suggested to be complex and influences the expression of sigma factors (Yakhnin et al., 2011).

Chaperone proteins, such as DnaK and GroEL, are also involved in stress response in *L. monocytogenes*. DnaK and GroEL are up-regulated when *L. monocytogenes* was exposed to bacteriocin enterocin AS-48 (Caballero Gómez et al., 2013) and NaCl (Duché et al., 2002).

1.5. Filamentation of *L. monocytogenes*

Filament formation is a phenomenon found in various bacterial species, including *E. coli* (Brandi et al. 1989; Lorian and Atkinson 1975; Jones et al., 2004, 2003, 2002, 2008), *Salmonella* (Pratt et al., 2012), *Bacillus* spp. (Breakefield and Landman, 1973) and *L. monocytogenes* (Vail et al., 2012), when cells cannot form septa during binary fission. Filament formation results from insufficient energy (Jones et al., 2006) and blockage of DNA replication. It is viewed as "economy growth" of cells; i.e., cells avoid the energy expenditure of septation at a time when the cell may have low energy reserves (Mattick et al.,

2003). Filamentation is also observed when DNA replication is inhibited (Pratt et al., 2012) due to environmental stress.

In *L. monocytogenes*, filamentation is observed when cells are exposed to temperature perturbation, acid/alkaline and salt under laboratory conditions (Isom et al., 1995; Giotis et al., 2007; Vail, 2012). Depending on the stress, the proportion of filamented cells in a population can range from 10% (Vail et al., 2012) to over 90% (Alonzo et al., 2011). The longest filament of a *Listeria monocytogenes* was recorded at 96 μm (Rowan et al., 2000).

In RTE meats, refrigeration storage and preservatives can cause stress to cells of *L. monocytogenes*. To date, no research has reported that *L. monocytogenes* can form filaments *in situ*.

If filamentation of *L. monocytogenes* occurs on RTE meat, it could lead to problems for the food industry. Meat processors can classify RTE meats as Category 2B products, on which the growth of *L. monocytogenes* should be ≤0.5 log CFU/g throughout the stated shelf-life (Canadian Food Inspection Agency, 2012). To meet this stringent criterion, the actual number of *L. monocytogenes* needs to be accurately enumerated. The plate count method that can be used by meat industry to determine the number of *L. monocytogenes* on a product could underestimate the actual number of *L. monocytogenes* in a filament. On an agar plate, both a filament and a single cell of *L. monocytogenes* produce one colony. However, a filament of *L. monocytogenes* could potentially divide into multiple

individual cells when it is placed in favoured growth conditions *in vitro* (Vail, 2012).

Filamentation of *L. monocytogenes* may not be avoidable. Methods that are suggested to be more accurate than a plate count have been developed to determine the number of filamented cells include total protein concentration measurement (Mattick et al., 2003) and quantification of total nucleoids (Athale and Chaudhari, 2011). Filaments and single cells can also be counted by flow cytometry (Gant et al., 1993; Wickens et al., 2000). However, before these methods are standardized for application to commercial products, extensive testing and calibration are needed.

The molecular mechanisms of filament formation in *L. monocytogenes* have not been elucidated. Most studies that report the mechanism of filamentation are focused on *E. coli*, *B. subtilis* and *Salmonella*. Overall, alternative sigma factors (rpoS and σ^{54}) that participate in the global gene regulation, determine filamentation in *E. coli* (Powell and Court, 1998) and in *Salmonella* (Mattick et al., 2003).

Filamentation mainly results from incomplete assembly of the σ^{54}–dependent filamentous temperature sensitive (Fts) protein complex during cell division in *E. coli* (Buddelmeijer and Beckwith, 2002; Weiss DS, 2004; Karimova et al., 2005). One Fts protein, FtsZ, self-assembles into a ring structure at the future division site and interacts directly with the septal-specific peptidoglycan synthesis

machinery (Bi and Luktenhaus, 1991). The Fts proteins form a complex with Mur proteins that are involved in cell wall synthesis during cell division (Satta et al., 1994). The Fts proteins also interact with other cell division proteins, including MinD, MinE (in *E. coli*), and DivIVA, MinD (in *B. subtilis*). Both FtsZ and MinE are located at the division site, while MinD and DivIVA are located at the poles of the cell. In *E. coli*, MinD is inhibitory to FtsZ and ensures that the FtsZ ring is located at the correct site of division. MinE is located at the site of division and is inhibitory to MinD, so that the FtsZ ring only assembles at the site of division and nowhere else in the cell.

In *B. subtilis*, DivIVA plays the same role as MinE in *E. coli*; the difference between DivIVA and MinE is that MinE dissembles when the FtsZ ring forms, while DivIVA remains at the poles of the cell during the entire process (Jacobs and Shapiro, 1999). FtsZ also interacts with other cell division proteins FtsA, penicillin-binding protein PBP3 (encoded by gene *pbpB/ftsI*) and cell division inhibitor SulA (Vicente and Errington, 1996). SulA can induce filamentation in *E. coli* (Justice et al., 2006) and *S. enterica* (Humphrey et al., 2011).

The regulation of FtsZ is complex as it involves the interaction with many cell division proteins. FtsZ interacts directly with FtsA (Dai and Lutkenhaus, 1992; Dewar et al., 1992), FtsQ (Descoteaux and Drapeau, 1987), FtsW (Khattar et al., 1994), PBP3 (Ayala et al., 1988; Bi and Lutkenhaus, 1990) that also interacts with FtsA (Tormo et al., 1986), SulA (Bi and Lutkenhaus, 1993; Higashitani et al., 1995), ZipA, and MinC, which interacts with MinD/E (Ward and Lutkenhaus,

1985; de Boer et al., 1989, 1990, 1992). In *L. monocytogenes*, similar Fts, Mur, Min and Div proteins are present (Gilmour et al., 2010). However, no research has been reported that explains the role of these proteins in filamentation of *L. monocytogenes*.

Enzymes that produce ATP and NADPH are also responsible for filamentation in *E. coli* (Dudkina et al., 2011). Both cell division and cell wall synthesis require energy, and energy production in bacterial cells involves glycolysis and the pentose phosphate pathway. Although the role of glycolysis and the pentose phosphate pathway in filamentation in *L. monocytogenes* have not been determined, it is possible that cells minimize energy expenditure by decreasing the activity of key enzymes in these pathways.

Overall, filamentation occurs when bacterial cells undergo energy depletion due to stress and cell division is incomplete. Filamentation is characterized by the loss of septation due to the down-regulation of genes involved in cell division and cell-wall synthesis, which are regulated by alternative sigma factors. In the future, more accurate methods for cell count need to be developed and validated for the food industry to monitor the number of *L. monocytogenes* in RTE meat products.

1.6. Transcriptomic analysis: qRT-PCR, microarray and RNA-seq

Transcriptomic analysis is particularly useful for the study of regulatory mechanisms for gene expression in bacterial species. Three methods that are commonly applied in transcriptomic studies of bacteria include quantitative real

time polymerase chain reaction (qRT-PCR), microarray and RNA sequencing (RNA-seq). These methods involve the reverse transcription of RNA to cDNA. However, the scope of use and robustness of these methods are different. The qRT-PCR method is suitable for quantification of expression of genes with known nucleotide sequences, and primers specifically targeting the genes need to be designed for the analysis. Compared with qRT-PCR, microarray and RNA-seq methods are more robust for the analysis of the global transcriptome in bacteria. For microarray experiments, it is important that the genome sequences of the bacteria are known. Microarray experiments use hybridization of fluorescently labelled, sheared cDNA from the bacteria and nucleotide probes that are immobilized on a platform. The hybridization efficiency, sensitivity and specificity largely depend on the quality of RNA, labelling methods, hybridization buffer and the specificity of the probes (MAQC Consortium et al., 2006). The quantification of gene expression is carried out by generating digital images from fluorescent signals of each hybridized probe-cDNA sequence.

RNA-seq is a next-generation sequencing technique that quantifies the expression of genes based on the frequency of retrieval of the cDNA fragments of the same nucleotide sequences. RNA-seq has several advantages over the microarray method. In RNA-seq, probes for hybridization are not needed, so the accuracy of the experiments is not affected by the design of the probes or reagents that are applied for hybridization. The genome sequences of bacteria can either be known or unknown (Birol et al., 2009), since the quantification process in RNA-

seq does not require the design of probes based on genomic sequences. Moreover, RNA-seq does not have upper limit of quantification since the quantification is based on frequency of nucleotide sequences or genes retrieved rather than fluorescent intensity as in a microarray analysis. A complete review of RNA-seq was summarized by Wang et al. in 2009.

RNA-seq is widely used in transcriptomic research in bacteria including *L. monocytogenes* (Cossart and Archambaud, 2009). RNA-seq was used to reveal the alteration in global gene expression when *L. monocytogenes* was exposed to environmental stress, such as biocide (Casey et al., 2014), and nutrient-depletion at the stationary phase (Oliver et al., 2009).

1.7. Research objectives

The research focused on the impact of sodium reduction in ready-to-eat (RTE) meat products on the autochthonous microbiota and physiology of *L. monocytogenes.* The long-term objective of this study was to identify the potential problems in microbiological safety and quality associated with sodium reduction, so that the interventions could be improved to ensure the safety and quality of the sodium-reduced RTE meats.

The short-term objectives of this study were achieved by examination of six aspects relevant to sodium reduction in RTE meat: **1)** characterization of spoilage microorganisms on regular and sodium-reduced retail RTE meats; **2)** the effect of sodium concentration on the growth of autochthonous microbiota and

L. monocytogenes on ham; **3)** the cellular response of *L. monocytogenes* to sublethal dose of bacteriocin (carnocyclin A) in a low sodium environment; **4)** the cellular response of *L. monocytogenes* to the preservatives sodium lactate and sodium diacetate *in vitro*; **5)** the impact of sodium lactate and sodium diacetate on the gene expression and filamentation of *L. monocytogenes in vitro*; and **6)** characterization of the environmental factors that would induce filament formation of *L. monocytogenes in vitro* and *in situ*.

1.8. References

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2. Culture-Dependent and Culture-Independent Study of Spoilage Microorganisms on RTE Meat Products

2.1. Introduction

Meat processors have made sodium-reduced ready-to-eat (RTE) meat products available as a result of concern that excess consumption of sodium can lead to health problems such as cardiovascular disease and high blood pressure (Taormina, 2010). Compared to regular sodium RTE meat, sodium-reduced products have 25-50% less sodium. According to the Health Canada's Sodium Reduction Strategy (2010), the sodium content of food products should be gradually reduced to the targeted levels to achieve a mean sodium intake of 2,300 mg per person per day by the year 2016. The specified sodium level target for fully cooked RTE meats is 800 mg per 100 g of product (sales weighted average; Health Canada, CA). One role of sodium chloride in processed meat is to decrease the water activity, thereby inhibiting microbial growth. Thus, sodium reduction could increase the risk of survival and growth of spoilage and pathogenic microorganisms on these products. Spoilage bacteria on vacuum packaged RTE meats include *Carnobacterium* spp., *Brochothrix* spp. (Stanley et al., 1981), *Lactobacillus* spp. (Korkeala and Björkroth, 1997) and *Leuconostoc* spp. (Johansson et al., 2011). *Leuconostoc* spp. produce exopolysaccharides (EPS) when grown in media containing sucrose (Johansson et al., 2011). However, the relationship between sodium level and the diversity of spoilage microorganisms on RTE meat products is uncharacterized.

Meat spoilage microorganisms can be studied using culture-dependent or independent methods. Culture-dependent methods allow the isolation and subsequent identification of microorganisms that prevail in the microbiota of a RTE meat product. Culture-independent methods such as 16S rDNA amplification (Chenoll et al., 2007) followed by Denaturing Gradient Gel Electrophoresis (DGGE) can provide information on the genera of the microorganisms present on the meat product. 16S rDNA bands from various microbial species can be separated on the denaturing gradient gel; the banding patterns are representative of the species presented on each meat product. In this study, both culture-dependent and culture-independent methods were used to compare the microbiota of sodium-reduced RTE meats and their regular sodium counterparts.

2.2. Materials and Methods

2.2.1. Products and Storage conditions

Six regular and 17 sodium-reduced packaged meat products labelled with 23 different commercial names were purchased throughout the year from local grocery stores in Edmonton, AB, Canada. The 17 sodium-reduced products represented all sodium-reduced products available in the marketplace at the time of the study. The 6 regular sodium products were the regular sodium counterpart of 6 of the sodium-reduced products. All products were stored at 7ºC, to mimic temperature abuse of retail meats, until the best before date stated on the package. For each product, 3 replicate packages with different best before dates were

analyzed. Products used in this study are listed in Table 2.1 and include various types of RTE meat, such as sliced deli meats, sausages, wieners, smokies and RTE bacon. A sodium-reduced product was considered a product that contained 720 or less mg sodium per 100 g of product, which was 30% less sodium than average sodium content in RTE meat products (1029 mg/ 100 g) in the year 2009 (Health Canada, 2010), or a product labeled as "sodium-reduced."

2.2.2. Microbiological analysis

For the enumeration of the surface microbiota of the products, samples were rinsed with 0.1% Bacto™ Peptone (Difco; Becton, Dickinson and Company, Sparks, MD) following vigorous massage of the products for 1 min and 10-fold serial dilutions of the wash were plated on Plate Count Agar (PCA; BD-Canada) for total viable counts (TVC) on both All-Purpose-Tween agar (APT; BD-Canada) and de Man, Rogosa and Sharpe (MRS; BD-Canada) agar for presumptive LAB counts, on Streptomycin Thallous Acetate Actidione agar (STAA; Oxoid Ltd., England) for counts of *Brochothrix* spp., and on Violet Red Bile Agar with Glucose (VRBG; BD-Canada) for counts of *Enterobacteriaceae*. All agar plates were stored aerobically. APT, MRS, and PCA plates were stored at 25°C for 72 h, STAA at 25°C for 48 h and VRBG at 37°C for 24 h prior to enumeration. Counts were converted to log CFU/cm^2.

2.2.3. Extraction of the sample total DNA

A 10 mL aliquot of the peptone wash from the sampling was used for extraction of total DNA. The peptone wash was centrifuged at 5,000 *x* g for 10 min at 22°C. Total DNA was extracted from the pellet using a DNeasy Blood and Tissue Kit (Qiagen, CA) following the Gram-positive bacteria protocol provided by the manufacturer.

2.2.4. RAPD analysis and 16S rDNA sequencing of culturable species

Up to 10 colonies were isolated from each sample. Total DNA was extracted from the isolated microorganisms and used as a template for Random Amplified Polymorphic DNA (RAPD) analysis. The amplifications were done according to Fontana (2005) and Aymerich (2005) for primer RAPD2, and R5, respectively. Strain identification by 16S rDNA sequencing was done as described by Miller et al. (2010). The genomic DNA was purified using a DNeasy Blood & Tissue Kit (QIAGEN, CA). The 16S rDNA was amplified using primers 616V (5'-AGAGTTTGATYMTGGCTC-3') and 630R (5'-CAKAAAGGAGGTGATCC-3') (Lehner et al., 2004). PCR products were sequenced using a BigDye Terminator Sequencing Kit in a DNA Analyzer (model 3730; Applied Biosystems, CA) according to the manufacturer's instructions. BLAST analysis of the DNA sequences was carried out with the 16S rDNA data available in the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.2.5. Genus-specific PCR

Total DNA from each meat sample was used as the template for the PCR amplification of the partial 16S rDNA of the surface microbiota. Primers HDA1-GC clamp and HDA2 (Walter et al., 2000) were used and the PCR program was set at: 94°C for 5 min; 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s; and 72°C for 30 min for a final extension.

2.2.6. PCR and construction of DGGE ladders

For the construction of DGGE ladders, 6 bacterial genera isolated from retail products (2.2.5.) were chosen as model microorganisms: *Pseudomonas fluorescens* B103, *Carnobacterium maltaromaticum* E102, *Lactobacillus* spp. (*L. sakei* F201 and *L. curvatus* C106), *Leuconostoc* spp. (*L. gelidum* C101 and *L. gasicomitatum* C302), *Staphylococcus warneri* B303 and *Brochothrix thermosphacta* P107. Total DNA was isolated using DNeasy Blood & Tissue Kit (Qiagen, CA) following the manufacturer's guide and primers HDA1-GC clamp and HDA2 were used to amplify partial 16S rDNA.

2.2.7. DGGE-sequencing analysis

PCR-amplified partial 16S rDNA from each meat sample was subjected to DGGE analysis. The gels were prepared with 8% polyacrylamide, and 22-55% denaturing gradient urea/formamide. Electrophoresis was carried out in 1x TAE buffer at 60 °C for 3.5 h at 170 V followed by staining with SYBR safe DNA gel stain (Invitrogen, CA).

DGGE gels were visualized under UV light and bands visible under Safe Imager™ blue-light transilluminator (Molecular Probes, USA) were excised, DNA bands were amplified by PCR as described above to obtain a higher concentration for the subsequent cloning, where DNA bands were ligated into pGEM®-T Easy vectors (Promega, CA) and cloned into *E. coli* DH5α cells following the manufacturer's instructions. Bacterial universal primers T7 (5'-AATACGACTCACTATAG-3') and SP6 (5'-ATTTAGGTGACACTATAG-3') were used for colony PCR. Primer T7 was used as the sequencing primer of the insert. The sequences of the 16S rDNAs were compared to the RDP database (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp).

2.2.8. Exopolysaccharide production and characterization

L. gasicomitatum C302 isolated from a sodium-reduced product was grown in APT agar supplemented with 5% (w/v) sucrose at 22°C.

The EPS was harvested from the surface of the agar plate and diluted ~ 1:5 with deionized H_2O. The sample was filtered through an Amicon® Ultra-15 Centrifugal Filter Device (Millipore Corp., CA) molecular weight cut-off of 10, 000 Da by centrifugation at 5,000 *x g* at room temperature. The EPS was concentrated to a final volume of 200 µL. A 100 µL aliquot was diluted to a final volume of 1 mL with ddH_2O for molecular weight determination; the rest of the sample was hydrolyzed in 2M H_2SO_4 at 80°C for 2 h and diluted 1 in 10 in ddH_2O for identification of the monosaccharide units by HPLC with an Aminex 87H column (BioRad, CA) at 70°C and a flow rate of 0.4 mL min (Schwab et al.,

2008). Commercially available monosaccharides (glucose, fructose, fucose, mannose, galactose, *N*-acetylgalactosamine) were used as standards (Sigma, CA). Size of the polysaccharides was determined by HPLC using a Superdex 200 Column (GE Healthcare, Baie d'Urfe, Canada) column with commercial levan as standard for comparison (Wang et al., 2010).

2.3. Results and Discussion

2.3.1. Microbiological analysis of RTE meat products

In this study, 23 low sodium meat products with 6 regular sodium counterparts from the same processor (Table 2.1) were purchased and stored at slightly temperature abusive conditions until the best before date indicated on the package. This was done to allow the growth of the surface microbiota for isolation by culture-dependent methods.

Table 2.1 Sodium contents and microbial counts (log CFU/cm^2) of RTE meat products at the best before date.

Product	Processor	Na content [mg/100g product]	Total viable counts[a]	Presumptive LAB	*Brochothrix* spp.	Group
chicken breast sliced, reduced Na	P1	424	8.5 ± 0.3	8.5 ± 0.2	2.2 (1)	1
chicken breast sliced, regular Na	P1	830	8.0 ± 0.3	8.5 ± 0.2	5.7 (2)	1
ham, sliced, reduced Na	P1	667	7.7 (2)	8.0 (2)	ND	2
ham, sliced, regular Na	P1	1090	3.5 (2)	3.1 (2)	3.0 (2)	2
smokies, reduced Na	P1	614	3.2 (2)	3.2 (2)	ND	2
smokies, flavored, reduced Na	P1	627	7.1 (1)	7.5 (1)	ND	2
turkey breast sliced, reduced Na	P2	394	7.3 ± 0.9	7.3 ± 0.8	3.3 (1)	1
turkey breast sliced, regular Na	P2	924	8.5 ± 0.1	8.5 ± 0.0	ND	1
turkey breast sliced, flavored, reduced Na	P2	466	8.9 ± 0.2	8.9 ± 0.3	2.3 (1)	1
turkey breast sliced, flavored, reduced Na	P3	682	3.1 (1)	2.5 (1)	ND	2
turkey hot dog, reduced Na	P3	661	ND	ND	ND	3
ham sliced, reduced Na	P4	585	5.0 (2)	4.9 (2)	ND	2
ham, sliced, flavored, reduced Na	P4	585	7.1 (1)	7.1 (1)	ND	2
turkey pepperoni, reduced Na	P5	750	ND	ND	ND	3
turkey pepperoni spicy, reduced Na	P5	750	ND	ND	ND	3
chicken breast, sliced, reduced Na	P6	660	5.6 (1)	5.6 (1)	ND	2
sausage, reduced Na	P7	712	7.0 (1)	7.8 (1)	ND	2
sausage, regular Na	P7	1200	ND	ND	ND	3
wiener, reduced Na	P8	687	5.0 (2)	5.1 (2)	2.2 (1)	2
hot dog, reduced Na	P9	526	ND	ND	ND	3
hot dog, regular Na	P9	868	2.6 (1)	2.8 (1)	ND	2
turkey bacon, reduced Na	P10	627	2.0 (1)	2.7 (1)	ND	2
turkey bacon, regular Na	P10	1000	1.8 (1)	1.8 (1)	ND	2

[a] Counts are shown as a mean ± standard deviation; n=3. Numbers in brackets indicate the number of samples with counts above the detection limit for a product where microbes were not present in every sample.

Na=sodium; ND indicates that counts on all samples of the product were below the detection limit of 20 CFU/mL[2].

Products were grouped into 3 groups based on their microbial load and the number of samples with detectable microbiota. Products with a high density (> log 8 CFU/cm^2) of bacteria in every sample, were assigned to Group 1. Group 1 contained 3 reduced sodium products and 2 regular sodium products. All products in this group were sliced RTE meats. Group 2 contained products where microbes were present in at least one replicate and the total load of bacteria ranged from 2 – 7 log CFU/ml. This group contained 10 reduced sodium products and 3 regular sodium products. All types of products were represented in this group. Three products from this group may have been subjected to high hydrostatic pressure (HHP) as their TVC and LAB counts were between 1.8 – 2.8 log CFU/cm^2. Products with counts below the detection limit in all 3 replicates were classified into Group 3. Group 3 contained different 5 product types, including 4 reduced sodium products, 1 regular sodium product, and 4 products that had been subjected to high pressure processing according to the claim on the manufacturer's website. No *Enterobacteriaceae* were found on any of the products analyzed.

Differences in microbial load between regular and sodium-reduced products in Group 1 were not detected when bacteria were cultured from the products. Sliced chicken or turkey breasts products from Group 1 had the highest microbial load (Group 1). Different types of products (ham, smokies) produced by the same manufacturer did not contain high levels of bacteria and were in Group 2. One sodium-reduced, sliced turkey product and a sodium-reduced sliced chicken

breast were the only sliced turkey or chicken breast products with bacteria detected on only 1 sample. The counts of the other 2 samples were below the detection limit. These products were manufactured at a processing facility that is known to use high pressure processing (HHP), although no label declaration about the use of HHP was made for these products.

In this study 150 bacterial strains were isolated from the RTE meat products out of which 49% of strains were *Leuconostoc* with the most prevalent species *Leuconostoc gelidum* (24%, Table 2.2). Twenty four percent of strains were carnobacteria (18% *Carnobacterium maltaromaticum*), 17% were *Brochothrix thermosphacta* and 6% were lactobacilli (Table 2.2). Among all of the isolates, *Leuconostoc* were the most common in both regular and reduced sodium products (54% and 45%, respectively).

Table 2.2 Summary of bacteria isolated from RTE meat products.

