Pressure resistance of *Escherichia coli* and *Listeria monocytogenes*

by

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Abstract

High pressure processing (HPP) is a non-thermal processing technology aimed to inactivate bacteria and improve the shelf life of food. Commercial parameters for ready-to-eat (RTE) meat products are 600 MPa for 3-7 min at cold or room temperatures. Many regulatory agencies require a 5-log reduction of pathogenic bacteria when foods are treated with HPP. The inactivation of pathogens is dependent on several factors including the food matrix. The aim of the research was to investigate impact of carbohydrate and fat on pressure resistance of *Escherichia coli* in beef and yogurt. To determine the effect of fat, four ground beef and five yogurt samples with different fat content were inoculated with resistant or sensitive strains of *E. coli* and treated at 600 MPa, 20 or 30°