Bacteria	Number of isolated strains		
	Overall (150 strains)	Reduced Na products (97 strains)	Regular Na products (57 strains)
Leuconostoc gelidum	39	15	24
C. maltaromaticum	27	19	8
Carnobacterium sp.	9	9	0
Brochothrix thermosphacta	26	10	16
Leuconostoc gasicomitatum	15	12	2
Leuconostoc carnosum	12	7	5
Leuconostoc mesenteroides	7	7	0
Lactobacillus sakei	6	4	2
Lactobacillus curvatus	5	5	0
Staphylococcus warneri	2	2	0
Lactobacillus curvatus/sakei	1	1	0
Enterococcus malodoratus	1	1	0

Audenaert et al. (2010) also found *Leuconostoc*, in particular *L. carnosum,* as the most prevalent psychrotrophic organism on modified atmosphere packaged meat products with bacterial counts ranged from 6 to 8 CFU/g. A greater proportion of carnobacteria and a lower incidence of *B. thermosphacta* were isolated from reduced sodium products. Carnobacteria and *Leuconostoc* were found on 69% of reduced sodium products of Groups 1 and 2. On regular sodium products of Groups 1 and 2, *Leuconostoc* were found on 80% of products, while carnobacteria only on 20%. Furthermore, the microbial community isolated from sodium-reduced products had more species diversity than that isolated from regular sodium products.

In products with a high density of bacteria (Group 1), *Leuconostoc* were the dominant species of bacteria (62%) while in Group 2 products, carnobacteria (43%) were the most commonly isolated species (Table 2.3).

Table 2.3 Bacteria isolated from RTE meats products in Group 1 and Group 2.

Bacteria	Number of isolated strains	
	Group 1	Group 2
Leuconostoc gelidum	29	10
Brochothrix thermosphacta	15	11
Leuconostoc carnosum	11	1
Carnobacterium maltaromaticum	8	19
Carnobacterium sp.	1	8
Leuconostoc gasicomitatum	7	8
Leuconostoc mesenteroides	7	0
Lactobacillus curvatus	4	1
Staphylococcus warneri	2	0
Lactobacillus curvatus / sakei	1	0
Lactobacillus sakei	1	4
Enterococcus malodoratus	1	0

Culture-dependent analysis of microbiota was conducted on regular and sodium-reduced products manufactured at the same facility. In all cases, regular sodium products had a narrower spectrum of bacterial species than reduced sodium products (Table 2.4).

2.3.2. Culture-independent comparison of reduced and regular sodium counterpart products

Culture-independent comparison of microbiota of reduced and regular sodium products manufactured was done to demonstrate differences in the total bacterial population of products and to assess the role of processing environment on the diversity of microbial species. DGGE-PCR showed similar trends seen with the culture-dependent analysis. A variation in the genera of the surface microbiota between the triplicate samples of each product was observed in both the culture-dependent and culture-independent study. For products manufactured at Plant P1 (Fig. 2.1), there was no consistency in the banding patterns of any of the products among the triplicate samples except for some of the products from Plant P2 (last three lanes on the right, Fig. 2.1), and the same trend was found for products from Plant P9 (Fig. 2.2). Despite the narrow spectrum of bacterial species identified by the culture-dependent study, more genera were isolated from regular sodium products than from their reduced sodium counterparts.

Table 2.4 Comparison of bacterial species isolated from regular and sodium-reduced RTE meat products by culture-dependent methods.

Sodium-reduced products		**Regular sodium product**	
Bacteria	**Number of isolates**	**Bacteria**	**Number of isolates**
Group 1		**Group 1**	
Leuconostoc mesenteroides	7	*Brochothrix thermosphacta*	9
Leuconostoc gasicomitatum	3	*C. maltaromaticum*	4
Leuconostoc carnosum	6	*Leuconostoc gelidum*	23
C. maltaromaticum	4	*Leuconostoc carnosum*	3
Brochothrix thermosphacta	4	*Leuconostoc gasicomitatum*	1
Enterococcus malodoratus	1		
Carnobacterium sp.	1	*Leuconostoc carnosum*	2
Staphylococcus warneri	2		
Lactobacillus sakei	1		
Group 2		**Group 2**	
Leuconostoc gasicomitatum	4	*Brochothrix thermosphacta*	7
Leuconostoc gelidum	3	*Leuconostoc gelidum*	2
Leuconostoc carnosum	1	*Lactobacillus sakei*	2
N/A	0	*Leuconostoc gasicomitatum*	1
		C. maltaromaticum	1

N/A not assigned to a genus.

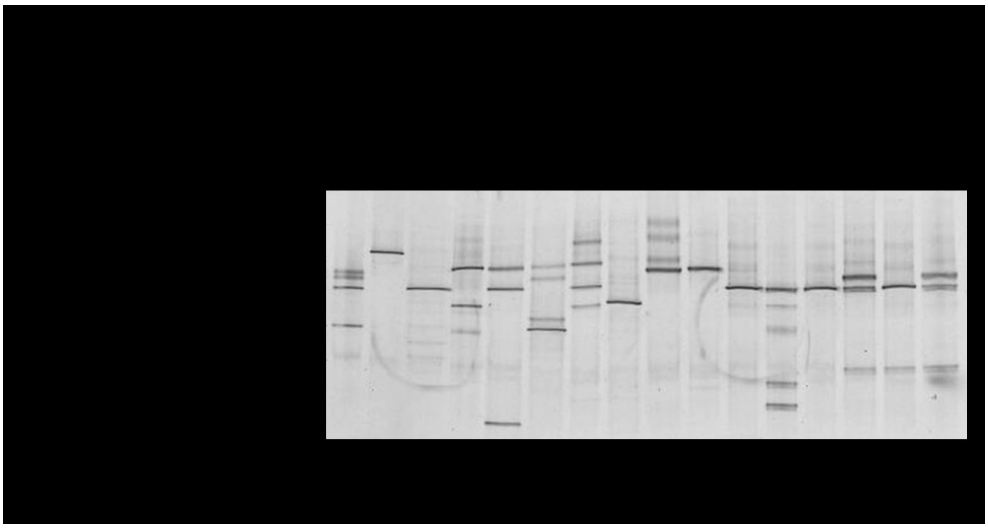

Figure 2.1 PCR-DGGE profiles of the surface microbiota of regular (Reg) and sodium-reduced (Re'd) vacuum packaged RTE meats from processing plants P1 and P2, which were stored at 7°C until the best before date. DNA bands: PCR products using total DNA as template and HDA1-GC and HDA2 as primers. Gel staining: SYBR® Safe.

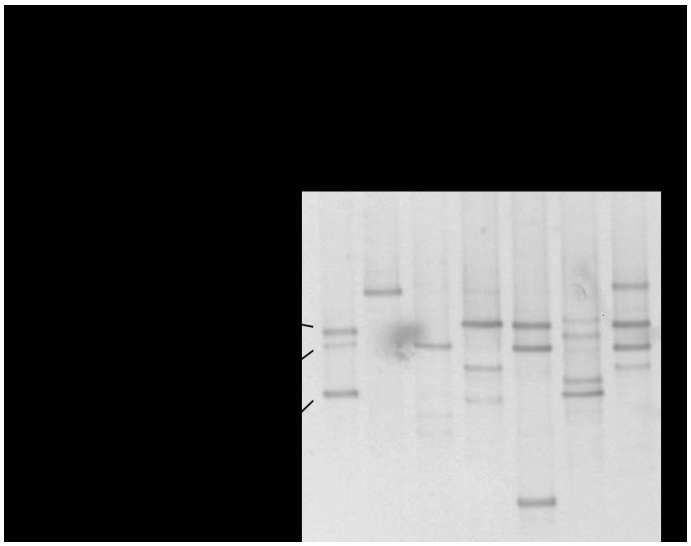

Figure 2.2 PCR-DGGE profiles of the surface microbiota of regular (Reg) and sodium-reduced (Re'd) vacuum packaged RTE meats from processing plant P9, which were stored at 7°C until the best before date. DNA bands: PCR products using total DNA as template and HDA1-GC and HDA2 as primers. Gel staining: SYBR® Safe.

Carnobacterium spp. were prevalent on regular sodium products from Plant P1. *Leuconostoc* spp. were found on both regular and sodium-reduced products, but not on all of the products tested. For products manufactured at Plant P2 (Fig. 2.1), *Leuconostoc* spp. were found in 6 out of 9 products tested, regardless of the sodium concentration. *Leuconostoc* spp. were found on both regular and sodium-reduced products from Plant P9, but not all the products contained these species (Fig. 2.2). The bands at the bottom of the gels in Fig. 2.1 and 2.2 were visible under the blue-light transilluminator but could not be amplified by PCR.

The genera of bacteria present on the meat samples seems to be specific to processing facilities (Fig. 2.1 and 2.2). *Carnobacterium* spp., *Leuconostoc* spp.

and *Brochothrix* spp. were commonly found in products from Plant P1. *Leuconostoc* spp. and *Brochothrix* spp. were commonly found in Plant P9 and *Carnobacterium* spp. and *Leuconostoc* spp. were commonly found in products from Plant P2. However, higher number of replicates plus samples from processing environment would need to be screened to accurately assess the exact role of the processing environment. Identification of genera on the surface of RTE meats by a culture independent method did not reveal additional species to that found by the culture dependent method.

Culture dependent (cell counts-16S rDNA sequencing) and culture independent (DGGE-PCR) comparison of microbiota of tested products showed a higher variety of bacterial species in low sodium RTE meat products. Culture dependent analysis revealed a higher sodium concentration could limit the diversity of bacterial species on regular sodium products. The most prevalent bacteria among all isolates were *L. gelidum*, *C. maltaromaticum*, *B. thermosphacta*, and *L. gasicomitatum* and *L. carnosum*. *L. gasicomitatum* (Vihavainen and Björkroth, 2009) and *B. thermosphacta* (Pennacchia et al., 2011) have been associated with food spoilage. Previous studies suggest that *L. gasicomitatum* spoils meat by slime formation and CO_2 production in vacuum-packaged sausages (Vihavainen et al., 2008). *Carnobacterium* spp. can spoil food (Leisner et al., 2007), but some *Carnobacterium* species can secrete antimicrobial compounds (Martin-Visscher et al., 2008) that will inhibit the growth of other closely related organisms such as lactic acid bacteria and *L. monocytogenes*.

2.3.3. Exopolysaccharide production and characterization

To screen whether the *Leuconostoc* spp. produce an EPS that had not been reported in literature, strains of *Leuconostoc* isolates were grown on sucrose-supplemented agar. EPS that had distinguishable morphology (colour or viscosity) was further analyzed for its size and types of monosaccharide units.

L. gasicomitatum C302 grown on APT agar supplemented with 5% sucrose at room temperature was able to produce exopolysaccharide (EPS). It produced a transparent, gel-like EPS.

HPLC analyses of the EPS showed that the monomers of the EPS are fructose (Figure 2.3) and the molecular weight of the EPS is equal to that of levan (Figure 2.4), which is a β-2, 6-fructan that has been previously found to be produced by *Leuconostoc* spp. (Holzapfel and Schillinger, 1992; Holt and Cote, 1998). Note that the peak (elution time: 50 min) could be from the carbohydrates in the growth medium since the EPS was not fully purified.

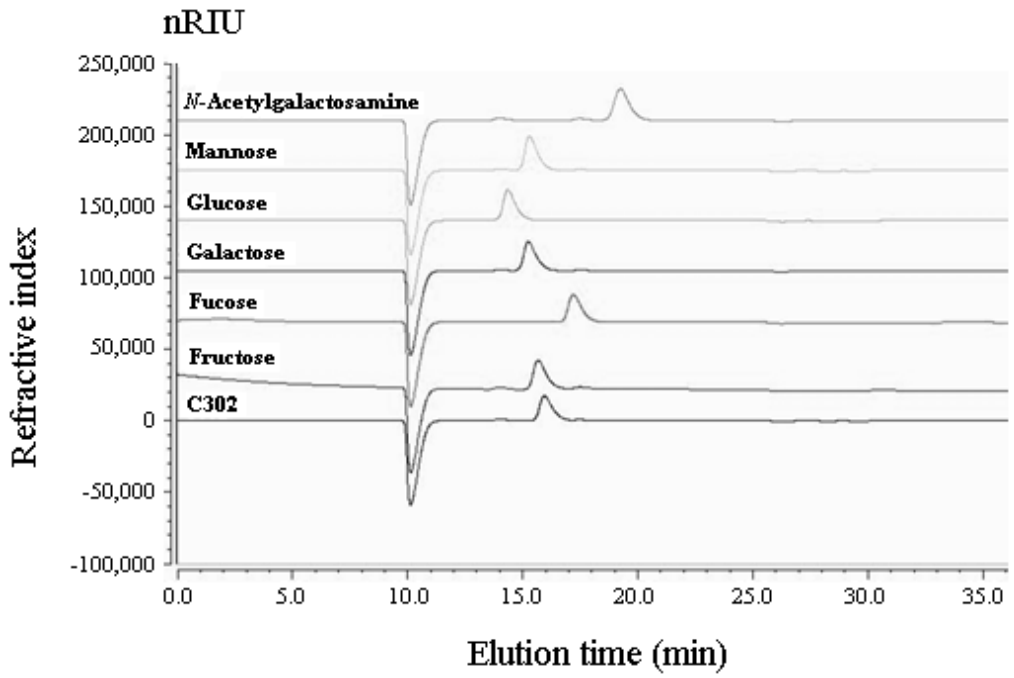

Figure 2.3 HPLC chromatograms of hydrolyzed EPS (monosaccharide unit) from *L. gasicomitatum* C302 ("C302") and monosaccharide standards.

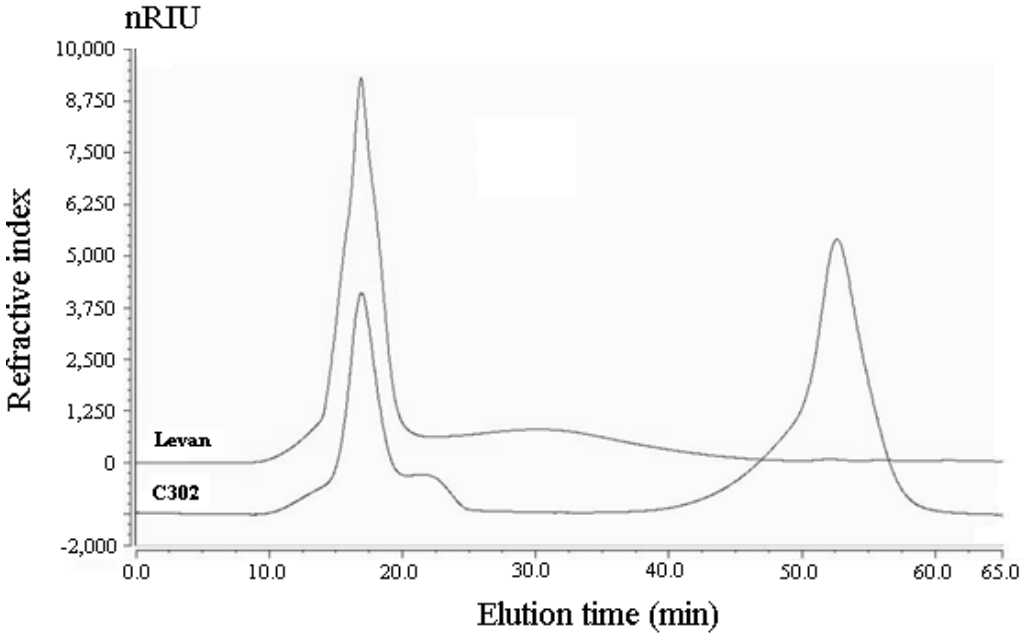

Figure 2.4 HPLC chromatograms of EPS from *L. gasicomitatum* C302 ("C302"). and standard (levan).

2.4. Conclusions

This work is the first report utilizing both culture-dependent and culture-independent (DGGE) methods to examine the microbiota of regular and reduced sodium meat products from the retail market and subjected to slight temperature abuse condition. This was done to mimic the product temperature during consumer handling. The diversity of microbial species in retail RTE meats under 23 different brand names was profiled. The meat products tested included sodium-reduced and the ones with regular concentration of NaCl, according to the labels. Culture-dependent analysis of the retail meat products identified *L. gelidum*, *C. maltaromaticum*, *B. thermosphacta*, and *L. gasicomitatum* and *L. carnosum* as the most prevalent microorganisms in RTE meats. Culture-independent analysis confirmed that *Carnobacterium* spp. and *Leuconostoc* spp. were the most prevalent microbial species in both regular and sodium-reduced RTE meats. The packaged sliced RTE meats were found as a group of products with the highest load of bacteria. HHP processed products were among the products with the lowest number of bacteria. This research demonstrated that the sodium-reduced meat products had more diverse bacterial species than the regular sodium counterpart product. HPLC analysis showed that *L. gasicomitatum* C302 isolated from sodium-reduced RTE meat produced EPS (levan) on sucrose-supplemented solid medium.

Knowledge of the microbiota on RTE meats is an initial step towards understanding the factors that influence the shelf life of these products. The

isolation of *Leuconostoc* spp. from most of the RTE meats implicates that production of EPS could be a major concern regarding the quality of these products. The presence of high counts of *Leuconostoc* spp. in meats could also impact the growth of other microorganisms, including *L. monocytogenes*, which is discussed in the following chapter.

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3.* Growth of *Listeria monocytogenes* on Sodium-Reduced Ham

3.1. Introduction

The sodium content in meat may influence the growth of *L. monocytogenes*. A change in NaCl concentration influences the water activity (a_w) of the environment, which affects the growth of microorganisms. Sodium ions interact with bacterial proteins (Häse et al., 2001), which could have an impact on the growth of *Listeria*. The presence of an autochthonous microbiota might also influence the growth of *Listeria* and its susceptibility to changes in NaCl concentrations. To date, there have not been any *in situ* studies specifically evaluating the effect of sodium and autochthonous microbiota on the growth of *L. monocytogenes*.

In Chapter 2 of this thesis it was unclear how the sodium concentration claimed by manufacturers could influence the diversity of microbiota on the RTE products. To accurately assess whether sodium reduction causes changes in the growth of *L. monocytogenes*, it would be necessary to produce products formulated with specific concentrations of total NaCl. This research aims to

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* A version of this chapter entitled "Growth of *Listeria monocytogenes* on sodium reduced ham" was published in the Proceedings of the 58th International Congress of Meat Science and Technology (12-17 August 2012) by Xiaoji Liu, Petr Miller, Phyllis J. Shand and Lynn M. McMullen.

provide insight on the growth of *L. monocytogenes* on sodium-reduced RTE meat products. The methods and models used in this study can be validated for future research monitoring microbial survival, growth and metabolic activity.

3.2. Materials and Methods

3.2.1. Bacterial strains and media

The strains used in this study were isolated from vacuum packaged sliced deli meats purchased from local retail stores. These included: *Brochothrix thermosphacta* P107, *Carnobacterium maltaromaticum* E102, *Lactobacillus sakei* F201, *Leuconostoc gasicomitatum* C302 and *Leuconostoc gelidum* C101. Frozen stocks of these cultures were maintained in 40% glycerol at -80°C. These strains were cultured from frozen stocks into All Purpose Tween broth (APT, BD-Canada, ON) and incubated overnight at 22°C. Strains of *L. monocytogenes* included *L. monocytogenes* FS13, FS14, FS19 and ATCC 7644. These strains were cultured from frozen stocks into Tryptic Soy Broth (TSB, BD-Canada) and incubated overnight at 37°C. Prior to use in experiments, the autochthonous bacteria and *L. monocytogenes* were grown overnight in appropriate media at 22°C.

To ensure that the growth of *L. monocytogenes* was not inhibited by the autochthonous strains used in this study, deferred inhibition assays were done (Ahn and Stiles, 1990). Strains of autochthonous bacteria were spotted (5 µL) onto APT agar and the plates were incubated at 22°C overnight. Soft APT agar

(7.5% agar) was inoculated (0.1%) with strains of *L. monocytogenes* and gently mixed. The soft agar was gently poured onto the plate with the autochthonous bacteria. Plates were incubated at 22°C and examined for zones of inhibition.

3.2.2. Ham production

Chopped-and-formed ham with 0.85%, 1.10% and 1.35% total NaCl (calculation based on formulation; details in Appendix A) was manufactured to examine the effect of sodium chloride and autochthonous microbiota on the growth of *L. monocytogenes*.

Pork leg meat was obtained from a federally inspected meat processing facility. Sodium chloride, sodium erythorbate, dextrose monohydrate, sodium tripolyphosphate (STPP) and Prague powder (94% NaCl and 6% $NaNO_2$) were provided by Griffith Laboratories™, Canada. Nylon casings (75 mm diameter) were purchased from UniPac (AB, Canada).

Pork leg meat was held at 4°C overnight and cut into ca. 4 cm x 4 cm x 4 cm cubes. The meat was ground through a 0.32 cm plate (Mini-matic; HollyBerk Sales, AB). Meat and other ingredients [ice (20% of meat weight), sodium tripolyphosphate (0.6% of meat weight), sodium erythorbate (0.1% of meat weight), dextrose (3% of meat weight), Prague powder (0.46% of meat weight), NaCl (variable depending on final sodium concentration)] were combined and tumbled (VAS-40, Glass®; Germany) under vacuum (80 kPa) at 4°C for 10 min and held for 1 h under vacuum. Meat batter was stuffed into casings and stored

overnight at 4°C. The hams were cooked in water (98°C) for 10 min and at 80°C until the internal temperature reached 71°C. Hams were chilled on ice for 10 min and held at 4°C for further cooling. Cooking losses of all ham products were < 3%. After overnight cooling, hams were sliced (2 mm thick x 33 cm^2) and inoculated immediately.

3.2.3. Inoculation and packaging

Overnight cultures of *L. monocytogenes* and/or the autochthonous microbiota were centrifuged at 4,000 *x g* for 10 min at 22°C. Cell pellets were suspended in 0.85% (w/w) sterile saline and diluted with saline. Individual slices of ham were inoculated with saline (100 μL), a cocktail of autochthonous bacteria (4.0 log CFU/slice), a cocktail of *L. monocytogenes* (3.0 log CFU/slice) or both the autochthonous cocktail and the cocktail of *L. monocytogenes*. Inocula were spread on the surface of the ham with a spreader. Ham slices were placed individually in 23.5 cm x 16.5 cm plastic vacuum packs [Oxygen transmission rate: 52 cc/m^2 (24 h/dry @ 23°C); Allied Pak Inc. Scarborough, ON, Canada] and vacuum packaged (Multivac C200; Pemberton and Associates, ON, Canada) and stored at 4°C.

3.2.4. Water activity and pH measurement

The water activity of the hams was measured using an AquaLab CX-2 water activity meter (Decagon Devices Inc., WA, USA) following manufacturer's instructions. The pH values of inoculated ham slices were measured at each storage time using an Accumet® surface pH electrode (Fisher Scientific, Canada).

3.2.5. Sampling

After 1, 4, 7, 15, 22, 28 and 36 days of storage, sterile 0.85% saline was added to each package and the packaged was stomached for 2 min. Serial dilutions were prepared in saline and samples were plated onto APT agar or PALCAM (Oxoid Ltd, ON, Canada) agar containing selective supplement (Oxoid). Cell counts were determined after 48 h incubation of APT plates at 22°C and 24 h incubation of PALCAM agar at 37°C.

3.2.6. Determination of the length of lag-phase

The logistic function (Zwietering et al., 1990) was used to model the lag time of *L. monocytogenes* on ham formulated with different concentrations of NaCl.

3.2.7. Statistical analysis

All experiments were replicated three times including production of ham. Analysis of variance and Tukey's multiple range test were used to determine significant main or interaction effects ($P < 0.05$) [IBM SPSS Statistics Version 21 (IBM Canada Ltd. ON, Canada)].

3.3. Results and Discussion

To investigate the effect of the concentration of sodium chloride on the growth of *L. monocytogenes*, hams with different concentrations of total sodium chloride were manufactured, and inoculated with cocktail of *L. monocytogenes*. No bacteria were detected on the control samples throughout the experiment. Counts of *L. monocytogenes* were determined (Fig. 3.1) and based on the logistic

model, the length of the lag phase of *L. monocytogenes* on samples formulated with 0.85, 1.10 and 1.35% NaCl was 4±0, 7±2 and 14±1 days, respectively. All of the ham products in this study were classified as sodium-reduced based on the definition by Health Canada (regular ham products have 2-3% NaCl in the formulation). There was no significant difference in the a_w of the hams formulated with various concentrations of NaCl. The a_w of all samples was between 0.982 and 0.985, which was contradictory to what is suggested in the literature that low sodium meats could have a higher water activity that can increase the growth of microorganisms (Troller, 1986). The pH values of all ham products dropped from 6.50 to 5.50 at the end of storage. The reduction of sodium chloride in hams reduces the concentration of Cl^- ions in the meat. Chloride ions inhibit the growth of *L. monocytogenes* on RTE meats such as cooked ham (Samapundo et al., 2013). The difference in the length of the lag phase could be a result of differences in Cl^- concentration.

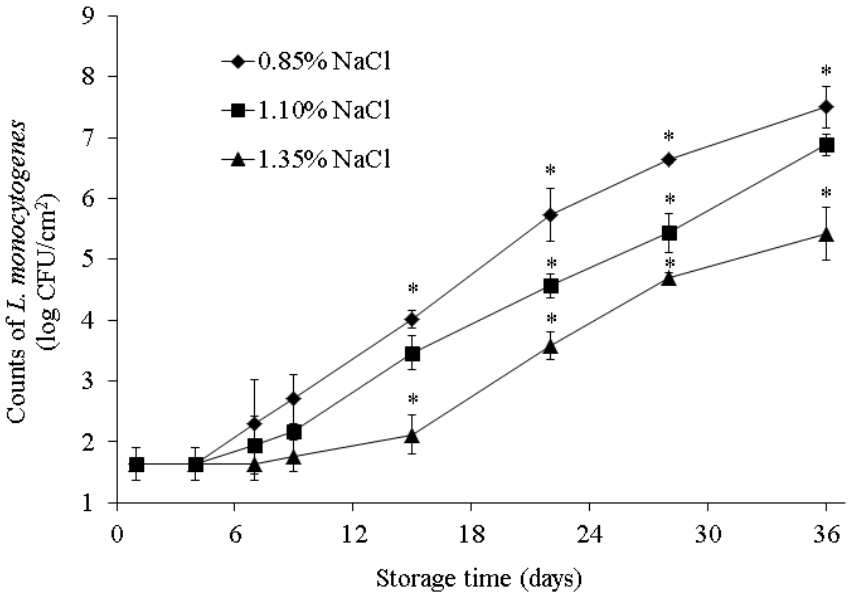

Figure 3.1 Mean log counts of *L. monocytogenes* on vacuum packaged ham formulated with 0.85, 1.10 or 1.35% NaCl and stored at 4ºC. (Mean ± SD, n=3). * indicates significant difference ($P<0.05$) among treatments at a given time.

The concentration of NaCl did not affect the maximum population of the autochthonous microbiota (Fig. 3.2). The count of autochthonous microbiota during log-phase growth rate, however, was significantly higher when growing on ham with the lowest concentration of NaCl.

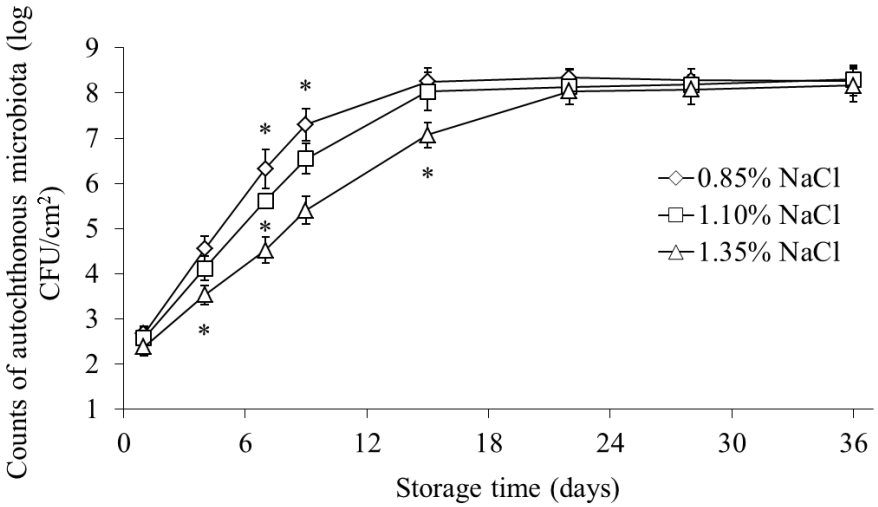

Figure 3.2 Mean log counts of autochthonous microbiota on vacuum packaged ham formulated with 0.85, 1.10 or 1.35% NaCl and stored at 4ºC. (Mean ± SD, n=3). * indicates significant difference ($P<0.05$) among treatments at a given time.

The effect of autochthonous microbiota on the growth of *L. monocytogenes* on sodium-reduced ham is shown in Fig. 3.3. The length of the lag phase, determined with the logistic function was 6±5, 7±3, 7±2 days on ham with 0.85, 1.1 and 1.35% NaCl, respectively. The length of the lag phase for *L. monocytogenes* was not influenced by the sodium chloride levels in the ham when the autochthonous microbiota was present (Fig. 3.1). At the end of the 36 days of storage, samples in which the authochthonous microbiota was present had a lower maximum population of *L. monocytogenes* (Fig. 3.3) compared to when the autochthonous microbiota was absent (Fig. 3.1). None of the strains used in the autochthonous microbiota cocktail exhibited antimicrobial effect against each other or produced bacteriocins active against *L. monocytogenes* (data not shown). However,

previous research suggests that autochthonous microorganisms could compete for nutrients and affect the growth of *L. monocytogenes* (Dallas et al., 1991). Metabolites other than antimicrobial peptides, such as lactic, formic and acetic acids, could also play a role in the inhibition of *L. monocytogenes*.

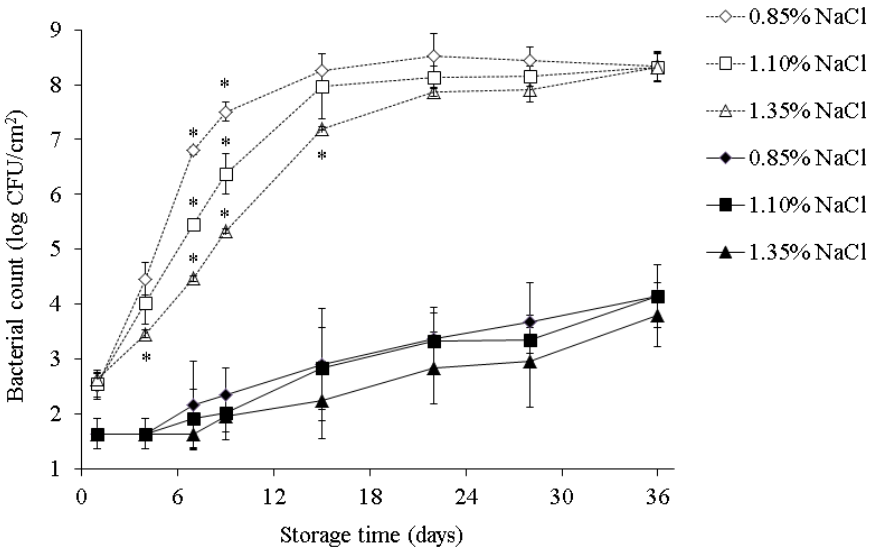

Figure 3.3 Mean log counts of total cells (dotted lines) and *L. monocytogenes* (solid lines) on vacuum packaged ham formulated with 0.85, 1.10 or 1.35% NaCl and stored at 4ºC. (Mean ± SD, n=3). * indicates significant difference ($P<0.05$) among treatments at a given time.

3.4. Conclusions

The growth of *L. monocytogenes* was influenced by both NaCl and autochthonous microbiota. The length of lag-phase of *L. monocytogenes* was shorter on ham with 0.85% NaCl than that with 1.10 or 1.35% NaCl. The presence of an autochthonous microbiota minimized the impact of lower NaCl

concentrations. On ham products with the lowest NaCl (0.85%), the maximum population of *L. monocytogenes* was reduced > 3 log CFU/cm^2 compared to when the autochthonous microbiota was absent.

The NaCl concentrations of ham used in this study (0.85, 1.10 and 1.35%) were close to the specifications for sodium reduction in RTE meats (Health Canada, 2010). This experiment was the first research that has carefully evaluated the growth of *L. monocytogenes* in RTE meat products. Prior to this study, it was anticipated that these formulations would impact the ability of *L. monocytogenes* to grow on these products but this has not been carefully documented. This study will provide guidance to improve the microbial safety of sodium-reduced RTE meat products.

This study showed that autochthonous microbiota had a significant effect on the growth of *L. monocytogenes*. The use of protective bacteria with antimicrobial properties could potentially extend the shelf life and/or improve a safety of sodium-reduced RTE meat products.

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4. Effects of Sodium Lactate and Sodium Diacetate on the Growth and Filamentation of *L. monocytogenes* 08-5923 *in situ*

4.1. Introduction

Traditionally, sodium serves as a preservative in meat products. A reduction in sodium may lead to an increase in the growth of food spoilage and pathogenic bacteria (Doyle & Glass, 2010). For sodium-reduced RTE vacuum-packaged processed meats, the concern is the potential growth of *Listeria monocytogenes* (Häse et al., 2001). In Canada, an average of 0.37 cases of listeriosis per 100,000 population is reported annually (Public Health Agency of Canada, 2012).

Preservatives, including sodium lactate and sodium diacetate, have been used to control the growth of pathogenic bacteria in RTE meats (Health Canada, 2013). In products with greater than 1% sodium, the presence of sodium lactate and sodium diacetate control the growth of *L. monocytogenes* in vacuum-packaged frankfurters (Bedie et al., 2001), vacuum-packaged turkey Bologna (Wederquist et al., 1995), vacuum-packaged wieners, and cooked bratwurst (Glass et al., 2002). No studies have reported the effectiveness of these antimicrobials to control the growth of *L. monocytogenes* on low sodium RTE meats.

Growth of pathogenic bacteria is also influenced by the presence of the autochthonous microbiota, such as lactic acid bacteria (LAB). Previous studies suggest that the growth of pathogens such as *L. monocytogenes* (Mattila-Sandholm & Skyttä, 1991; Upton 1995), *Staphylococcus aureus*, *Salmonella*

enterica serovar Enteritidis and *Yersinia enterocolitica* (Nielsen & Zeuthen, 1985) was inhibited by the presence of LAB. The combined effect of an autochthonous microbiota and reduction of sodium chloride on the growth of *L. monocytogenes* has not yet been reported.

The purpose of this study was to examine the effect of different concentrations of total sodium, sodium lactate and sodium diacetate on the growth of *L. monocytogenes* on ham. The strain of *L. monocytogenes* was involved in an outbreak of listeriosis in Canada in 2008 (Gilmour et al., 2010).

4.2. Materials and Methods

4.2.1. Bacterial strains and cultures

L. monocytogenes 08-5923 was cultured from frozen stocks in Tryptic Soy Broth (TSB, BD-Canada) and incubated overnight at 37°C. Prior to use in experiments, *L. monocytogenes* was grown overnight in appropriate media at 22°C. PALCAM (Oxoid Ltd, ON, Canada) agar containing selective supplement (Oxoid) was used for enumeration of *Listeria*.

4.2.2. Ham production

Chopped-and-formed ham with 0.6 and 1% total sodium, with/without 1.4% (w/w) sodium lactate and 0.1% (w/w) sodium diacetate (NaL/DA) were manufactured according to the procedures outlined in Chapter 3 of this thesis. Each ham slice was 3 mm thick and had a surface area of 15 cm^2.

4.2.3. Inoculation and packaging

An overnight culture of *L. monocytogenes* 08-5923 was centrifuged at 4,000 *x g* for 10 min at 22°C. Cell pellets were suspended in 0.85% (w/w) sterile saline and diluted with saline. Individual slices of ham were inoculated with saline (50 µL) or *L. monocytogenes* (5.0 log CFU/slice). Inocula were spread on the surface of the ham with a spreader. Ham slices were placed individually in 23.5 cm x 16.5 cm plastic vacuum packs [Oxygen transmission rate: 52 cc/m^2 (24 h/dry @ 23°C); Allied Pak Inc. Scarborough, ON, Canada] and vacuum packaged (Multivac C200; Pemberton and Associates, ON, Canada) and stored at 4°C.

4.2.4. Cell counts

Sterile saline was added to each package in an appropriate volume to make serial dilutions of the cells of *L. monocytogenes* in the package and the mixture was massaged vigorously by hand for 1 min. Serial dilutions were prepared and samples were plated onto APT or PALCAM agars. Cells were enumerated on APT agar after 48 h incubation at 22°C and on PALCAM agar containing selective supplement after 24 h incubation at 37°C.

4.2.5. Determination of the length of the lag-phase

The logistic function (Zwietering et al., 1990) was used to model the lag time of *L. monocytogenes* on ham formulated with different concentrations of total sodium in the presence and absence of NaL/DA.

4.2.6. Cell morphology

Ten mL of 1% (v/v) formaldehyde was added to packages of inoculated ham slices to fix (maintain the morphology) *L. monocytogenes* prior to microscopy analysis. Cells were examined under a Zeiss microscope with Axiocam MRm (CarlZeiss Inc., Oberkochen, Germany) at 1,000 *x* magnification.

4.2.7. Water activity and pH measurement

The water activity of the hams was measured using an AquaLab CX-2 water activity meter (Decagon Devices Inc., WA, USA) following manufacturer's instructions. The pH values of inoculated ham slices were measured at each storage time using an Accumet® surface pH electrode (Fisher Scientific, Canada).

4.2.8. Statistical analysis

All experiments were replicated three times including production of ham. Analysis of variance and Tukey's multiple range test were used to determine significant main or interaction effects ($P < 0.05$) [IBM SPSS Statistics Version 21 (IBM Canada Ltd. ON, Canada)].

4.3. Results and Discussion

4.3.1. Effect of NaL/DA on the growth of *L. monocytogenes* on ham formulated with different sodium concentrations

To study the effect of NaL/DA on the growth of *L. monocytogenes*, *L. monocytogenes* was inoculated onto ham products manufactured with different

sodium concentrations with and without NaL/DA. The length of the lag phase of *L. monocytogenes* on ham with 0.6% total sodium was 11±2 without NaL/DA and 25±2 d with NaL/DA. The length of the lag phase of *L. monocytogenes* on ham with 1% total sodium was 26±3 without NaL/DA and 32±4 d with NaL/DA. The addition of NaL/DA in low salt meats used in our study significantly ($P<0.05$) reduced the maximum population of *L. monocytogenes* by 2 logs by the end of storage (Fig. 4.1). The addition of NaL/DA to low sodium products did not provide the same antimicrobial effect as was observed in higher sodium meats. The presence of NaL/DA in higher salt RTE meats prevented (Glass et al., 2002) or reduced the growth by 5 log CFU/g (Thompson et al., 2008) of *L. monocytogenes*.

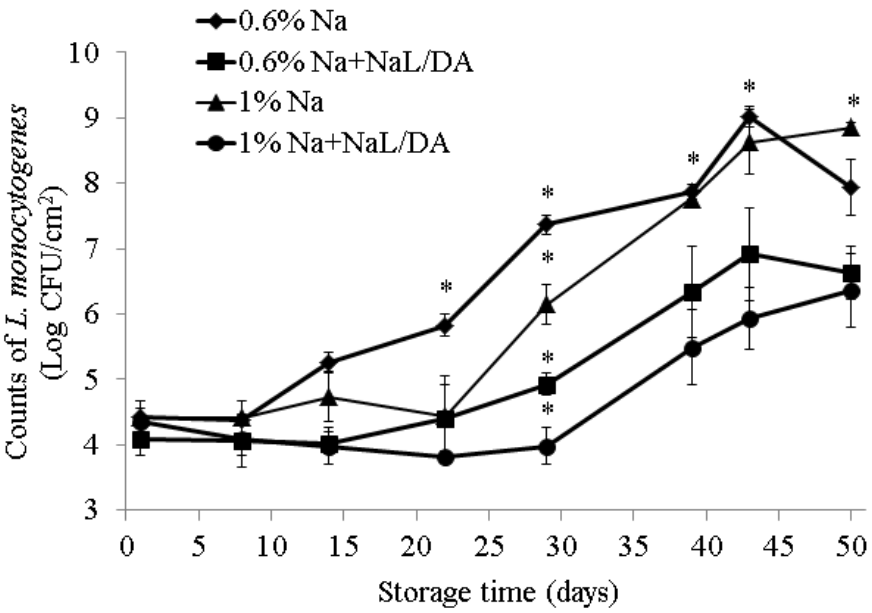


Figure 4.1 Growth of *L. monocytogenes* 08-5923 on ham formulated with 0.6% and 1% total sodium with or without NaL/DA and stored at 4ºC. (Mean ± SD, n=3). * indicates significant difference ($P<0.05$) among treatments at a given time.

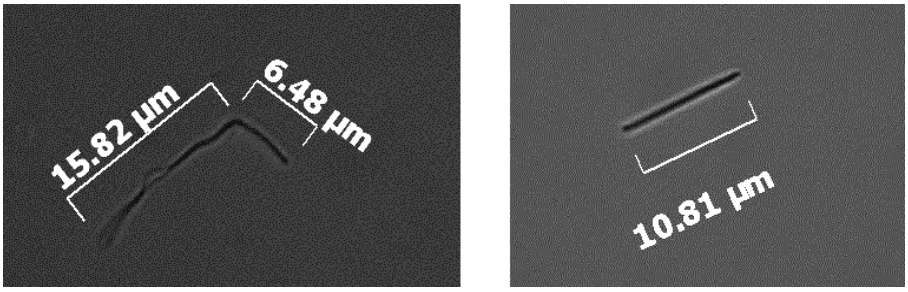
The combination of NaL/DA was less effective in controlling the growth of *L. monocytogenes* in sodium-reduced RTE meats than regular RTE meats. Previous studies on higher salt RTE meats have shown that ≥ 1% sodium lactate plus ≥ 0.1% diacetate prevent growth of *L. monocytogenes* for 8 weeks on wieners stored at 4.5°C (Glass et al., 2002). To better control the growth of *L. monocytogenes* in sodium-reduced RTE meats, the combined use of sodium lactate and diacetate with other antimicrobials such as bacteriocins (Carlson et al., 2007), or with smoke or high hydrostatic pressure processing (Toepfl et al., 2006) may be necessary.

The surface pH of all inoculated ham products dropped from 6.5 to 5-5.5 as a result of growth of *L. monocytogenes*. The decrease in pH may result from the production of lactic acid from metabolism of *L. monocytogenes* under anaerobic conditions (Romick et al., 1996).When pH decreases, more lactate and acetate will be present in a protonated form. Previous studies suggest that the undissociated form of lactate and acetate inhibit the growth of *Listeria* spp. (Ostling and Lindgren, 1993; Janssen et al., 2007). Thus, the pH decrease in ham may lead to the decrease in the effectiveness of NaL/DA to inhibit *L. monocytogenes*.

The a_w of all ham products was the same (0.97).

4.3.2. Cell morphology

This study was the first isolation of filaments of *L. monocytogenes* from meat products. Filaments of *L. monocytogenes* 08-5923 were observed on ham both with and without sodium lactate-diacetate (Figure 4.2). It could be the cold storage temperature (4°C) that resulted in filamentation of *L. monocytogenes*. Previous research (Vail, 2012) showed that *L. monocytogenes* could form filaments at cold temperature *in vitro*.



(a) (b)

Figure 4.2 Filaments of *L. monocytogenes* 08-5923 under light microscope (1,000 *x* magnification). (a) Isolated after 7 weeks of storage from ham formulated with 0.6% sodium without preservatives ; (b) Isolated after 6 weeks of storage from ham formulated with 0.6% sodium and NaL/DA.

4.4. Conclusions

This study examined the effect of NaL/DA on the growth of *L. monocytogenes* in sodium-reduced ham products. The combination of sodium chloride and NaL/DA resulted in an increase in the length of lag phase of *L. monocytogenes* 08-5923 by 3 weeks, compared to ham with less sodium (0.6% total) and without NaL/DA. Besides the concentration of sodium and the addition of NaL/DA, the effect of a_w or pH on the growth of *L. monocytogenes* 08-5923 was not found.

However, the concentrations of NaL/DA in this experiment, which was currently recommended by industry on regular sodium RTE meats, did not provide the same control of the growth of *L. monocytogenes* in sodium-reduced

ham products as is reported in the literature for RTE meats with higher concentrations of sodium. Therefore, other measures, such as increasing the concentrations of NaL/DA, or combining the use of antimicrobials, need to be taken by the industry to prevent the growth of *L. monocytogenes* on low sodium RTE products.

This is the first report demonstrating that filamentation of *L. monocytogenes* can occur on a meat product (*in situ*). Filaments of *L. monocytogenes* were isolated from sodium-reduced ham products (0.6% total Na) with and without NaL/DA. Filamentation of *L. monocytogenes* could be a result of refrigeration temperature (4°C). Discovery of filaments of *L. monocytogenes* on meat products led to the in-depth study of the stress and filamentation of *L. monocytogenes* in Chapter 7.

4.5. References

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5.* Cellular Response of *Listeria monocytogenes* 08-5923 to a Sublethal Dose of Carnocyclin A

5.1. Introduction

Listeria monocytogenes has been responsible for numerous foodborne illness outbreaks as a result of consumption of contaminated ready-to-eat meat (RTE) products (Hof, 2003). A listeriosis outbreak from a single manufacturer in Canada in 2008 resulted in 22 deaths and 57 confirmed positive cases (Public Health Agency of Canada, 2009).

To control the growth of *L. monocytogenes*, bacteriocin-containing antimicrobials such as Micocin® (CanBiocin Inc.) can be used in RTE meat products. One active compound in Micocin®, carnocyclin A (CCLA), is a 5.9 kDa cyclic, class IIc bacteriocin (Martin-Visscher et al., 2008) that has strong antilisterial activity. CCLA forms ion channels in the membrane and results in dissipation of membrane potential, which leads to cell death (Gong et al., 2009). Neither the stress response of *Listeria* to CCLA nor the development of resistance in *Listeria* has been elucidated. Previous research focuses on class IIa bacteriocins, which cause cell surface alterations in *Listeria* (Vadyvaloo et al., 2004) and in particular, result in cell lysis by targeting the mannose-specific

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* A version of this chapter entitled "Stress and adaptation of *Listeria monocytogenes* 08-5923 exposed to a sublethal dose of carnocyclin A" by Xiaoji Liu, Urmila Basu, Petr Miller and Lynn M. McMullen has been published by Applied Environmental Microbiology. 80:3835–3841.

phosphotransferase (PTS) system (Tessema et al., 2009; Kjos et al., 2011). The disruption of the mannose-specific PTS system plays a role in bacteriocin resistance (Diep et al., 2007; Gravesen et al., 2002). Alternative sigma factors, such as σ^B (sigB; Oliver et al., 2010; Sue et al., 2004; Wemekamp-Kamphuis et al., 2004) and σ^{54} (rpoN; Robichon et al., 1997), are also involved in resistance to class IIa bacteriocin. These alternative sigma factors are also involved in other environmental stresses such as cold-shock, acid and osmotolerance in *Listeria* (Ferreira et al., 2001; Okada et al., 2006).

It is postulated that CCLA can trigger alteration in the expression levels of genes that are also regulated by σ^B/σ^{54}. The differential expression of genes, proteins and morphology of *L. monocytogenes* 08-5923, one of the two strains that were involved in the 2008 listeriosis outbreak in Canada (genomic sequence NC_013768; Gilmour et al., 2010), in the presence and absence of CCLA were compared.

5.2. Materials and Methods

5.2.1. Bacterial strains and cultures

L. monocytogenes 08-5923 was grown in Tryptic Soy Broth (TSB; Becton Dickinson Canada, ON) at 21°C for 24 h prior to use. *Carnobacterium maltaromaticum* ATCC PTA-5313 (formerly *Carnobacterium maltaromaticum* UAL307) was grown in All-Purpose-Tween (APT; BD-Canada) broth 21°C.

5.2.2. CCLA isolation and purification

CCLA was isolated from an overnight culture of 1 L *C. maltaromaticum* ATCC PTA-5313 grown at 21°C and purified according to previous methods (Martin-Visscher et al., 2008). Cell-free supernatant was concentrated using Sep-Pak® C18 cartridges (Waters Corporation, MA) pre-washed with 2-propanol containing 0.1% trifluoroacetic acid (TFA, Sigma-Aldrich, Oakville, ON), conditioned with 0.1% TFA in H_2O and eluted in 40% to 70% 2-propanol in H_2O with 0.1% TFA. The 2-propanol was removed by RotaVAP (Büchi Corp., DE) at 35ºC. Active peptides were separated by reverse-phase HPLC using a C8 column (5-m particle size, 4.6-250-mm Vydac 208TP54) and a linear gradient of 20% to 50% aqueous IPA containing 0.1% (TFA over 50 min at a flow rate of 1 ml/min and detection at 220 nm. CCLA had retention time of 45.2 to 47.9 min.

5.2.3. RNA isolation

Twenty-five ml of log-phase *L. monocytogenes* 08-5923 [optical density (OD) of 0.2 at 600 nm] was grown in TSB or in TSB with 7 μg/ml CCLA (1/10 of minimal inhibitory concentration for *L. monocytogenes* 08-5923) for 4 h at 21°C (final OD_{600nm} = 0.5) to obtain cells that were at the mid-log phase of growth. RNAprotect® Bacteria Reagent (Qiagen Inc., ON, Canada) was added to the cell culture according to the manufacturer's instructions. Total RNA was isolated using the RNeasy mini kit (Qiagen Inc.) and treated with DNase I (New England Biolabs Ltd., ON, Canada) on-column according to the manufacturers' protocol. RNA quantity was measured using a Nanodrop 1000 Spectrophotometer (Thermo

Fisher Scientific, ON, Canada), and the samples were stored at -80^{o}C. RNA quality and quantity were assessed on the Agilent 2100 bioanalyzer (G2938C, Agilent Technologies Inc., ON, Canada) using Agilent Nano 6000 Assay and the RNA with integrity number (RIN) > 7.0 was used for subsequent microarray experiments. For quantitative reverse transcriptase real time PCR (qRT-PCR), total RNA was isolated as above from cells in lag phase (15 min), mid-log phase (4 h) and early stationary phase (30 h).

5.2.4. Gene expression microarrays and data analysis

The *L. monocytogenes* EGD-e 385 K gene expression microarray from Roche NimbleGen including all the 2857 annotated open reading frames (ORFs) of the genome, was used. The cDNA was synthesized from RNA extracted from three independent biological repetitions (SOP#M007, SOP#M008; J. Craig Venter™ Institute [http://pfgrc.jcvi.org/index.php/microarray/protocols]) and labeled with Cy3 Mono-Reactive Dye (GE Healthcare Life Sciences, ON, Canada). Hybridization of the labeled cDNA probes using the NimbleGen hybridization station and washing were carried out according to the NimbleGen protocol. Microarray raw data was pre-processed and normalized by NimbleScan (version 2.6; Roche NimbleGen [www.nimblegen.com]).

GeneSifter® software (trial version; Geospiza, Inc. [www.genesifter.net]; Reimers, 2010) was used for the analysis of normalized results. The statistical analyses were the average of three independent samples (CCLA-treated *L. monocytogenes* 08-5923 vs. untreated) with a cut off of twofold change in

expression level and P values ($P<0.05$) to determine differentially expressed genes. Genes that showed 2-fold or greater change in expression levels were selected for subsequent qRT-PCR.

5.2.5. Total cytosoluble protein identification

L. monocytogenes 08-5923 was grown in TSB or in TSB with CCLA at room temperature for 4 h. Cells were pelleted by centrifugation at 10,600 x g for 1 min and washed once with 1 x SigmaFAST™ protease inhibitor cocktail tablet (Sigma-Aldrich Ltd., ON, Canada) reconstituted in ddH_2O. Cell pellets were re-suspended in the fresh cocktail, mixed 1:1 (v:v) with 0.1 mm Zirconia-silica beads (BioSpec Inc., OK) and lysed by bead beating for 45 s a total of 3 times. The cell envelope was removed by centrifugation at 10,600 x g for 2 min. Concentration of the total soluble protein was determined by Bio-Rad protein assay following the manufacturer's instructions (Bio-Rad Laboratories Ltd., ON, Canada).

The total soluble proteins were separated by SDS-PAGE with 16% polyacrylamide (Schägger and von Jagow, 1987). Five µg of proteins from the untreated and treated *Listeria* were loaded into each lane and subjected to electrophoresis at 130 V for 80 min. The gel was fixed (45 min in a solution containing 50% methanol and 10% acetic acid) and protein bands were visualized by EZBlue™ gel staining (Sigma-Aldrich Ltd.). The density of protein bands was measured by AlphaEase FluorChem™ densitometry (Alpha Innotech; Fisher Scientific) to identified differentially expressed proteins.

The differentially expressed proteins in *L. monocytogenes* 08-5923 grown with or without CCLA were analyzed by the Mass Spectrometry Facility, Department of Chemistry, University of Alberta. Protein bands that were visibly differentially expressed were excised from SDS-PAGE gel, in-gel digested by trypsin (Sigma-Aldrich Ltd.) and analyzed by Ultra-performance liquid chromatography coupled to a quadrupole time-of-flight mass spectrometer (UPLC-ESI-Q-TOF-MS) (Q-TOF Premier; Waters, MA). Proteins were identified from mass spectrometry data using Mascot search engine (http://www.matrixscience.com/search_form_select.html).

5.2.6. qRT-PCR and data analysis

The qRT-PCR experiments were performed to validate the differential expression of genes at different times of exposure to CCLA. The experimental design included three biological replicates including the control (cells grown in TSB) and treated (TSB with 7 µg/ml CCLA); and two technical replicates within each biological replicate. Genes that were ≥ 2-fold up- or down-regulated ($p<0.05$) from the microarray experiments, as well as genes encoding the differentially expressed proteins were quantified using SYBR Green (Invitrogen, ON, Canada). Primers (Table 5.1 and 5.2) were designed (Primer3 [http://frodo.wi.mit.edu/primer3/]) and tested for specificity with genomic DNA prior to the analysis. Total RNA was isolated and reverse-transcribed into cDNA using reverse transcriptase SuperScript® III (Promega, WI) according to the manufacturer's protocol. Quantification of the transcripts were carried out (7500

Fast Real-Time PCR system, Applied Biosystems, CA) using QuantiFast® SYBR® Green PCR Kit (Qiagen Inc.). Relative quantification values were obtained using a comparative threshold cycle method ($\Delta\Delta C_T$; 0 and Schmittgen, 2001). Housekeeping gene *rpoB* was selected as the reference. The C_T slope method was used to validate the internal control (*rpoB*). The *rpoB* was amplified from 10-fold serial dilutions of genomic DNA prepared from *L. monocytogenes*. The C_T slope was linear and the qRT-PCR efficiency of *rpoB* was 100.9%. One-tail unpaired *t*-test was performed for determination of the significance ($P<0.05$) in differential expression of genes.

Table 5.1 Primers used in qRT-PCR for confirmation of the genes that were found differentially expressed in microarray experiment.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
ftsE	TATGCGATGGAAGTGGTTGA	CTCACCACCGGAAAGTTCAT
ftsX	AATGGTTGGATGACCTTTGC	CGTTGCAAGCTTGTTCATGT
rpoB	GAAATCTGGGTTCGTCGTGT	GCTACGTTTGGACGTTGGTT
fliF	TCTACATGAACACGCCCAAA	AAAATGTTGCCGCTTTTGTC
clpE	AGCAAACTTTGGGTCGAATG	GTTCACGGTTTGCTTGGTTT
baiE	ACAAGCAAAGGCAGACATCC	CAATAAAGCCATGCCCAGTT
deoR	CAGGCCTTGAGCAAAATGAT	ATAATACGCGCGATTGGAAG
hisC	TGCGAAACGTTTTGAACAAG	CAGCACCAGAACGCGTAATA
flgD	AAATGGCGCAACTTTCCTTA	CGCCGTTTAGTGAAACACCT
yitT	TGGATTTTCCAGCGAATACC	ATCACTACCGCCAGTTGTCC
tgl	GACAAGCCGAGTTTCAAAGC	CGGCATCTGTGACAGTTTGT
lmo0867	CAATCGGCTATCTCGCTTTT	TGCGATAATTGCCATCGTTA
ywzB	ACTTTTTGGGCACTTCAAGC	CCTAACACAATCGAAATAATCACAA
lmo1796	CACCCGGAAGACGTTGTTAT	ACGAATCGCTTTCGCAATAC
ftsW	GGGATCGCTAGTCTGATTGC	TACCAAGCATCATCGACAGC
lmo2311	GCTCAATGCGTTTATGACGA	CGCTGGCAAATAATCTCGTT
trn	CCGAAAAGAGTCGCGATAAT	TGCTGCATAGGTGGATGCTA
hrcA	GGGATATTCCCGATGGTCTT	TCTTGTGGGCCCTAGGAGTA
abc	AATGAGAGCGCCAATTATGG	TGCGCGATACCTTCTACAAA
fmt	AGAAGCCGACCTGCTTGTAA	GTCAAGCAGCGCGTAGTGTA
lmo2317	CCCTTTTCAACCGTGATGTT	GCTCACGAACCCTTTCCATA
mfs	GGGGAAAAAGCTGGAATTGT	TTGCAATCGTTGGCATGTAT
malP	CAATTGGATGGAGCGAAAAT	CGCCATTACGGTGAATTTCT
mptC	ATTCCAGCTGCAGCACTTTT	CAACGGCAACTACCATTCCT
mbl	GCAATTCGTGAAGTTGCTGA	ACCGGAAGGCTCAAAAATCT
gidA	GGCGTGTGTAAAGGCGTTAT	GGTTATTTGGACCGCTTGAA
rodA	CTTCCGCTTGGTCTTGTAGC	CAATGAGCGCAATCGAACTA
divIVA	TCCGTGAGCGTTTACGTATG	CGCAAGTTCTGTCGCATCTA
murZ	GCGATTTATTTACGGGCAGA	TTGCACGAACAGCTGCTAAC
fliM	GTGTGACATCACCGAACGAC	TCTTCCACCGAGAAAAATGG
motB	TTCATGGAAGCGATCATTCA	CGCACCATGATTTCTACACG
mptAB	ACGTTTCGGCAATACCAAAG	ACAGAGTGAGCCATGGAACC
murE	TTATGGATCTCCGACCCAAG	ACGATACATCGTGCCAATCA
pdp	AGTGAAAATCGCACCGAAAG	GCAATAATTCCGAGGCATGT
agrB	CTCCGGCAGACACAGAAAGT	TGCGAATGGTATTAGCAACG
bglK	CCGAAAAATGGCTTGGTAAA	CCGCGAACTAATTCACCATT
cheA	CGACGTTCCGAATTGAGATT	CGTGAACGTGTTGAATGTCC
celC	TTGCAGCTATTCGTGACACC	CATACCAGATCCGCCGTAGT
dacB2	ACCAAAAAGCGAGAGAAGCA	GGCATTTTCTTGCAACCATT
celA	ATTTCGAGGAAGCAGAAGCA	TTCCACTTTTTCACCGGAAG
celB	TGGATATCGTGATGCTTGGA	CGGCTTTCCCGTTTAACATA

Table 5.2 Primers used in qRT-PCR for confirmation of the down-regulation of proteins.

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
alaS	GGTAGCTGCTGGAAACGAAG	TTTCGCTCTCGATTGTTCCT
adhE	CAAGCTGGCTTCAAAGTTCC	ACGATCGAATGCTTCTGCTT
pflA	CCCACTTAACCCAGAAGCAG	TACCACCGTTGATAGCACCA
pnpA	TATTCACTCGTGGCCAAACA	GCCAGTTTCCCCAACACTAA
pykA	AAGTGCTGCAGTTGTTGTGG	TTGCATCTTTAGCACCAACG
gnd	GGAAGAGAATGCGGACAAAA	ATCTGTAGCATCGCCAGCTT
fri	GGCGAACAAATGGATGAAGT	GCTTCTTCTACGCTCGCATT
rpsK	TACTCGTAAACGCCGTGTGA	TAGGGAACCTGCACTTGACC
rpsH	AGTAAGCCAGGTTTGCGTGT	TTTAGCACGGGCTTCTTTGT
rplU	TGAAACAGGTGGGAAACAAA	TAGCGGAATCTCCACCTACG
rplL	AATTTGGCGTAACTGCTGCT	ACCAGTGATTTCACGAACCA
hup	GCAGCGAAAGCAGTAGAAGC	GTACGAGGGTTACGGCCTTT
rpsT	TTGATGAAGCAGCTGCAAAC	CGAGCAGCATTGTTTTTGTG
csp	GAAAAAGGCTTCGGTTTCATC	ATTCAACGCTTTGACCTTCG

5.2.7. Cell morphology examination

Cells of *L. monocytogenes* 08-5923 grown with or without CCLA were fixed with 10% (v/v) formaldehyde and pelleted by centrifugation at 11,000 *x g* for 15 sec. The cell pellet was re-suspended in 0.1 M phosphate buffer containing 2.5% glutaraldehyde and 2% paraformaldehyde, stained with 2% sodium phosphotungstate (PTA) and examined using a Transmission electrom microscope (TEM) (Philips Morgagni 268; FEI™, OR) operating at 80 kV.

5.3. Results and Discussion

5.3.1. Functional classification of differentially expressed genes

Global cDNA microarray experiments were performed to compare the expression levels of genes in untreated and treated (CCLA, 4 h) *L. monocytogenes*. Global differential gene expression changes in response to CCLA were determined using high-density NimbleGene microarray for *L. monocytogenes* of 2857 genes. Forty genes were found to be differentially expressed with the threshold of a minimum two-fold difference ($p<0.05$) (Table 5.3). These genes were grouped into several functional categories including cell division, cell wall synthesis, motility, transport and translation (Table 5.3).

Table 5.3 Identification of differentially expressed genes in CCLA-treated (4 h) *L. monocytogenes* 08-5923 represented as fold change (n=3, $p<0.05$). +: up-regulation, -: down-regulation

Gene name	Gene annotation	Product	Function	Fold change
clpE	*lmo0997*	ATP-dependent protease	Cell division	+3.69
ftsW	*lmo1071*	cell division protein FtsW		-2.14
divIVA	*lmo2020*	cell division initiation protein		-2.01
rodA	*lmo2428*	similar to cell division proteins RodA		-2.64
ftsX	*lmo2506*	cell division transport system permease protein		-2.08
ftsE	*lmo2507*	cell division transport system ATP-binding protein		-2.32
mbl	*lmo2525*	rod shape-determining protein MreB		-2.03
pdp	*lmo0835*	peptidoglycan binding protein	Cell wall synthesis	-2.21
murE	*lmo2038*	UDP-N-acetylmuramoylalanyl-D-glutamate-2,6-diaminopimelate ligase		-2.02
murZ	*lmo2552*	UDP-N-acetylglucosamine 1-carboxyvinyltransferase		-2.41
dacB2	*lmo1855*	D-alanyl-D-alanine carboxypeptidase		-2.41
abc	*lmo0767*	ABC transporter, permease protein	Membrane functions	+2.13
mfs	*lmo1250*	Major Facilitator Superfamily		+2.05
ywzB	*lmo2527*	similar to *B. subtilis* YwzB protein		+2.37
mptAB	*lmo0096*	PTS system, mannose-specific IIA & IIB component		+2.14
mptC	*lmo0097*	PTS system, mannose-specific IIC component		-2.29
celA	*lmo2765*	PTS system, cellobiose-specific IIA component		+2.35
celB	*lmo2762*	PTS system, cellobiose-specific IIB component		+2.10
celC	*lmo2763*	PTS system, cellobiose-specific IIC component		+2.81
deoR	*lmo2107*	similar to *DeoR* family transcriptional regulator	Gene regulation	+2.28
hrcA	*lmo1475*	heat-inducible transcription repressor		+2.43
agrB	*lmo0048*	similar to *Staphylococcus* two-component sensor histidine kinase AgrB		+3.18
fmt	*lmo1823*	methionyl-tRNA formyltransferase	Nucleotide & protein synthesis	-2.07
hisC	*lmo1925*	histidinol-phosphate aminotransferase		-2.26
gidA	*lmo2810*	tRNA uridine 5-carboxymethylaminomethyl modification enzyme GidA		-2.61
tgl	*lmo0717*	transglycosylase	Metabolism	-2.01
baiE	*lmo0754*	bile acid 7-alpha dehydratase		-2.74
trn	*lmo1903*	thioredoxin		-2.21
yitT	*lmo1909*	YitT family protein		-2.05
malP	*lmo2121*	maltose phosphorylase		+2.01
bglK	*lmo2764*	beta-glucoside kinase		+2.85
flgD	*lmo0696*	flagella basal body rod modification protein	Motility	-2.70
fliF	*lmo0713*	flagella MS-ring protein		-2.20
motB	*lmo0686*	chemotaxis protein		-2.23
fliM	*lmo0699*	flagella motor switch protein		-2.26
cheA	*lmo0692*	two-component sensor histidine kinase		-2.82
lmo0867	*lmo0867*	hypothetical protein	Unknown	-2.39
lmo1796	*lmo1796*	hypothetical protein		-2.33
lmo2311	*lmo2311*	hypothetical protein		+2.25
lmo2317	*lmo2317*	hypothetical protein		+2.07

5.3.2. Differential expression of total soluble proteins

To gain further insight, the protein expression patterns of whole-cell lysate from *L. monocytogenes* with and without exposure to CCLA (4 h) were compared using SDS-PAGE. Out of 11 protein bands that were visibly different in density (Fig. 5.1) between the lysate from the CCLA-treated and untreated cells there were 14 proteins identified by UPLC-ESI-Q-TOF-MS. The proteins were involved in glycolysis (PykA), pyruvate metabolism (AdhE and PflA), stress-response (Csp protein), DNA replication (Hup), translation (Rps and Rpl proteins) and purine/pyrimidine biosynthesis (PnpA) (Table 5.4). The down-regulation of PykA in treated *L. monocytogenes* confirmed the observed down-regulation of the gene encoding this protein as shown in Figure 5.2 (panel G).

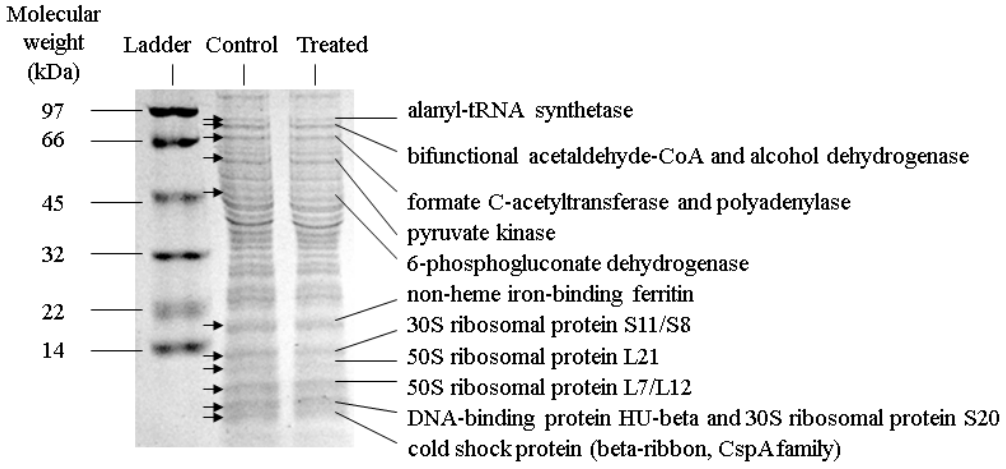

Figure 5.1 Differential production of proteins by *L. monocytogenes* 08-5923; treated with CCLA for 4 h and control shown on a SDS-PAGE gel. Proteins expressed differentially are indicated by arrows.

Table 5.4 Identification of differentially expressed proteins by MS.

Protein name	Product	Calculated pI	Nominal molecular mass (Da)	Differential expression
AlaS	Alanyl-tRNA synthetase	5.13	92938	down
AdhE	Bifunctional acetaldehyde-CoA/alcohol dehydrogenase	6.48	95135	down
PflA	Formate C-acetyltransferase	5.41	83950	down
PnpA	Polyadenylase	5.23	79780	down
PykA	Pyruvate kinase	5.39	62673	down
Gnd	6-phosphogluconate dehydrogenase	5.11	52497	down
Fri	Non-heme iron-binding ferritin	4.86	18036	down
RpsK	30S ribosomal protein S11	11.40	13834	down
RpsH	30S ribosomal protein S8	9.48	14635	down
RplU	50S ribosomal protein L21	9.57	11207	down
RplL	50S ribosomal protein L7/L12	4.54	12462	down
Hup	DNA-binding protein HU-beta	9.65	9876	down
RpsT	30S ribosomal protein S20	10.70	9163	down
Csp	Cold-shock protein CspA family	4.45	7261	down

5.3.3. Confirmation of differentially expressed genes in *L. monocytogenes*

To validate results from microarray experiments and to investigate the gene expression changes over time, a total of 54 genes (40 differentially expressed genes from the microarray experiment and 14 genes encoding proteins differentially expressed identified from SDS-PAGE; Fig. 5.2), were selected for qRT-PCR and gene expression levels were quantified following treatment with CCLA after 15 min (lag phase), 4 h (log phase) and 30 h (early stationary phase). For a number of the genes, expression was significantly ($P<0.05$) up-regulated after 15 min of exposure to CCLA and down-regulated after 4 h and 30 h.

The up-regulation of genes encoding proteins involved in metabolism and membrane functions was observed upon 15 min of exposure to a sublethal dose of

CCLA. Genes encoding proteins involved in metabolism included bile acid 7-alpha dehydratase (BaiE), thioredoxin (Trn) and pyruvate kinase (PykA) were significantly up-regulated ($P<0.05$). The genes involved in cell wall synthesis (*murZ* and *dacB2*) were up-regulated ($P<0.05$). Genes involved in cell division, including *clpE* and *divIVA*, were up-regulated, while *malP* was down-regulated in cells exposed to CCLA for 15 min. After 4 h exposure to CCLA, genes involved in cell division (*clpE*, *ftsW, divIVA*, *rodA*, *ftsX*, and *ftsE*), cell wall synthesis (*mbl, murE, murZ*), flagella synthesis (*flgD, motB, cheA*) and metabolism (*pykA* and *gnd*) were significantly down-regulated. Up-regulation of *lmo2311*, which is regulated by σ^{54} (Arous et al., 2004), was observed. After the cells were exposed to CCLA for 30 h, more genes were down-regulated compared to those after 4 h of exposure. These genes were involved in cell division, cell wall synthesis, membrane function, transcription regulation, metabolism and motility (Fig. 5.2). Both the mannose-specific PTS genes (*mpt* genes) and the cellobiose-specific PTS genes such as *celA*, *celB* and *celC* were down-regulated ($p<0.05$).

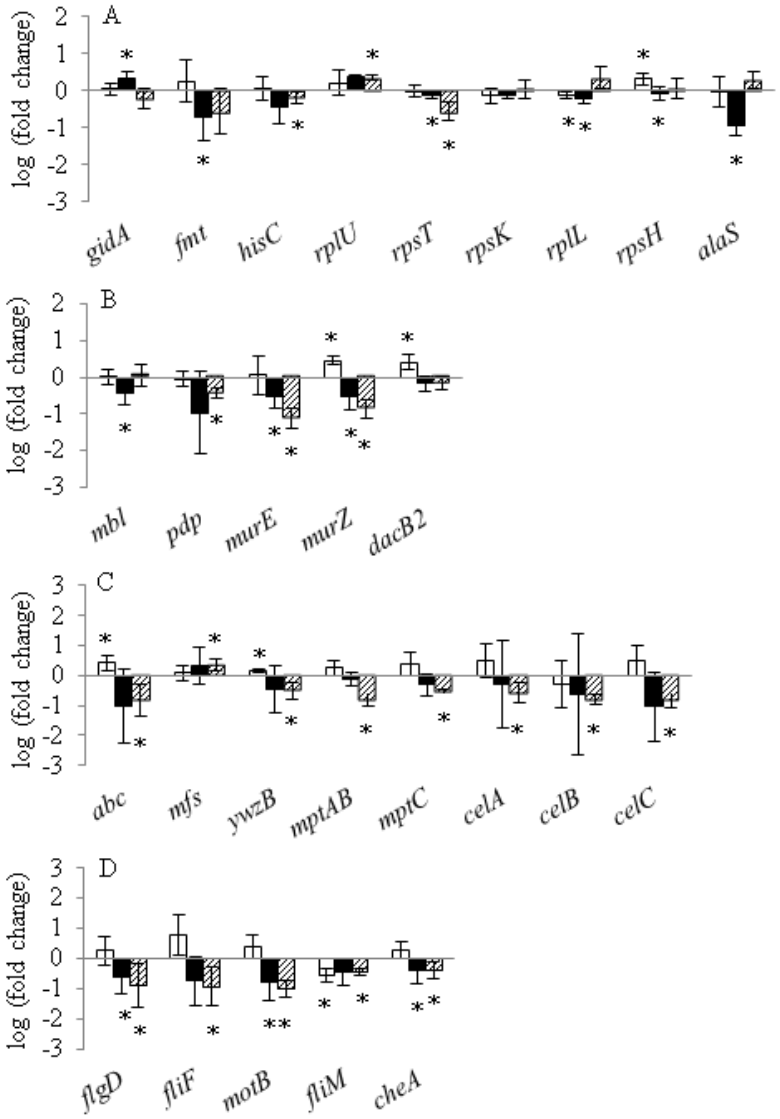

Figure 5.2 Log fold changes of genes in *L. monocytogenes* 08-5923 exposed to CCLA for 15 min (□); 4 h (■); and 30 h (▨). Untreated samples were used as a reference. Genes were grouped according to their biological function. A- nucleotide/protein synthesis; B- cell wall synthesis; C- membrane functions; D- motility; E- transcription regulation; F- cell division; G- metabolism; H- unknown functions. Note different Y axis for C and D. Data expressed as log (fold change). Mean ± SD (n=3). * indicates genes significantly up- or down-regulated at $P<0.05$.

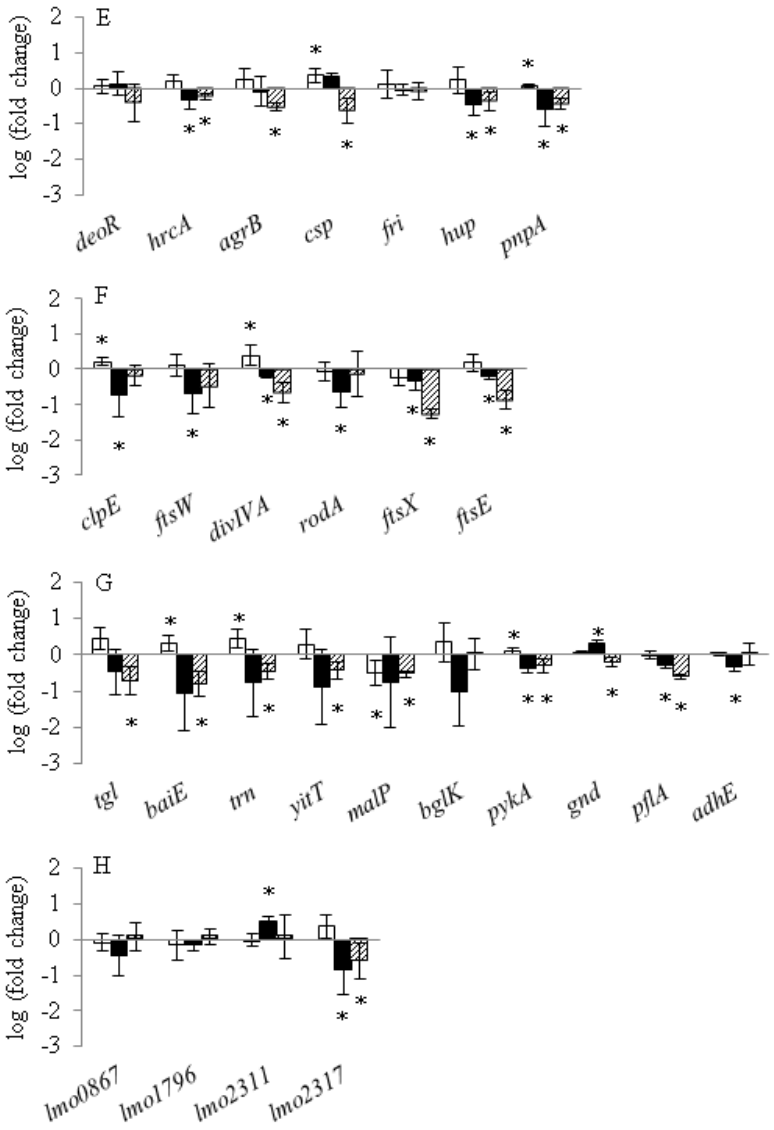

Figure 5.2 continued.

The up-regulation of σ^{54}-regulated *mptAB* and *cel* detected by cDNA microarray after 4 h exposure to CCLA could indicate an immediate adaptive response of *Listeria* cells as the mannose-specific (Diep et al., 2007) and cellobiose-specific PTS systems are receptors for bacteriocins (Campelo et al.,

2011) and the up-regulation of mannose-specific PTS system has been observed in bacteriocin-sensitive cells (Kjos et al., 2009; Ramnath et al., 2004). Gong et al (Gong et al., 2009) demonstrated that CCLA forms ion-selective channels in lipid bilayers, which may indicate that CCLA interaction with membranes is not receptor mediated. However, our microarray results suggested that both the mannose-specific and cellobiose-specific PTS systems may be receptors for CCLA. The down-regulation of the mannose-specific and cellobiose-specific PTS systems observed by qRT-PCR analysis after 30 h exposure to CCLA demonstrates that *Listeria* limits the expression of genes to minimize the production of the PTS targets to CCLA. This down-regulation of the PTS targets could play a role in adaptation to CCLA as these targets have been suggested to play a role in resistance to Class II bacteriocins (Williams et al., 2005; Giotis et al., 2008; Kjos et al., 2011).

The expression of genes involved in cell wall synthesis (*mur*) followed the same pattern as that of the PTS systems after 30 h exposure to CCLA. Although the function of Mur proteins in antimicrobial stress response of *Listeria* has not been described in the literature, Mur is the target of antibiotics in *Staphylococcus aureus* (O'Neill et al., 2009).

Absence of flagella in *Listeria* cells exposed to CCLA for 30 h could be as a result inhibition of flagella synthesis because the basal body of the flagella may not develop properly as genes encoding various components of flagella, including MS-ring (*fliF*), motor switch (*fliM*), motor rotation (*motB*) and rod synthesis

(*flgD*) were down-regulated. In addition, *cheA,* a chemotaxis gene responsible for virulence (0) was down-regulated. Flagellar motility is also important for biofilm formation of *L. monocytogenes* (Lemon et al., 2007). Others have found that stress from antimicrobials can inhibit the synthesis of flagella in *E. coli* (Burt et al., 2007) but the impact of bacteriocins on flagella synthesis in *L. monocytogenes* has not been described in the literature. Lack of flagella can inhibit adhesion of *Listeria* (Tresse et al., 2006) and may impact its ability to form biofilms.

The cell division gene *ftsE* was up-regulated when *L. monocytogenes* was exposed to CCLA for 15 min. High pressure for 15 min also up-regulates *ftsE* in *Listeria* (Bowman et al., 2008). After 4 h exposure to CCLA, *ftsE* was down-regulated, along with *ftsX* and *ftsW*. Gene *ftsE* in *L. monocytogenes* is regulated by σ^B (Shin et al., 2010), which is a general stress response regulator. Various stresses such as liquid smoke (Guilbaud et al., 2008), cold (Mattila et al., 2012), antibiotic (Nielsen et al., 2012; Shin et al., 2010), and heat (van der Veen et al., 2007) also down-regulate *fts* genes in *Listeria*. The up or down-regulation of *fts* genes may depend on the type of stress and time of exposure.

5.3.4. Cell morphology

The morphology of untreated and treated (CCLA, 4 h and 30 h) *L. monocytogenes* 08-5923 was compared using TEM to examine whether exposure to CCLA could result in visible damage to the cell structure. No morphological changes were observed after 4 h treatment of *L. monocytogenes* 08-5923 with CCLA (Fig. 5.3). However, after 30 h the cells had no flagella attached to the cell

surface (Fig. 5.3). This corresponds with down-regulation of motility genes observed previously.

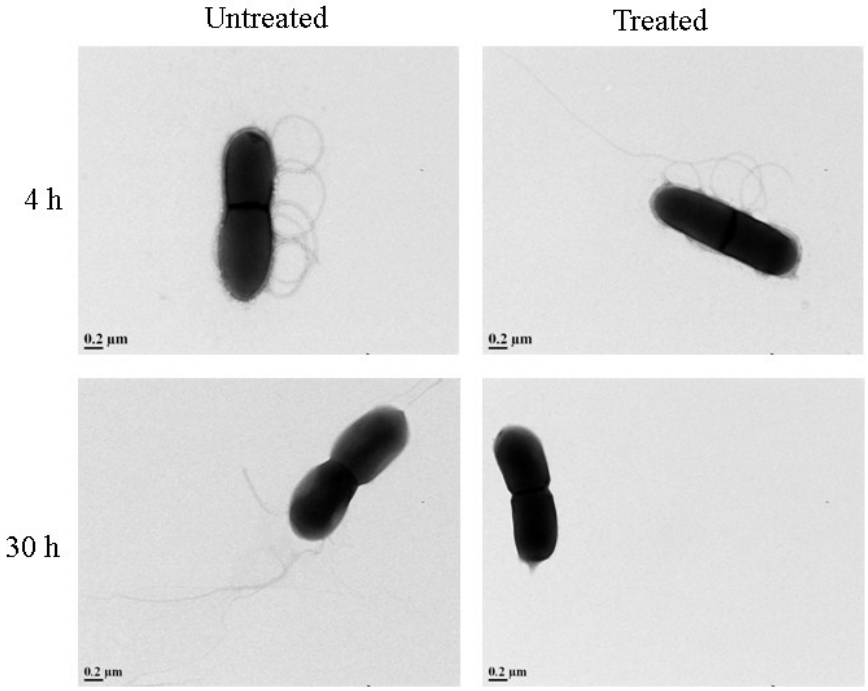

Figure 5.3 TEM of *L. monocytogenes* 08-5923 cells in the presence (Treated, 4 and 30 h) and absence (Untreated) of CCLA (28k x magnification).

5.4. Conclusions

Commercial preservatives that are used to control the growth of *L. monocytogenes* in RTE meats include Micocin®, which contain carnocyclin A, along with two class IIa bacteriocins, carnocyclin BM1 and piscicolin 126. Although the stress responses of *L. monocytogenes* to class IIa bacteriocins have been characterized, the response to class IIc cyclic bacteriocins has not been studied to date. Our study described the cellular alteration and adaptation of *L.*

monocytogenes when exposed to a sublethal dose of a class IIc bacteriocin, which provides insight to the general stress response and resistance mechanisms to bacteriocins.

Treatment of *L. monocytogenes* 08-5923 with CCLA resulted in differential expression of genes that are known to be involved in cell division, cell wall synthesis, membrane function, transcriptional regulation, nucleotide and protein synthesis, metabolism, and cell motility. The PTS function, cell wall synthesis, motility, and cell division genes were affected the most. The down-regulation of these genes was not because of inhibition of cell growth, as at each time point, the OD_{600nm} of treated and untreated cells were comparable (data not shown). No filamentation of cells was observed. This indicates that the down-regulation of these genes was due to the adaptation to CCLA.

In summary, a sublethal dose of carnocyclin A targeted the PTS systems of *Listeria* and cells began to repair injury by up-regulation of genes involved in cell wall synthesis. After 4 h, cells gradually became desensitized to CCLA and started to decrease activities such as cell division, cell wall synthesis and motility. After 30 h, cells adapted to CCLA through a reduction of several metabolic processes and motility functions. Adaptation to bacteriocins could affect the sensitivity to antimicrobials that are used to control the growth of *L. monocytogenes*.

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6. Transcriptomic analysis of *Listeria monocytogenes* 08-5923 in the presence of sodium lactate and sodium diacetate

6.1. Introduction

Sodium lactate and sodium diacetate are commonly used as food preservatives to control the growth of *Listeria monocytogenes*. A combination of 3% sodium lactate and 0.25% sodium diacetate [concentrations permitted by the U.S. Department of Agriculture Food Safety and Inspection Service] effectively control the growth of *L. monocytogenes* in vacuum-packaged frankfurters during refrigeration storage (Bedie et al., 2001). A mixture of sodium lactate and sodium diacetate (NaL/DA) also controls the growth of *L. monocytogenes* on vacuum-packaged wieners and cooked bratwurst (Glass et al., 2002).

At the molecular level, the responses of *L. monocytogenes* to various salts are different. Exposure to NaCl results in differential expression of DnaK, EF-Tu, GbuA and CcpA (Duché et al., 2002; Bae et al., 2012). A mixture of NaL/DA could specifically shut down the expression of DNA-binding protein in *Listeria* (Mbandi et al., 2007). Previous studies suggest that these proteins also cross-protect against alkaline stress (Giotis et al., 2010) and acid stress (Bergholz et al, 2012; Koutsoumanis et al., 2003). The cross-protection is suggested from the regulation of gene expression by RNA polymerase factors (Giotis et al., 2008; Bergholz et al., 2012). To reveal the differential expression of these sigma factors as well as other relevant genes in *L. monocytogenes* under stress, next-generation

RNA sequencing (RNA-seq) was performed to profile the transcriptomic events occurring in *L. monocytogenes* in response to NaL/DA.

This study reports the first transcriptome study using RNA-seq technology to determine the impact of NaL/DA on gene expression in *L. monocytogenes*. Previously RNA-seq has been applied to study the transcriptomes of both prokaryotes and eukaryotes (Mortazavi et al., 2008; Wang et al., 2009; Oszalok and Milos, 2011). In this study, RNA-seq was combined with microscopy and flow cytometry analyses to correlate the gene expression with phenotypic characteristics (elongation and filamentation) of *L. monocytogenes*. *L. monocytogenes* 08-5923 that was involved in a foodborne disease outbreak in Canada (Gilmore et al., 2010) was chosen for use in this study.

6.2. Materials and Methods

6.2.1. Bacterial strain and culture

L. monocytogenes 08-5923 was cultured from a frozen stock culture stored at -80°C in 40% glycerol and grown in Tryptic Soy Broth (TSB) at 22°C for 48 h and subcultured (1% inoculum) into 50 mL of fresh TSB (control) or TSB with 1.4% sodium lactate and 0.1% sodium diacetate (treated). Cells from three independent biological replicates were grown at 37°C for 24 h. The treatments were conducted at the optimal growth temperature of *L. monocytogenes* (37°C) rather than refrigeration storage temperature of meat products to examine specifically the effect of NaL/DA on the physiology of *L. monocytogenes* 08-5923.

6.2.2. Light microscopy

To examine the morphology of *L. monocytogenes*, cells were fixed by mixing culture with 10% (v/v) formaldehyde in phosphate buffered saline to achieve the final concentration of 1% (v/v) formaldehyde. A drop of culture-formaldehyde mixture was examined under a Zeiss microscope with Axiocam MRm (CarlZeiss Inc., Oberkochen, Germany).

6.2.3. Flow cytometry

All cells were fixed using 1% (v/v) formaldehyde prior to flow cytometry analysis. Fluorescent dye, SYTO® BC (Invitrogen, Canada) was added to samples according to manufacturer's instructions. Forward scatter (FSC), side scatter and fluorescence intensity of each sample (10,000 cells) were recorded by a FACS Calibur flow cytometer (BD-Canada). Data were analyzed using BD CellQuest Pro™ software (BD-Canada). Student's *t*-tests were performed to evaluate the differences in the mean FSC of the 10,000 cells in each sample.

6.2.4. Filamentation of *Listeria* in the presence of sodium lactate or sodium acetate

L. monocytogenes 08-5923 was grown in TSB at 37°C in the absence or presence of sodium lactate or sodium acetate. Filamentation of *L. monocytogenes* 08-5923 was monitored by flow cytometry after 8 and 24 h of inoculation.

6.2.5. Total RNA isolation

RNAprotect® Reagent (Qiagen, ON) was added to a culture grown for either 8 or 24 h and total RNA was isolated using the RNeasy® mini kit (Qiagen, ON)

following treatment with DNase I (New England Biolabs, ON) on-column according to the manufacturers' protocols. RNA quality and quantity was assessed on the Agilent 2100 bioanalyzer (G2938C, Agilent Technologies, CA) using Agilent Nano 6000 Assay for prokaryotes. The RNA with integrity number (RIN) > 8.0 was used for subsequent library preparation for the RNA-seq experiment.

6.2.6. Quantitative real time PCR to determine expression of *ftsE* in *Listeria* exposed to sodium lactate, sodium acetate and acetic acid

To confirm the differential expression of *ftsE* gene, total RNA was extracted from 5 mL of *L. monocytogenes* 08-5923 exposed to preservatives for 8 or 24 h and the expression levels of *fts* genes were measured by quantitative real time PCR (qRT-PCR). Expression level of *ftsE* was quantified using SYBR Green (Invitrogen, ON). Primers (*ftsE*-forward: 5'-TATGCGATGGAAGTGGTTGA-3'; *ftsE*-reverse: 5'-CTCACCACCGGAAAGTTCAT-3') were designed using Primer 3 (http://frodo.wi.mit.edu/primer3/) and tested for specificity with genomic DNA prior to the analysis. Total RNA was reverse-transcribed into cDNA using reverse transcriptase SuperScript® III (Promega, WI) according to the manufacturer's protocol. Quantification of the transcripts was carried out on a 7500 Fast Real-Time PCR system (Applied Biosystems, ON) using QuantiFast® SYBR Green PCR Kit (Qiagen, ON). Relative quantification values were obtained using the comparative threshold cycle method ($\Delta\Delta C_T$).The housekeeping gene *rpoB* was selected as the reference (primers: *rpoB*-forward: 5'-GAAATCTGGGTTCGTCGTGT-3'; *rpoB*-reverse: 5'-

GCTACGTTTGGACGTTGGTT-3'). Unpaired *t*-test was performed for determination of the significance in differential expression of genes.

6.2.7. mRNA enrichment and construction of cDNA libraries for RNA-seq

The rRNA and sRNA from the total RNA extracted from *L. monocytogenes* 08-5923 were removed by Terminator™ 5′-Phosphate-Dependent Exonuclease (Epicentre®, WI) according to the manufacturer's instructions. Enriched mRNA was purified following the RNA Cleanup protocol using the RNeasy® mini kit (Qiagen, ON).

Two hundred ng of mRNA from each control and treated sample was used to construct a cDNA library following the TruSeq™ RNA Sample Preparation Guide (Low Throughput protocol; illumina®, CA) using the TruSeq RNA Sample Preparation Kits v2, Set A (illumina®, CA). The mRNA was fragmented at 94°C for 8 min, reverse-transcribed into ds cDNA using SuperScript™ III Reverse Transcriptase (Invitrogen, ON) and Second Strand Master Mix (illumina®, CA). End repair of the double stranded cDNA was performed using 3' to 5' exonuclease (End Repair Mix; illumina®, CA). The 3' ends were adenylated (A-Tailing mix; illumina®, CA) and adapters were ligated (indexing) onto the ends of the ds cDNA. Adapter-ligated ds cDNAs were enriched by 15 cycles of PCR reaction with an annealing temperature of 60°C (illumina®, CA).The quality and quantity of the cDNA libraries was assessed on Agilent 2200 TapeStation System using the High Sensitivity D1K ScreenTape (Agilent Technologies Inc., Germany).

6.2.8. RNA-seq and data analysis

Sequencing of the cDNA libraries was performed at the High Throughput Genomics Center (Seattle, WA) using Illumina 36 bps paired-end RNA-seq. Mapping of reads to genome (*L. monocytogenes* 08-5923, NC_013768.1) and assembly of transcripts were performed using software Rockhopper (McClure et al., 2013). To estimate the level of transcription for each gene, the number of reads that mapped within each annotated coding sequence (CDS) was determined. Transcript boundaries including 5′ and 3′ untranslated regions (UTRs) were identified. To normalize for the variation in transcript length and sequence yield, RPKM (reads per kilobase per million) values (Mortazavi et al., 2008) were calculated using the formula:

$$RPKM = 10^9 * C / (N * L)$$

C = counts of mapped fragments, L = length of transcript and N = total count of mapped fragments.

RPKM values were log-transformed into $\log_2$ (RPKM).

6.2.9. Analysis of differential expression

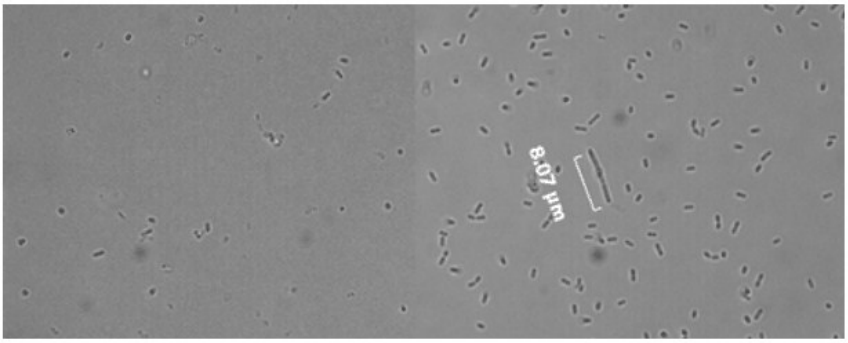
Differential expression was assessed using Rockhopper (McClure et al., 2013). The total read count was determined for each transcript by combining data from three biological replicate sequencing runs. Read counts, which are proportional to expression level were used to determine the abundance of each transcript. The expression levels of the control and treated samples were

compared to determine the fold change. A fold change > 1.5 was considered significantly differentially expressed in this study. The *q*-values were calculated and adjusted by Rockhopper (McClure et al., 2013) using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995) with the false discovery rate (FDR) < 1%. The *q*-values <0.01 are considered significant.

6.3. Results and Discussion

6.3.1. Cell morphology

No obvious morphological difference was observed in treated (24 h) and untreated *L. monocytogenes* 08-5923 under light microscope (Figure 6.1), except an 8-μm long filament was observed in the treated sample.



(a) (b)

Figure 6.1 *L. monocytogenes* 08-5923 under light microscope (1,000 *x* magnification). (a) untreated cells; (b) cells exposed to NaL/DA for 24 h.

However, flow cytometry data (Figure 6.2) showed that the average forward light scatter, which is proportional to the cell length of *L. monocytogenes* 08-5923, was significantly higher than those of untreated cells ($p<0.05$).

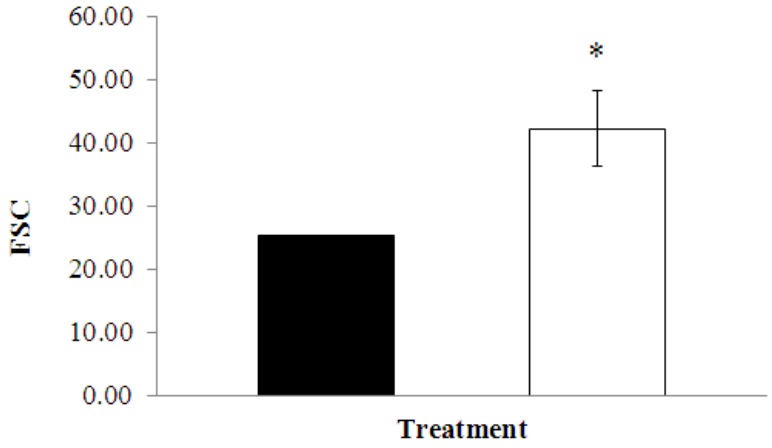


Figure 6.2 Forward light scatter (FSC) of cells of *L. monocytogenes* 08-5923 grown in the absence (■) and presence (□) of a combination of 1.4% sodium lactate and 0.1% sodium diacetate for 24 h (Mean ± SD, n=3). * indicates significant difference ($P<0.05$)

6.3.2. Differential expression of *ftsE* in filamented *Listeria*

Flow cytometry data showed that the average length of cells treated with NaL alone was significantly ($p<0.05$) longer than that of untreated at both 8 h and 24 h (Figure 6.3). The average length of sodium acetate treated cells was significantly ($p<0.05$) longer than that of untreated at 24 h but not 8 h. This observation indicates that the majority of the population of *L. monocytogenes* was in filamentous-morphology after 24 h when exposed to NaAc, and after 8 h when exposed to NaLac. The high standard deviation of FSC in *L. monocytogenes* after

24 h exposure to NaLac compared with after 8 h of the same treatment indicates a mixture of filamentous and normal cells of *L. monocytogenes* in the population.

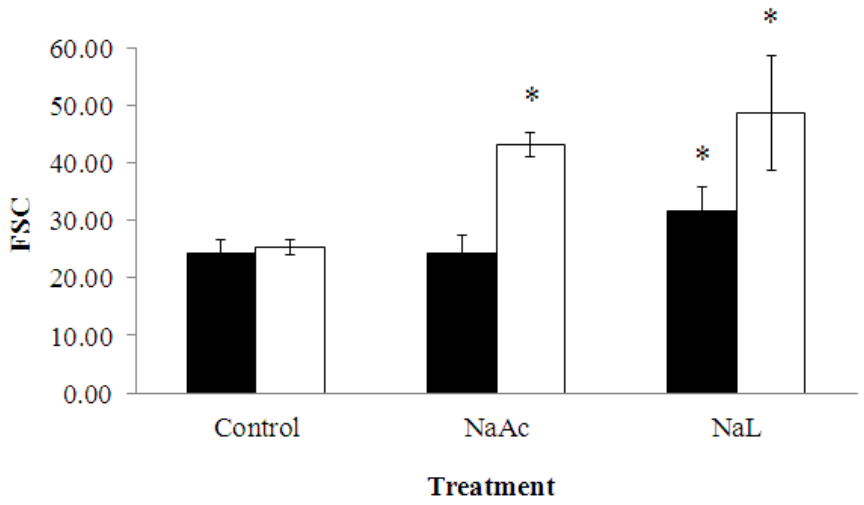


Figure 6.3 Forward light scatter (FSC) of cells of *L. monocytogenes* 08-5923 grown in the absence (■) and presence (□) of sodium acetate (NaAc) or sodium lactate (NaL) alone for 8 and 24 h (Mean ± SD, n=3). * indicates significant difference ($P<0.05$)

The expression level of *ftsE* in *L. monocytogenes* 08-5923 after 8 h exposure to sodium lactate was -3.03±0.10 (down-regulated) and 2.63±0.15 (up-regulated) after 24 h. In contrast, the expression level of *ftsE* in *L. monocytogenes* 08-5923 after 8 h exposure to sodium acetate was 1.29±0.43 and 2.16±0.92 (up-regulated) after 24 h. It is possible that *L. monocytogenes* were filamenting after 8 h exposure to NaLac (down-regulation of *ftsE*) and filaments started to divide after 24 h (up-regulation of *ftsE*), since a mixture of filamentous and normal cells of *L. monocytogenes* was present in the population.

6.3.3. Mapping of the data from Illumina-based RNA-seq libraries

Six cDNA libraries from 3 biological replicates of untreated and treated *L. monocytogenes* 08-5923 were sequenced. For each library, a total of 5.8 to 13.8 million reads were obtained. Successfully aligned reads covered 97% to 98% of the genome. Aligned reads consist of ~40% protein coding genes, 50% ribosomal RNAs and 10% unannotated regions (Table 6.1).

6.3.4. Quantification of transcript levels

Full details of the number of reads mapping to each gene and protein coding regions, 5'UTR, 3'UTR, together with $\log_2$ (RPKM) values are available in Appendix B. As previously reported for RNA-seq analysis of eukaryotic RNA (Wilhelm et al., 2008) and prokaryotic RNA (Perkins et al., 2009; Chaudhuri et al., 2011), the $\log_2$ (RPKM) values in replicates were in close agreement (example shown in Appendix B.1).

6.3.5. Differentially expressed genes and pathways

A total of 557 genes/regions were differentially expressed (q-value < 0.01). Most of these genes are involved in transcription regulation, metabolism and cell structure synthesis (Table 6.1).

Genes encoding stress proteins (Clp, heat shock proteins) were down-regulated >2-fold. All the *clp* genes (*clpB, clpC, clpE, clpP, clpX*) were down-regulated 2.38 to 5.00-fold. Genes encoding heat shock proteins (GroESL, DnaK) were down-regulated 2.78 to 4.35-fold. Genes encoding two-component systems

(*hisJ*, *lisK*, OmpR family gene, *resE*) were up-regulated 2.00 to 2.71-fold. Genes encoding RNA polymerase factors SigC was down-regulated 2.17-fold and SigH was up-regulated 2.43-fold. Genes encoding DNA-binding protein (Fus) and glycolytic enzymes (PykA, Eno, FbaA, Pgm) were down-regulated. The DNA repair gene (*radC*), cell division gene (*ftsE*) as well as the cell structure synthesis genes (flagella synthesis: *flgK*, *fliF*, *fliD*) were up-regulated (Table 6.2).

Table 6.1 Summary of aligned reads in RNA-seq to the reference genome (*L. monocytogenes* 08-5923). (C = untreated; T = treated)

Aligning sequencing reads C1		
Total reads:	5768534	
Successfully aligned reads:	5617884	97%
	Aligning (sense) to protein-coding genes:	28%
	Aligning (antisense) to protein-coding genes:	25%
	Aligning (sense) to ribosomal RNAs:	20%
	Aligning (antisense) to ribosomal RNAs:	19%
	Aligning (sense) to transfer RNAs:	0%
	Aligning (antisense) to transfer RNAs:	0%
	Aligning (sense) to miscellaneous RNAs:	0%
	Aligning (antisense) to miscellaneous RNAs:	0%
	Aligning to unannotated regions:	8%
Aligning sequencing reads C2		
Total reads:	13805387	
Successfully aligned reads:	13565918	98%
	Aligning (sense) to protein-coding genes:	24%
	Aligning (antisense) to protein-coding genes:	23%
	Aligning (sense) to ribosomal RNAs:	24%
	Aligning (antisense) to ribosomal RNAs:	23%
	Aligning (sense) to transfer RNAs:	0%
	Aligning (antisense) to transfer RNAs:	0%
	Aligning (sense) to miscellaneous RNAs:	0%
	Aligning (antisense) to miscellaneous RNAs:	0%
	Aligning to unannotated regions:	6%

Table 6.1 Continued.

Aligning sequencing reads C3		
Total reads:	8827575	
Successfully aligned reads:	8678299	98%
	Aligning (sense) to protein-coding genes:	22%
	Aligning (antisense) to protein-coding genes:	20%
	Aligning (sense) to ribosomal RNAs:	26%
	Aligning (antisense) to ribosomal RNAs:	25%
	Aligning (sense) to transfer RNAs:	0%
	Aligning (antisense) to transfer RNAs:	0%
	Aligning (sense) to miscellaneous RNAs:	0%
	Aligning (antisense) to miscellaneous RNAs:	0%
	Aligning to unannotated regions:	7%
Aligning sequencing reads T1		
Total reads:	8820739	
Successfully aligned reads:	8629819	98%
	Aligning (sense) to protein-coding genes:	20%
	Aligning (antisense) to protein-coding genes:	18%
	Aligning (sense) to ribosomal RNAs:	27%
	Aligning (antisense) to ribosomal RNAs:	27%
	Aligning (sense) to transfer RNAs:	0%
	Aligning (antisense) to transfer RNAs:	0%
	Aligning (sense) to miscellaneous RNAs:	0%
	Aligning (antisense) to miscellaneous RNAs:	0%
	Aligning to unannotated regions:	7%

Table 6.1 Continued.

Aligning sequencing reads T2		
Total reads:	6330773	
Successfully aligned reads:	6189324	98%
	Aligning (sense) to protein-coding genes:	28%
	Aligning (antisense) to protein-coding genes:	26%
	Aligning (sense) to ribosomal RNAs:	21%
	Aligning (antisense) to ribosomal RNAs:	20%
	Aligning (sense) to transfer RNAs:	0%
	Aligning (antisense) to transfer RNAs:	0%
	Aligning (sense) to miscellaneous RNAs:	0%
	Aligning (antisense) to miscellaneous RNAs:	0%
	Aligning to unannotated regions:	5%
Aligning sequencing reads T3		
Total reads:	7773793	
Successfully aligned reads:	7654328	98%
	Aligning (sense) to protein-coding genes:	11%
	Aligning (antisense) to protein-coding genes:	10%
	Aligning (sense) to ribosomal RNAs:	38%
	Aligning (antisense) to ribosomal RNAs:	36%
	Aligning (sense) to transfer RNAs:	0%
	Aligning (antisense) to transfer RNAs:	0%
	Aligning (sense) to miscellaneous RNAs:	0%
	Aligning (antisense) to miscellaneous RNAs:	0%
	Aligning to unannotated regions:	4%

Table 6.1 Continued.

Transcript boundaries		
	Number of 5'UTRs:	1237
	Number of 3'UTRs:	1156
	Number of predicted RNAs:	2536
	Number of predicted RNAs (not antisense):	274
	Number of predicted RNAs (antisense):	2262
Differential gene expression		
	Number of differentially expressed protein coding genes:	135
Likely operons		
	Number of gene-pairs predicted to be part of the same operon:	1457
	Number of predicted multi-gene operons:	631

Exposure of *L. monocytogenes* 08-5923 to sodium lactate for 8 h resulted in filamentation and down-regulation of *lmo2507* (*ftsE*). After 24 h, *ftsE* was up-regulated > 2-fold while septa in filaments started to form and the majority of cells under microscope were divided (data not shown). This suggests the down-regulation of *lmo2507* could be involved in filament formation in *L. monocytogenes*. We did not observe the same effects with sodium acetate in that filamentation was observed but *ftsE* was not down-regulated after 8 h of exposure. It may be that the effects of sodium acetate on expression of *ftsE* happened prior to 8 h.

Table 6.2 Summary of differentially expressed (>1.5-fold) protein-coding genes in *L. monocytogenes* 08-5923 exposed to a combination of NaL/DA (+: up-regulation; -: down-regulation).

Function	Gene/Product	Fold change
Stress proteins	*hrcA*	-6.25
	groES, groEL	-3.13, -2.78
	grpE	-5.26
	ATP-dependent proteases: *clpB, clpC, clpE, clpP, clpX*	-2.38, -2.78, -5.00, -5.00, -4.35
	Class I heat-shock proteins: *dnaK, dnaJ*	-4.35, -4.00
	ltrA (low temperature requirement protein)	+1.91
Two-component systems	*hisJ*, histidine ABC transporter	+2.55
	two-component system, OmpR family, sensor histidine kinase KdpD	+2.26
	lisK (two-component sensor histidine kinase)	+2.71
	resE (responding to environmental changes)	+2.00
Transcription regulation	RNA polymerase factors: *sigC*	-2.17
	sigH	+2.43
Protein translation	Elongation Factor Tu (EF-TU), Ts, G	-5.26, -3.57, -3.23
	fus (RNA-binding)	-5.26
	infA; translation initiation factor IF-1	-2.17
	DeoR family transcriptional regulator, fructose operon transcriptional repressor	-3.33

Table 6.2 Continued.

Biosynthesis	*argB* (acetylglutamate kinase)	+2.06
	ansB (asparagine synthase)	+1.82
	purA (adenylosuccinate synthetase)	+1.92
	cbiF, cbiK (cobolamin synthesis)	+1.65, +1.88
	LM5923_2254; pyridoxine biosynthesis protein	+2.06
	tyrS, tyrosyl-tRNA synthetase	+1.90
	purN	+1.77
	cysK (cysteine synthase A)	+1.60
Cell division	*ftsE*	+1.79
DNA-repair	LM5923_1599 (putative holliday junction resolvase)	-3.85
	radC	+1.95

Table 6.2 Continued.

	gap (GTPase-activating protein)	-4.35
	ATP-binding cassette, subfamily B, bacterial	-2.94
	phosphotransferase system enzyme I	-2.63
	trxA (thioredoxin)	-4.00
	LM5923_2555; Fe-S cluster assembly protein SufB	-3.13
	cysteine desulfuras /selenocysteine lyase; iron homeostasis	-2.50
	LM5923_2556; nitrogen fixation protein NifU	-3.33
	sufB, sufD (Fe-S cluster assembly protein)	-3.13, -2.33
Cellular function	LM5923_1323; metal ion-dependent L-alanoyl-D-glutamate peptidase	-2.56
	LM5923_2000; iron/zinc/copper transport system substrate-binding protein	-2.44
	fri (ferritin-like protein)	-2.44
	LM5923_1071; TS system, beta-glucosides-specific	+1.95
	LM5923_1880 (beta-glucosidase)	+1.98
	ctaA (cytochrome aa3 assembly protein)	+1.93
	gatB (carbon-nitrogen ligase activity, with glutamine as amido-N-donor)	+1.73
	bvrB (beta-glucoside-specific phosphotransferase)	+1.88
	recA (recombinase A)	-2.27

Table 6.2 Continued.

Metabolism	*hisC* (histidinol-phosphate aminotransferase)	+1.88
	ilvB (acetolactate synthase)	+1.92
	pgk (phosphoglycerate kinase)	-2.86
	aspartate alpha-decarboxylase, *panB, panC, panD*	-2.86, -2.17, -2.70
	pgm (phosphoglycerate mutase)	-3.70
	fructose-1-phosphate kinase	-3.23
	tpi (triosephosphate isomerase)	-4.00
	fbaA (fructose-bisphosphate aldolase)	-5.88
	fruA	-3.33
	fruB (fructose-1-phosphate kinase)	-3.03
	eno (enolase)	-3.70
	pyruvate formate lyase, *pflB, pflC*	-2.44
	pykA (pyruvate kinase)	-2.33
	3-methyl-2-oxobutanoate hydroxymethyltransferase	-2.44
	LM5923_0088; bifunctional glutamate--cysteine ligase/glutathione synthetase; glutathione metabolism	+2.22
	glyceraldehyde-3-phosphate dehydrogenase	-4.55
	ldh (lactate dehydrogenase)	-2.56
	pyridoxine biosynthesis protein	+2.06
	agmatine deiminase	+1.52
	phosphoglyceromutase	-3.33

Table 6.2 Continued.

Motility	*flgK* (flagellar hook-associated protein)	+1.81
	fliD (flagellar hook-associated protein), *fliF* (flagellar basal-body M-ring protein)	+1.81, +1.90
Resistance	small multidrug resistance protein, SMR family	-2.33
	mecA (methicillin resistant protein)	-3.33
	LM5923_1814 (small multidrug resistance protein, SMR family)	-2.33
Virulence factors	internalin *inlG, inlI*	+2.52, +2.16
	LM5923_1765; peptidoglycan linked protein (LPXTG)	+2.15
	mpl (Zinc metalloproteinase precursor)	+1.83

The down-regulation of the glycolytic genes such as *pykA* and the up-regulation of DNA-repair and flagella synthesis genes suggest that the cells of *L. monocytogenes* could have directed part of the energy for metabolic activities to repair DNA damage and cellular structures. This observation agrees with the previous findings on the inhibitory effect of lactate and diacetate on *L. monocytogenes* by reducing the production of energy (Stasiewicz et al., 2011).

The differentially expressed genes form a network represented in Fig 6.4, which involves two-component regulatory factors, sigma factors (Chaturongakul et al., 2011) and genes involved in many cellular functions. Sigma factor σ^B contributes to acid and osmotic stress of *Listeria* (Kazmierczak et al., 2003). SigC

was reported to be up-regulated in *Listeria* following cold/heat stress (Chan et al., 2008; Zhang et al., 2005). SigH has dual functions - it is involved in the transition from exponential phase to stationary phase; and it is also important for the growth of cells in a minimal medium and in cold/alkaline conditions (Chan et al., 2008; Chaturongakul et al., 2008; Rea et al., 2004).

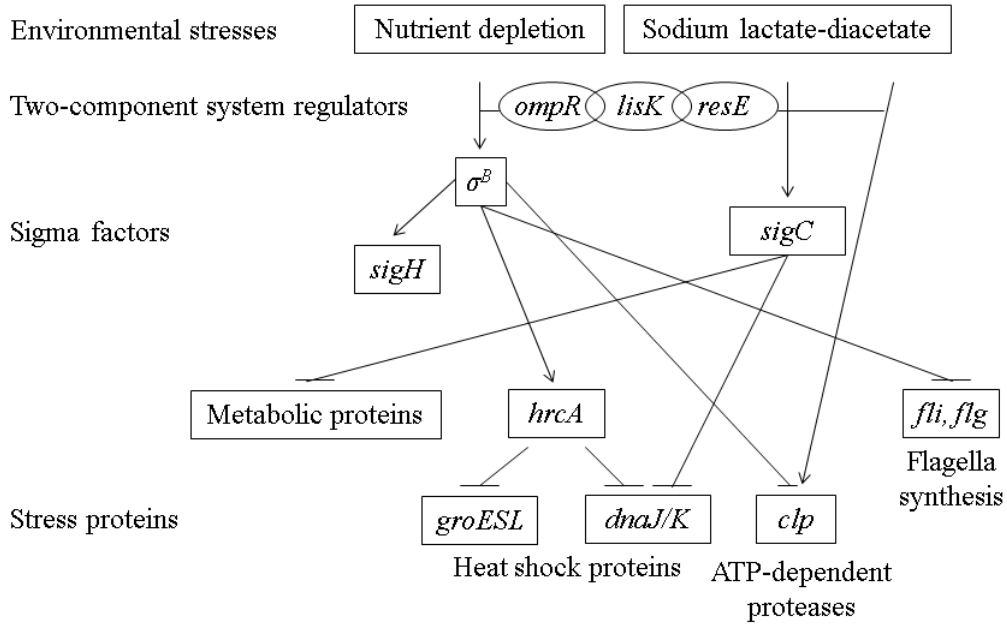


Figure 6.4 Proposed representation of *L. monocytogenes* 08-5923 stress response network.

Heat shock genes, *hrcA, grpE, dnaK, and dnaJ* are located within the *dnaK* operon (Homuth et al., 1997). The protein product, HrcA, is a repressor protein in the heat shock response and a negative transcriptional factor controlling the expression of the stress-specific operons *dnaK* and *groESL* in several bacteria (Hecker et al., 1996). Heat shock genes were up-regulated in *L. monocytogenes*

under salt (NaCl) stress (Duché et al., 2002; Chatterjee et al., 2006). Interestingly, contrary to previous studies, all these genes were down-regulated in this study clearly indicating that the response to NaCl is different from that of NaL/NaDA. Previous studies suggested that exposure of *Listeria* to NaCl increases the heat resistance, while exposure to sodium lactate decreases the heat resistance (McMahon et al., 1999; Juneja, 2003). Similarly, this study showed that exposure of *L. monocytogenes* cells to 1.4% sodium lactate and 0.1% sodium diacetate resulted in down-regulation of the genes encoding heat shock proteins that are required for survival of *L. monocytogenes* under thermal treatment.

Other stress genes, *clpC* and *clpP*, were also down-regulated in this study in the presence of sodium lactate-diacetate. Clp proteins are important for the growth of microorganisms under stress conditions. *Streptococcus mutans* lacking ClpP, but not strains lacking ClpC, was unable to grow under stress-inducing conditions (Lemos and Burne, 2002). The expression of *clpC*, *clpP* and *groESL* mentioned earlier, is negatively regulated by CtsR and HrcA as well as the sigma factors (Chaturongakul et al., 2011) but independently of σ^B (Hecker et al., 1996).

6.4. Conclusions

Overall, exposure to sodium lactate/acetate or a mixture of sodium lactate and sodium diacetate resulted in different stress responses of *L. monocytogenes*, either causing filament formation, or reduced glycolytic activity without filamentation. Glycolysis is one of the major pathways of energy production inside cell, and has been linked to filament formation in *E. coli*. *E. coli* treated with berberine, a plant

alkaloid, exhibits filamentous phenotype (Boberek et al., 2010). Berberine promotes glycolysis (Yin et al., 2008) and inhibits FtsZ ring formation during cell division (Domadia et al., 2008), which suggests an increased glycolytic activity is associated with filamentation in *E. coli*. This is consistent with that the cells of *L. monocytogenes* exposed to a mixture of sodium diacetate resulted in reduced glycolytic activity but not filamentation.

This study demonstrated that exposure of *L. monocytogenes* to preservative commonly used in sodium-reduced RTE meats resulted in filament formation *in vitro*. Prior to this thesis work, it is unknown whether *L. monocytogenes* can form filaments in the presence of preservatives *in situ*. If this happens, it could lead to underestimation of actual number of cells present in food, which has significant impact on the food safety risk and tolerance levels of *L. monocytogenes* set by regulatory agencies (Jones et al., 2013). Discovery of filament formation in *L. monocytogenes in situ* is discussed in Chapter 7.

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7. Filamentation of *Listeria monocytogenes* 08-5923

7.1. Introduction

Many factors, including storage conditions, antimicrobial agents, and NaCl, could cause stress in *L. monocytogenes* growing on meat products. A food preservative, Micocin™ X (CanBiocin Inc.), can be used on ready-to-eat meat (RTE) products to suppress the growth of *L. monocytogenes*. Micocin™ X is prepared from the cell free supernatant of *Carnobacterium maltaromaticum* UAL307 (CFS307) and it contains bacteriocins (carnobacteriocin BM1, piscicolin 126 and carnocyclin A) and various organic acids, including acetic and lactic acids as end products of *C. maltaromaticum* metabolism (Borch and Molin, 1989). A preliminary study by the current author showed that 1% (w/v) sodium/potassium salts of acetate, lactate, CFS307 or 0.1% (w/v) acetic/lactic acid could induce filamentation *in vitro* in *L. monocytogenes*. *L. monocytogenes* could form filaments up to 97 nm in length *in vitro* in the presence of sublethal dose of CFS307 (unpublished data).

This study examined the roles of the genes engaged in cell division and cell wall synthesis involved in filamentation of *L. monocytogenes* 08-5923, which is one of the strains involved in an outbreak of listeriosis in Canada in 2008 (Gilmour et al., 2010). *L. monocytogenes* was inoculated on RTE meat products in the presence and absence of preservatives (NaCl and sublethal dosage of CFS307) and stored for 3 months at 4°C. Filamentation of cells was monitored by flow

cytometry. The expression levels of genes encoding proteins and enzymes in cell division (*ftsX*), cell wall synthesis (UDP-N-acetylglucosamine 1-carboxyvinyltransferase (MurZ, E.C.2.5.1.7, *murZ*), ATP production (*pykA*) and NADPH generation (*gnd*) were measured by qRT-PCR to explore the mechanism behind filamentation on RTE meats.

7.2. Materials and Methods

7.2.1. Bacterial culture and *in vitro* filamentation induction

L. monocytogenes 08-5923 was cultured from a frozen stock culture stored at -80°C in 40% glycerol and grown at 22°C for 24 h in Tryptic Soy Broth (TSB; BD-Canada) or in TSB with one of the following 1% (w/v): NaCl/KCl, sodium/potassium lactate, sodium/potassium acetate and CFS307, or 0.1% (w/v) acetic acid or lactic acid. To obtain the CFS307, *C. maltaromaticum* UAL307 was obtained from the University of Alberta Food Microbiology Lactic Acid Bacteria Culture Collection and grown overnight All Purpose Tween broth (APT; BD-Canada) at 22°C. The culture supernatant was filtered (0.22 µm, Millipore®, CA).

7.2.2. Transmission electron microscopy

Cells of *L. monocytogenes* 08-5923 grown with or without CCLA were fixed with 10% (v/v) formaldehyde and pelleted by centrifugation at 11,000 *x g* for 15 sec. The cell pellet was re-suspended in 0.1 M phosphate buffer containing 2.5% glutaraldehyde and 2% paraformaldehyde, stained with 2% sodium

phosphotungstate and examined using a Transmission electron microscope (TEM) (Philips Morgagni 268; FEI™, OR) operating at 80 kV.

7.2.3. Manufacture of chopped and formed ham

Sliced pork ham with 0.55, 1.35 and 2.35% total NaCl was manufactured as described in Chapter 3, section 2.2. Each slice was 3 mm thick and had a surface area of ca. 15 cm^2.

7.2.4. Treatment, inoculation and packaging

C. maltaromaticum UAL307 was grown in APT broth at 22°C for 24 h. Cell-free supernatant (CFS307) was obtained by centrifuging the culture of UAL307 at 4,000 *x* g for 10 min and the supernatant was passed through a 0.22 μm filter (Millipore). In a preliminary experiment, a culture of *L. monocytogenes* 08-5923 was exposed to 1% (w/v) CFS307, or the same concentration of sodium/potassium acetate, sodium/potassium lactate, or 0.1% (w/v) acetic/lactic acid for 24 h at 22°C. Cell morphology was examined under light microscope. For the *in situ* experiment, 50 μL of saline (0.85% w/w) or CFS307 (100%, 1 in 5, 1 in 10 and 1 in 20 dilutions in saline) were spread over ham slices prior to inoculation and left air dried for 2 min. *L. monocytogenes* 08-5923 was cultured from frozen stock in Tryptic Soy Broth (TSB; BD-Canada) at 22°C and sub-cultured once before inoculation. Individual slices of ham were inoculated with *L. monocytogenes* 08-5923 (3.0 log CFU/slice). Ham slices were vacuum packaged individually (C200; Multivac) and stored at 4°C. Three replicate experiments were completed.

7.2.5. Cell counts

After 1, 2 and 3 months (4, 8, and 12 weeks, respectively) of storage, sterile 0.85% saline was added to each package followed by vigorous massage for 1 min to obtain microorganisms from the ham surface. Serial dilutions were prepared from this saline and samples were plated onto APT agar or PALCAM agar with antimicrobial supplement (Oxoid Ltd., CA). Cell counts were enumerated on APT agar after 48 h incubation at 22°C and on PALCAM agar after 24 h incubation at 37°C.

7.2.6. Flow cytometry

Cells were fixed by mixing culture with 10% (v/v) formaldehyde in phosphate buffered saline to achieve the final concentration of 1% (v/v) formaldehyde. SYTO® BC (Invitrogen, CA) fluorescence dye was added to samples according to manufacturer's instructions. Forward scatter (FSC), side scatter (SSC) and fluorescence intensity of from each sample (10,000 cells) were recorded by a FACSCalibur flow cytometer (BD-Canada). Data were analyzed using software BD CellQuest Pro™. Student's *t*-tests were performed to evaluate the differences in the mean FSC of the 10,000 cells in each sample.

7.2.7. Light microscopy

All cells were fixed using 1% (v/v) formaldehyde followed by Gram-stain. Non-filamented and filamented *Listeria* cells were observed using a Zeiss microscope with Axiocam MRm (CarlZeiss Inc., Oberkochen, Germany) to obtain images.

7.2.8. RNA isolation

RNAprotect® Bacteria Reagent (Qiagen, ON) (diluted 2:1 with saline) was added to the ham slice according to the manufacturer's instructions. Ten mL of diluted RNAprotect® was added to inoculated ham in vacuum packages and massaged vigorously for 1 min. Total RNA was isolated using the RNeasy mini kit (Qiagen) and treated with DNase I (New England Biolabs, ON) on-column according to the manufacturers' protocols. RNA quantity were measured using a Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific, AB), and the samples were stored at -80^{o}C.

7.2.9. qRT-PCR

Total RNA was reverse-transcribed into the first-strand cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA) according to the manufacturer's protocol. Primers targeting four genes (*ftsX*, *murZ*, *pykA* and *gnd*) (Table 4.1) were designed using Primer3 (http://frodo.wi.mit.edu/primer3/). All qRT-PCR experiments were performed on a 7500 Fast Real-Time PCR system (Applied Biosystems) using QuantiFast® SYBR® Green PCR Kit (Qiagen). Relative quantification of the genes was determined using the comparative threshold cycle method ($\Delta\Delta C_T$) with *rpoB* as the housekeeping gene and compared to the expression level of each gene in *L. monocytogenes* 08-5923 on ham with 0.2% NaCl without CFS307. Unpaired *t*-tests were performed for determination of the significance in differential expression of genes.

Table 7.1 Primers used in qRT-PCR for amplification of genes.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
rpoB	GAAATCTGGGTTCGTCGTGT	GCTACGTTTGGACGTTGGTT
ftsX	AATGGTTGGATGACCTTTGC	CGTTGCAAGCTTGTTCATGT
gnd	GGAAGAGAATGCGGACAAAA	ATCTGTAGCATCGCCAGCTT
murZ	GCGATTTATTTACGGGCAGA	TTGCACGAACAGCTGCTAAC
pykA	AAGTGCTGCAGTTGTTGTGG	TTGCATCTTTAGCACCAACG

7.3. Results and Discussion

7.3.1. *In vitro* study

In the preliminary study, *L. monocytogenes* 08-5923 was treated with CFS307 for 24 h. The majority of the *Listeria* population formed filaments (Figure 7.1).

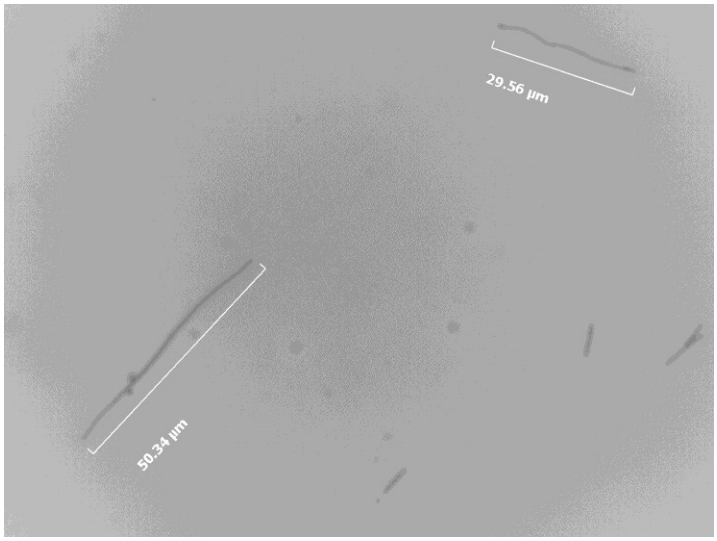


Figure 7.1 *L. monocytogenes* 08-5923 under light microscope (1000 *x* magnification). Cells were treated with CFS307 for 24 h at 22°C in TSB.

Under TEM, *L. monocytogenes* does not contain segments between the undivided daughter cells within the filaments and these filaments do not have flagella (Figure 7.2). The cell wall of filaments was rough compared to the smooth cell wall of a single cell. *L. monocytogenes* exposed to 1% (w/v) sodium/potassium acetate, sodium/potassium lactate, or 0.1% (w/v) acetic/lactic acid all formed filaments after 24 h at 22°C.

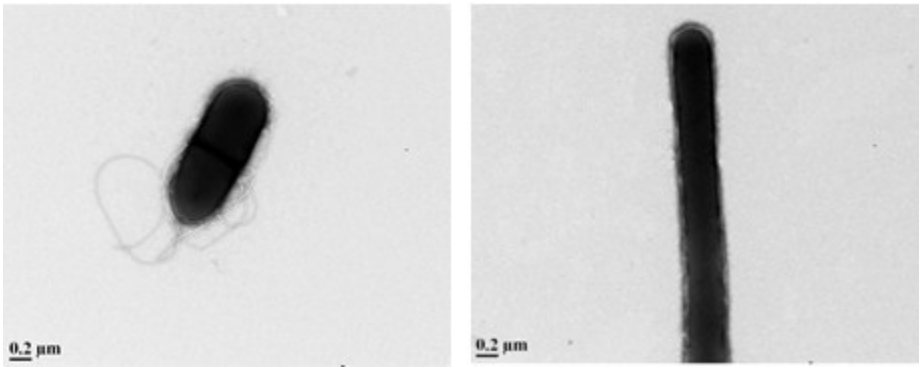


Figure 7.2 *L. monocytogenes* 08-5923 under TEM (28k *x* magnification). Left: normal cell (untreated); right: filament (exposed to CFS307).

7.3.2. Effect of varying concentrations of NaCl and CFS307 on cell growth, length and morphology

Following one month of storage, the cell counts of *L. monocytogenes* on ham with 0.55% total NaCl was significantly higher than on ham with 2.35% total NaCl ($p<0.05$, Figure 7.3). After 2 months of storage, the cell counts on ham with 0.55% and 2.35% total NaCl were not significantly different ($P>0.05$). After 90 days of storage (Figure 7.3) population of *L. monocytogenes* on all ham samples

increased from 2 log CFU/cm^2 (inoculation level) to approx. 8 log CFU/cm^2. No significant difference was observed in counts of *L. monocytogenes* or in the presence or absence of CFS307 on ham products with the same concentration of NaCl.

After 1 month of storage, the mean FSC (measurement related to cell length) in *L. monocytogenes* on ham with 2.35% NaCl (regardless of the presence of CFS307) was approximately twice the mean FSC of *L. monocytogenes* on ham with 0.55% NaCl without CFS307 (Figure 7.4). The mean FSC of *L. monocytogenes* on ham with/without CFS307 were not significantly different ($p>0.05$). The effect of NaCl and CFS307 on the mean FSC on *L. monocytogenes* was not additive as the cell length on ham with 2.35% NaCl and CFS307 was not significantly different from that on ham with 2.35% NaCl without CFS307 (Figure 7.4). A similar trend was observed after 2 months of storage, however, after 3 months of storage, the FSC of *L. monocytogenes* in most samples decreased as compared with the FSC from month 1 and month 2 (Figure 7.4).

Following each month of storage, the FSC increased significantly ($P<0.05$) with the increasing concentration of NaCl, but not with CFS307 added. This observation clearly suggests that NaCl induces filamentation in *L. monocytogenes* 08-5923, while sublethal dose of CFS307 had little or no effect on filament formation *in situ*.

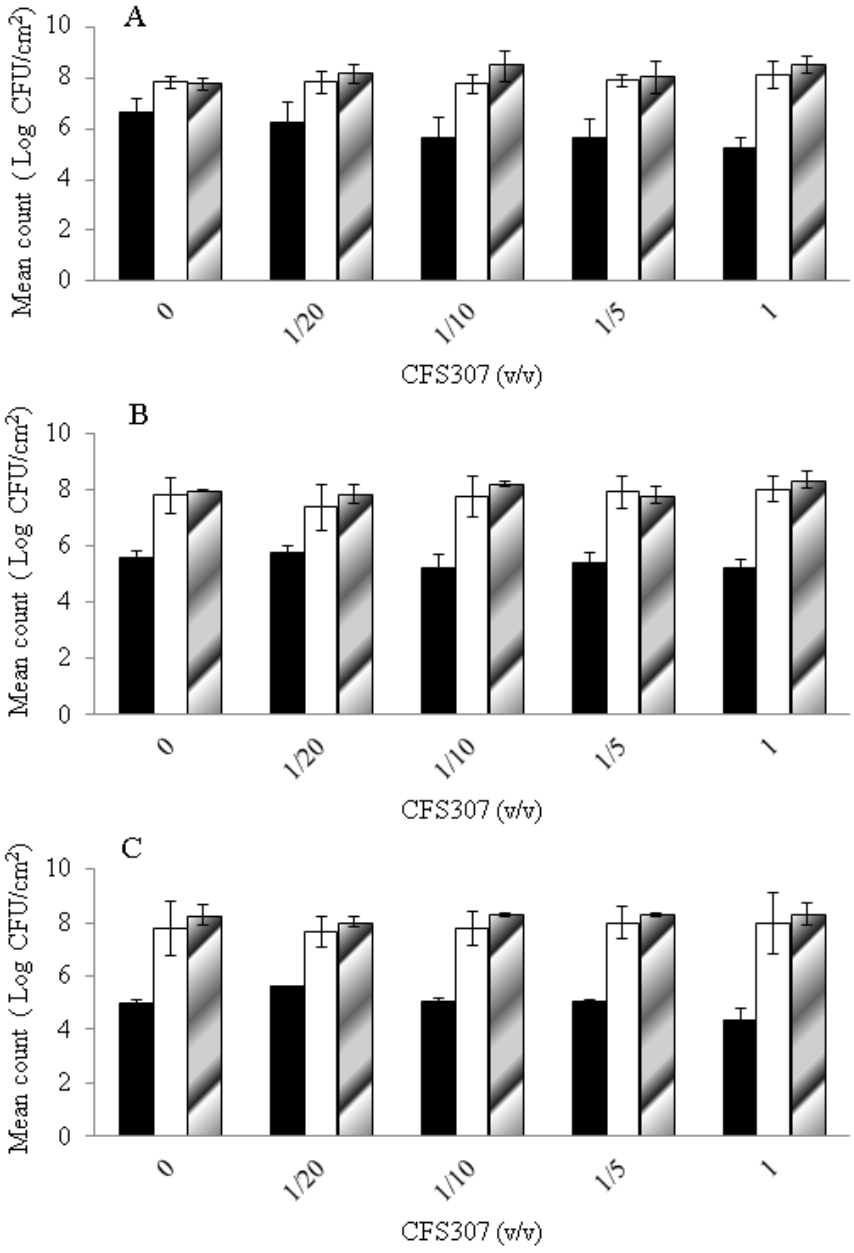


Figure 7.3 Growth of *L. monocytogenes* 08-5923 on ham with (A) 0.55%, (B) 1.35%, and with (C) 2.35% total NaCl at day 30 (black bars), day 60 (white bars) and at day 90 (stripped bars). The y-axis indicates the dilutions of CFS307 (0, 1/20, 1/10, 1/5 and undiluted). Data are presented as mean ± standard deviations (n=3).

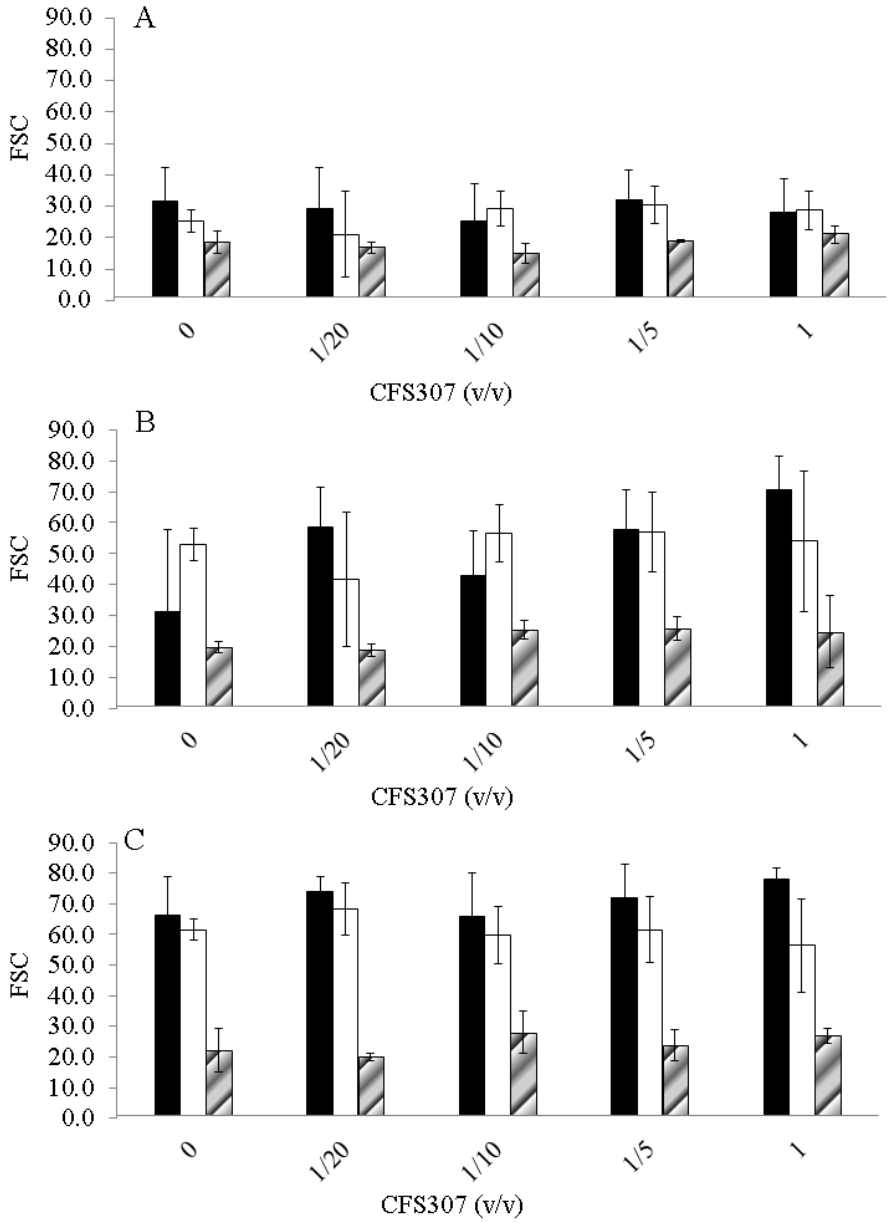


Figure 7.4 FSC of *L. monocytogenes* 08-5923 on ham with added CFS307. Section A, B, C represents ham with 0.55%, 1.35%, and 2.35% total NaCl respectively. Black bars - FSC at day 30, white bars - day 60, stripped bars – day 90. The y-axis indicates the dilutions of CFS307 (0, 1/20, 1/10, 1/5 and undiluted). Data are presented as mean ± standard deviations (n=3).

7.3.2. Gene expression changes in *L. monocytogenes* in response to NaCl and CFS307

The relative expression levels of all genes were compared to the expression level of particular gene of *L. monocytogenes* grown on ham with 0.55% total NaCl without CFS307. After 1 month of storage, down-regulation of *ftsX* and no change in *murZ* expression level as well as the significance in an increase in FSC (vs. control) was observed on ham with 2.35% of total NaCl (cells were filamenting) (Figure 7.5).

This study shows the engagement of *ftsX* in filamentation of *L. monocytogenes*. Previous studies on Fts proteins in *E. coli* found that under or overexpression of a subunit (e.g., FtsZ) in the Fts complex (Typas et al., 2012), or an imbalance of the ratio between these subunits (e.g., FtsZ/FtsA), resulted in filamentation in *E. coli* (Dai and Lutkenhaus, 1992; Dewar et al., 1992; Jeong and Lee, 2003). In addition, genes encoding the Fts protein complex, including *ftsE* and *ftsX* (*lmo2506*) are located in one operon together with *ftsY* (Gill and Salmond, 1986). FtsE and FtsX proteins build a complex with similar characteristics of an ABC-type transporter, and were shown to play a role in cell division and/or salt transport (de Leeuw et al., 1999).

These results suggest that *murZ* (cell wall synthesis gene) is involved in filamentation in *L. monocytogenes*. A previous study found that N-acetylmuramidase, which regulates cell wall degradation, could also be involved in filamentation of *L. monocytogenes* (Machata et al., 2005). It is possible that

peptidoglycan metabolism (synthesis and degradation) is related to filamentation in *L. monocytogenes*.

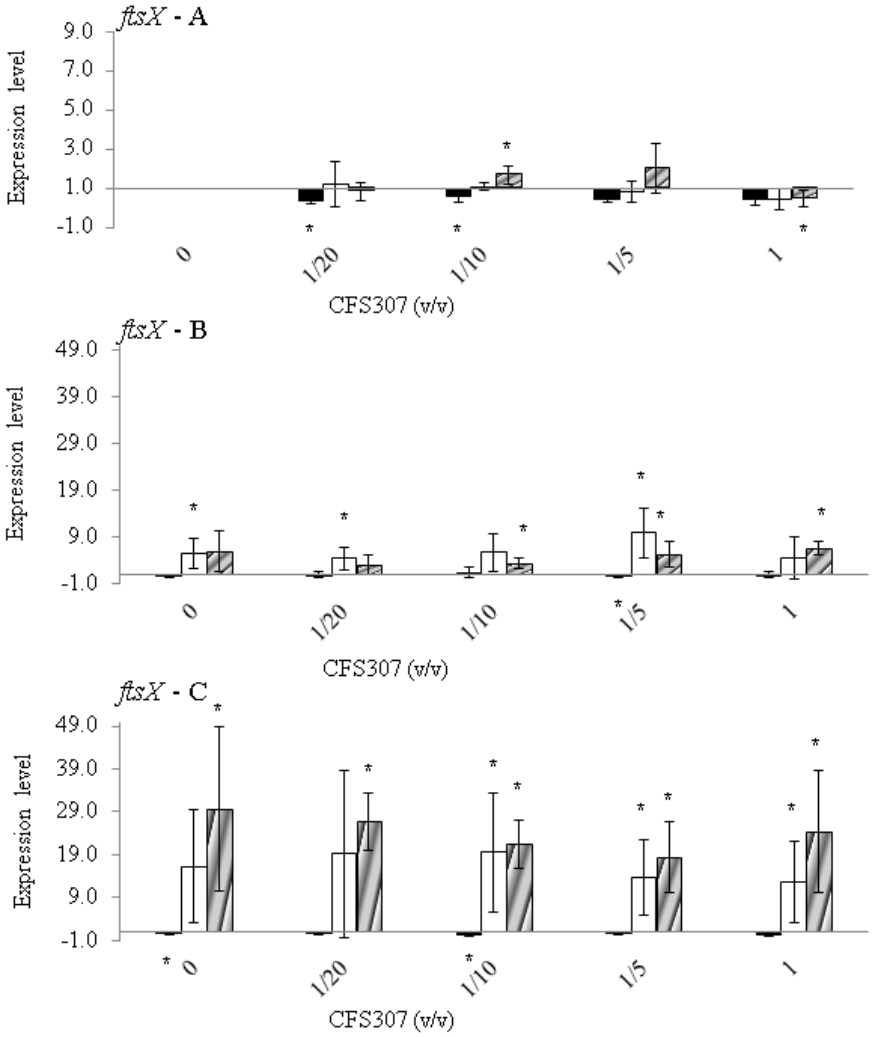


Figure 7.5 Expression levels of *ftsX* (*lmo2506*) and *murZ* (*lmo2552*) [Mean ± SD (n=3)] on ham with (A) 0.55%, (B) 1.35%, and with (C) 2.35% total NaCl at day 30 (black bars), day 60 (white bars) and at day 90 (stripped bars). The y-axis indicates the dilutions of CFS307 (0, 1/20, 1/10, 1/5 and undiluted). Note the scale of y-axis of *ftsX*-A. * indicates significant difference ($P<0.05$)

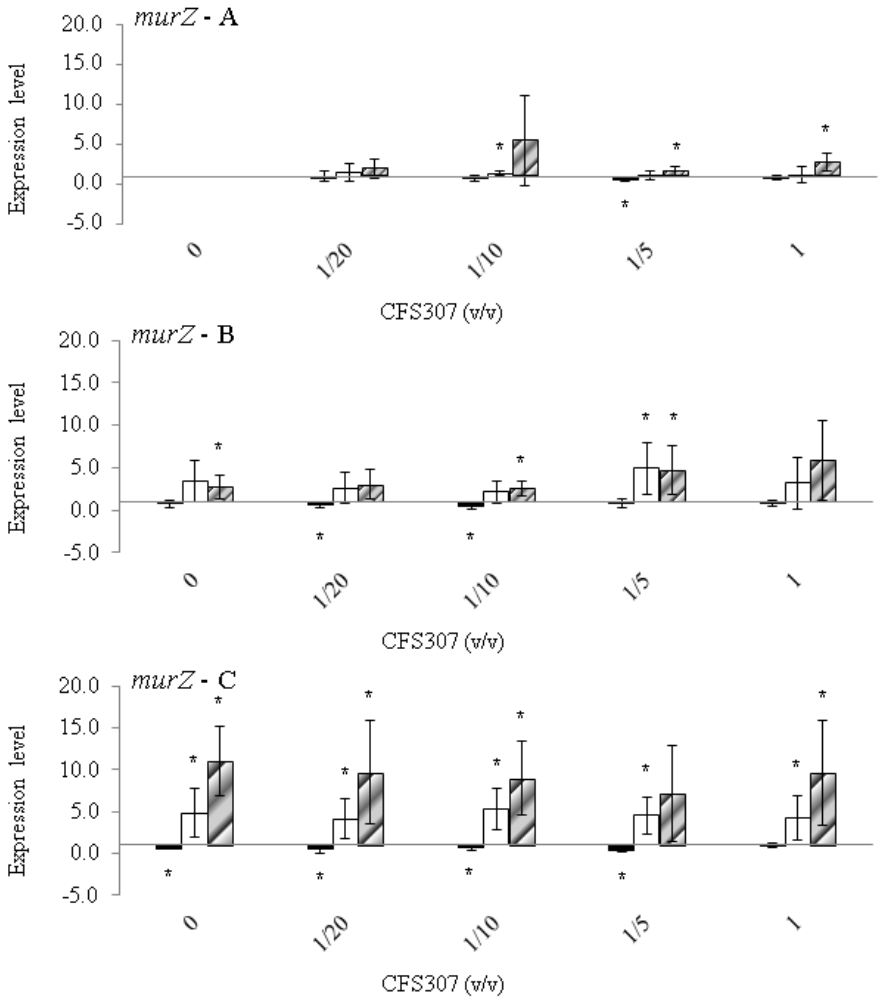


Figure 7.5 continued.

After 2 months of storage, the expression levels of both genes increased while the FSC decreased slightly and an increase in cell counts (population was growing) as well as the up-regulation of *gnd* gene was found (Figure 7.6). After 3 months of storage, the expression levels of *ftsX, murZ,* and *gnd* increased 35-, 7-fold, and 4-fold respectively and the FSC substantially decreased in comparison to samples analyzed after two months of storage while cells were dividing. The

increase in the expression levels of *ftsX, murZ, pykA* and *gnd* and the decrease in FSC of treated *L. monocytogenes* were followed by a slight increase in *Listeria* counts.

Previous studies observed the up-regulation of *pykA* and *gnd* in *L. monocytogenes* exposed to acid (Wemekamp-Kamphuis et al., 2004) and high pressure (Simpson and Gilmour, 1997). However, in our study only *gnd* was up-regulated on high salt products and *pykA* was up-regulated only on high salt products threated with CFS307 but on those products no significant changes in a cell length were found. It is possible that *pykA* and *gnd* are involved in the general stress response of *L. monocytogenes*.

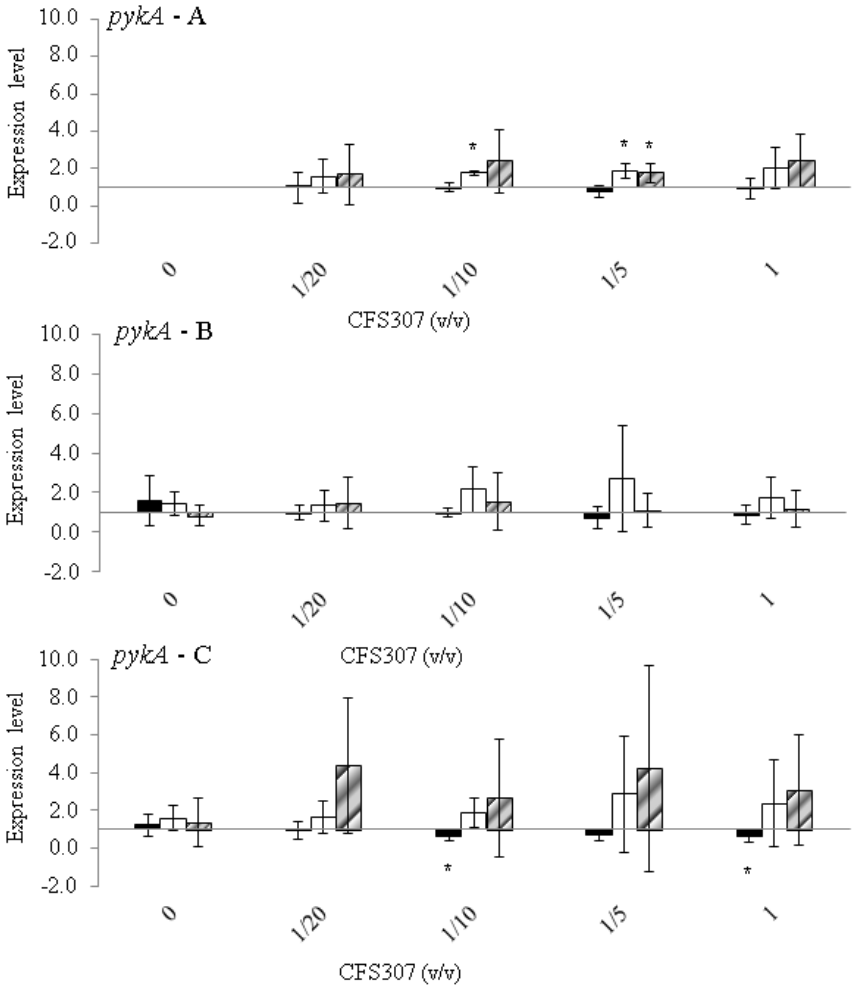


Figure 7.6 Expression levels of *pykA* (*lmo1570*) and *gnd* (*lmo1376*) [Mean ± SD (n=3)] on ham with (A) 0.55%, (B) 1.35%, and with (C) 2.35% total NaCl at day 30 (black bars), day 60 (white bars) and at day 90 (stripped bars). The y-axis indicates the dilutions of CFS307 (0, 1/20, 1/10, 1/5 and undiluted). * indicates significant difference ($P<0.05$).

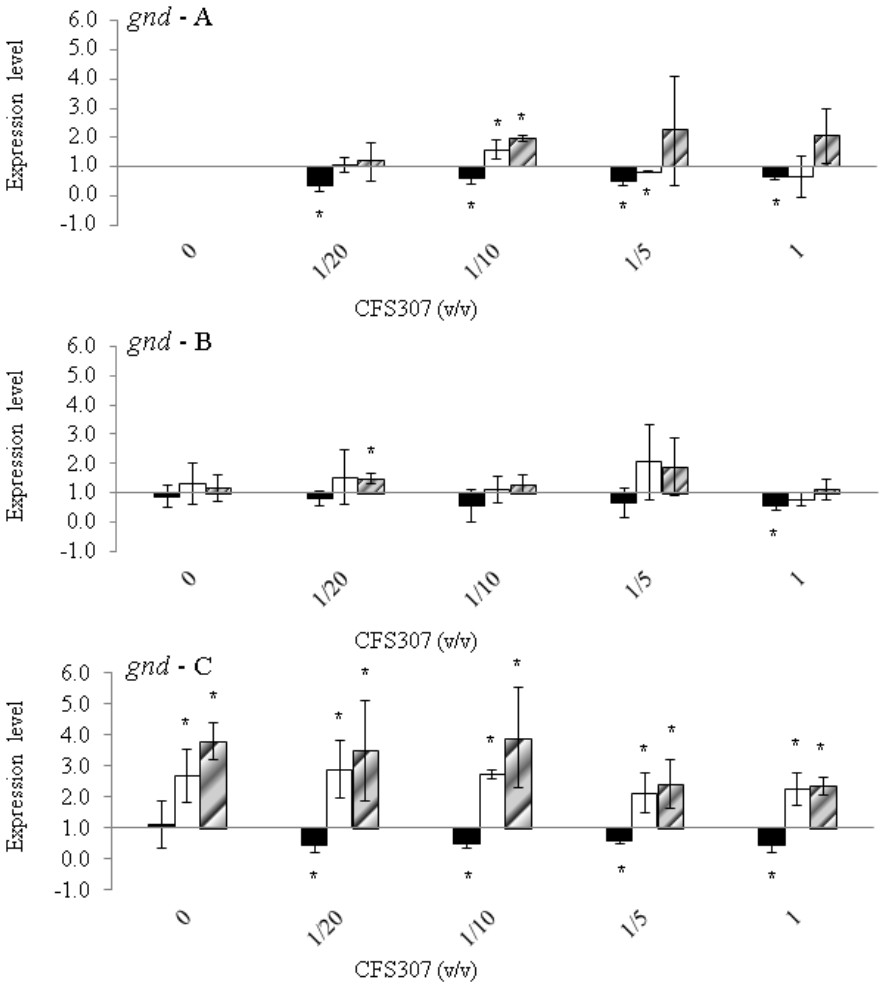
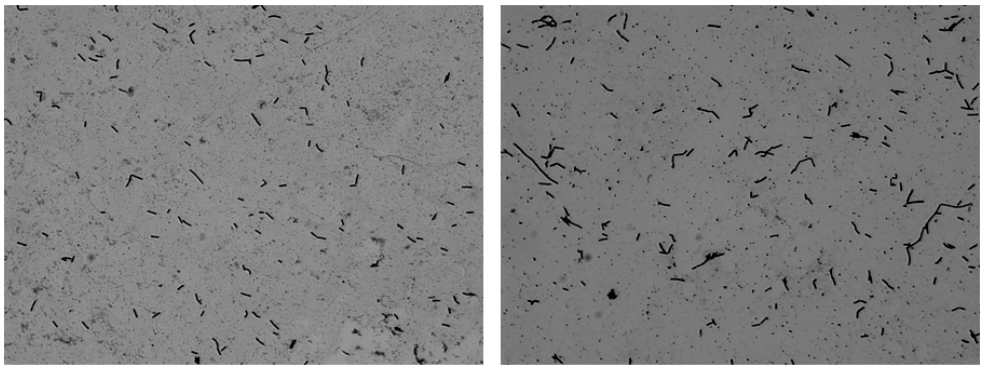


Figure 7.6 continued.

7.3.3. Cell morphology

Figure 7.7 shows examples of filamented and non-filamented *Listeria* from ham products with different concentrations of total NaCl.



(a) **(b)**

Figure 7.7 *L. monocytogenes* 08-5923 from ham under light microscope (100 *x* magnification).(a) *L. monocytogenes* 08-5923 on ham with 0.55% total NaCl after 2 months of storage; (b) *L. monocytogenes* 08-5923 on ham with 2.35% total NaCl after 2 months of storage.

After 2 months of storage, part of the cells in the population on ham with 0.55% NaCl was visibly shorter in length (Figure 7.7a) than the cells on ham with 2.35% NaCl (Figure 7.7b). Cell counts from samples (7.7a and 7.7b) on APT and on PALCAM were comparable, confirmed the microorganisms on Figure 7.7 are *L. monocytogenes*, not contamination from other microorganisms. This confirmed the observation in the difference in the flow cytometry measurement of mean FSC in *Listeria* on ham with 0.55% and 2.35% total NaCl. Vail et al. discovered that NaCl can induce filamentation in *L. monocytogenes in vitro* (Vail et al., 2012).

This study showed that *L. monocytogenes* can also form filaments in the presence of > 2% total NaCl on meat products.

7.4. Conclusions

Filamentation is a complex process that bacteria undergo when exposed to stress conditions. This study showed the involvement of genes (*ftsX*, *murZ*, *pykA* and *gnd*), which were linked with cell counts, cell length and cell morphology of *L. monocytogenes* 08-5923.

It is yet unknown why CFS307 could induce the filamentation of *L. monocytogenes* 08-5923 *in vitro* but not *in situ*. To gain further insight into global transcriptomic regulation leading to filamentation, cDNA microarray or RNA-seq experiments could be conducted to profile the differential expression of the genes in filamented *L. monocytogenes*.

This study describes the filamentation of *L. monocytogenes* 08-5923 on ham with high salt (2.35% total NaCl); filamentation of part of the *Listeria* population was observed on both CFS307 treated and untreated samples (under light microscope). An increase in FSC (compared to *Listeria* on ham with 0.55% total NaCl, no CFS307) was observed. During the first two months of storage, cells were dividing and an increase in cell counts was accompanied by a decrease in FSC.

The down-regulation of *ftsX* (cell division) and up-regulation *murZ* (peptidoglycan synthesis) is involved in filamentation. This study also suggests

that the genes encoding enzymes in generating ATP and NADPH, such as *pykA* (glycolysis) and *gnd* (pentose phosphate pathway), could participate in stress response of *L. monocytogenes* to NaCl and/or CFS307.

7.5. References

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8. General Discussion and Conclusions

Foodborne pathogens, especially *L. monocytogenes*, are a concern for the health of consumers. *L. monocytogenes* survives and grows at refrigeration temperature on RTE meats. The growth of *L. monocytogenes* is inhibited by preservatives, including NaCl. NaCl controls the growth of *L. monocytogenes* by reducing water activity a_w (Troller and Christian, 1978). With the reduction of sodium content in meat products to meet the guidelines of Sodium Reduction Strategy (Health Canada, 2010), there is the potential for an increase in the growth of *L. monocytogenes* on these products. The current work demonstrated that the length of lag phase of *L. monocytogenes* growing on aseptically-prepared ham with reduced NaCl (0.85% total) was reduced significantly compared with ham with higher concentrations of NaCl (1.35% total; Chapter 3). However, there was no significant difference in the a_w of the hams formulated with various concentrations of NaCl, which was contradictory to what is suggested in the literature that low sodium meats could have a higher water activity that can increase the growth of microorganisms (Troller, 1986). It could be that the reduction of NaCl in hams resulted in the reduction of the concentration of Cl^- ions. Chloride ions inhibit the growth of *L. monocytogenes* on RTE meats (Samapundo et al., 2013). These findings suggest that the observed difference in the length of the lag phase in *L. monocytogenes* growing on ham with various concentrations of NaCl could be a result of differences in the concentration of Cl^-.

The current work also showed that the growth of *L. monocytogenes* on RTE meats was influenced by the presence of autochthonous microorganisms – mostly lactic acid bacteria (Korkeala and Björkroth, 1997) and *Brochothrix* spp. (Jay, 2010). Some of these autochthonous microorganisms are spoilage microorganisms, which produce metabolites that cause quality defects in meats (Blickstad and Molin, 1984; Cai et al., 1998; Lyhs et al., 2004; Johansson et al., 2011). The present study described the production of EPS (levan) from the spoilage microorganism *L. gasicomitatum* C302 (Chapter 2).

Both the addition of NaCl and the presence of an autochthonous microbiota inhibited the growth of *L. monocytogenes* on ham products. The concentrations of NaCl used in this study (0.85%, 1.10% and 1.35%) were close to the specifications for sodium reduction in RTE meats. The length of the lag-phase of *L. monocytogenes* was significantly shorter on ham with 0.85% NaCl than that with 1.10% NaCl; however, this difference disappeared when an autochthonous microbiota was present. The presence of an autochthonous microbiota reduced the maximum population of *L. monocytogenes* at the end of the 36-day storage (Chapter 3).

To control the growth of *L. monocytogenes*, non-NaCl preservatives including organic acids, acetates, lactates, and bacteriocins or bacteriocin-producing organisms can be added to RTE meat (Health Canada, 2013; Levine and Fellers, 1940; Hwang et al., 2012; Mellefont and Ross, 2007; Sallam et al., 2007). These preservatives exert antilisterial effects through various mechanisms and cause

stress in *L. monocytogenes*. *L. monocytogenes* can adapt to a sublethal dose of these preservatives and develop resistance (Crandall and Montville, 1998; Gravesen et al., 2002; Vadyvaloo et al., 2004). Protective bacterial strains, which secrete antilisterial bacteriocins, have been suggested to limit the growth of *L. monocytogenes* (Duffes et al., 1999; Schöbitz et al., 1999; Greer and Dilts, 2006). The producer strain of commercially available bacteriocin preparation (Micocin®), *C. maltaromaticum* UAL307, secretes bacteriocins including CCLA, which induced stress in *L. monocytogenes* 08-5923 by down-regulation of genes or proteins that were involved in critical biological functions including cell division, cell wall synthesis, membrane function and metabolism (Chapter 5).

In addition to the growth of *L. monocytogenes*, the filamentation of *L. monocytogenes* is also an important aspect for the food industry. Filamentation of *L. monocytogenes* can potentially lead to underestimation of the actual population of *L. monocytogenes* on a food product. The meat industry intends to qualify RTE meats as Category 2B products, which according to the guidelines (Canadian Food Inspection Agency, 2012), the growth of *L. monocytogenes* should be ≤ 0.5 log CFU/g of product. Therefore, the filamentation of *L. monocytogenes* could potentially impact the accurate counts of *L. monocytogenes*. This study examined the effects of salts, acids and bacteriocins on the filamentation of *L. monocytogenes*. Salts (sodium and potassium salts of chlorides, acetates and lactates) and acids (formic acid and lactic acid) induced filamentation in *L. monocytogenes* 08-5923 *in vitro* (Chapter 7). The cell-free supernatant of *C.*

maltaromaticum UAL307 induced filamentation but not purified CCLA. It could be the salts and acids (the end metabolites of *C. maltaromaticum* UAL307) that induced filamentation but not CCLA from the cell-free supernatant of *C. maltaromaticum* UAL307. This research demonstrated that CCLA is a potent antilisterial peptide which at sublethal doses does not cause filamentation in *L. monocytogenes*.

The study of filamentation of *L. monocytogenes in situ* showed that *ftsX* was down-regulated in *L. monocytogenes* 08-5923 from ham with 2.35% total NaCl (high salt) where part of the population was in filament-form (Chapter 7). The *fts* genes are involved in cell division and filamentation in *E. coli* (de Leeuw et al., 1999; Weiss DS, 2004; Karimova et al., 2005). Prior to the current work, the role of *fts* in filamentation in *L. monocytogenes* have not been described. The screening of expression levels of different *fts* genes in the presence of various salts suggested that down-regulation of *fts* genes could be involved in filamentation (Chapter 7). However, to confirm the role of *fts* genes in filamentation, the construction of mutants with *fts* gene deleted to examine the phenotype is necessary – if deletion of the gene is not lethal. There are still gaps in the research on the mechanisms of filamentation of *L. monocytogenes*. For example, it is unknown whether there would be more genes in addition to *fts* that are critical for filament formation in *L. monocytogenes*.

Although CCLA did not induce filamentation, it causes other types of stress in *L. monocytogenes* 08-5923. The cDNA microarray study (Chapter 5) revealed that

the genes involved in cell division, cell wall synthesis and motility were up-regulated immediately (15 min) following exposure to CCLA. However, cells gradually (30 min) adapted to the sublethal stress by down-regulation of these genes. The absence of flagella was observed, as a result of down-regulation of the genes involved in assembly of flagella. The SDS-PAGE-LC-MS/MS study (Chapter 5) showed that proteins involved in protein and nucleotide synthesis were down-regulated, which is a sign of cellular stress – although in this study, the activities or the half-life of these key enzymes were not measured *in vivo* to elucidate whether it was the amount of the enzyme(s) produced in the cell or the activity of these enzyme(s) that resulted in the absence of flagella.

In addition to the factors that influence the growth of *L. monocytogenes* described in the current work, other factors, such as refrigeration temperature (Walker et al., 1990) and pH (Wijtzes et al., 1993; Tienungoon et al., 2000), influence the growth of *L. monocytogenes* on RTE meats. In this research, all of the control (*L. monocytogenes* grown on ham) and the treated samples (*L. monocytogenes* grown on ham with addition of preservatives or authchthonous microbiota) were held at the same temperature. There was no significant difference in the pH values of the control and the treated samples at any given sampling time. This study did not examine the effect of packaging (i.e., aerobic/vacuum/modified atmosphere packaging; low/high permeability) on the growth of *L. monocytogenes*. The growth of *L. monocytogenes* can be limited by using low-permeability film, regardless of gaseous atmospheres (Tsigarida et al.,

2000). All the control and treated samples throughout the experiments in the current work were packaged using the same material. Therefore, the effects from variations in the pH/temperature or packaging material between the control and the treated samples in the current work on experimental results were eliminated.

This research examined the growth, filamentation, gene expression and protein production of *L. monocytogenes* in the presence of food preservatives (NaCl, NaL/DA and CCLA) and/or autochthonous microbiota *in vitro* and *in situ*. More diverse microbial species were found on retail RTE meats labelled as sodium-reduced products than regular salt RTE meats. *Carnobacterium* spp. and *Leuconostoc* spp. were the most prevalent species on both sodium-reduced and regular retail RTE meats. The growth of *L. monocytogenes* on RTE meats was inhibited by the addition of preservatives and the presence of an autochthonous microbiota. The transcriptomic and proteomic responses of *L. monocytogenes* exposed to a sublethal dose of CCLA was profiled for the first time; and compared with the stress response to NaL/DA. CCLA and NaL/DA induced stress in *L. monocytogenes* by targeting different gene/protein regulatory pathways: CCLA mainly targeted genes involved in membrane functions such as sugar-specific transporter, cell wall synthesis and flagella synthesis; while NaLac/DA targeted genes encoding cytosoluble stress proteins such as heat shock proteins. The current work reports for the first time that *L. monocytogenes* could form filaments both *in vitro* and *in situ*. The morphology of filamented and non-filamentous cells of *L. monocytogenes* was examined under TEM. Preservatives

induced filamentation in *L. monocytogenes in vitro* but not CCLA. The qRT-PCR study of *L. monocytogenes* grown on ham products with/without NaL/DA and different concentrations of NaCl showed that down-regulation of cell division gene *ftsX* and up-regulation peptidoglycan synthesis gene *murZ* could be involved in filamentation.

Overall, this research examined the effect of reduction in the concentrations of Na/NaCl, the presence of autochthonous microbiota and the addition of preservatives on the growth, morphology, gene expression of *L. monocytogenes*. Understanding the growth and genetics of foodborne pathogens and authochthonous microbiota in sodium-reduced environments is important since these microorganisms are directly related to the shelf-life, safety and quality of the RTE meat products. Unlike many studies reported in the literature, the current experiments were conducted both *in vitro* and *in situ*. This work connected the *in vitro* and *in situ* studies with the studies on *L. monocytogenes* and authochthonous microbiota to the prevalence of these microorganisms on RTE meats from retail market. The results of these experiments would help to better understand the impact of sodium reduction on the physiology of foodborne pathogen in various environmental conditions relevant to food processing or retail market.

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APPENDIX A

Formulation of Ham Products

A.1. Formulation for a 4.62 kg batch (% out of 100/total batch weight)

3.72 kg lean pork leg (80.52%)

0.744 kg ice (16.10%)

22.68 g STPP (0.49%)

NaCl (% variable depending on experimental design)

17.01 g Prague powder #1 (0.37%; contains 94% NaCl, 6% $NaNO_2$)

3.78 g sodium erythorbate (0.08%)

113.4 g dextrose (2.45%)

A.2. Calculation of sodium concentration

3 kg lean meat

0.6 kg ice

19 g STPP

NaCl:

Sodium diacetate (0.1%): 4 g

Sodium lactate (60% solution; 1.4%): 92 g

14 g Prague powder

3 g sodium erythorbate

93 g dextrose

If making products (3.8 kg batch) containing 0.6% total Na (22.8 g Na): the 1.4% (92 g) sodium lactate and 0.1% (4 g) sodium diacetate would give: [Na] from sodium lactate and diacetate = 92 g *60% (liquid form)*23/112.06 + 4 g *23/142.08= 11.33 g + 0.65 g = 11.98 g

[Na] from STPP = 19 g * (23/367.864) = 1.19 g;

[Na] from Prague powder #1 (6% sodium nitrite and 94% table salt) = 14 g*6% * (23/69) + 14 g*94% * (23/58.44) = 0.28 g + 5.18 g= 5.46 g;

[Na] from fresh pork leg meat = 3 kg*(55mg/100g) = 1.65 g;

[Na] from sodium erythorbate = 3 g * (23/198.11) = 0.35 g;

[Na] from non-NaCl sources = 11.98 g + 1.19 g + 5.46 g + 1.65 g + 0.35 g = 20.63 g;

[Na] from non-NaCl sources excluding diacetate/lactate: 20.63 g – 11.98 g = 8.65 g.

Treatments (6.2.8):

1) Negative control for Low: target ≤ health-check 655 mg/100g [Sept 2011] (0.600% [Na])

i.e., add (22.8 g – 8.65 g)*58.44/23 = 36 g NaCl per 3.8 kg batch

2) Low: 0.600% with lactate-diacetate

i.e., add (22.8 g – 20.63 g)*58.44/23 = 5.5 g NaCl per 3.8 kg batch

3) Negative control for Regular: (1% [Na]; 38 g Na)

i.e., add (38 g – 8.65 g)*58.44/23 = 74.6 g NaCl per 3.8 kg batch

4) Regular: 1% [Na]

i.e., add (38 g – 20.63 g)*58.44/23 = 44 g NaCl per 3.8 kg batch

APPENDIX B

B.1. Replicate comparison of RPKM for coding sequences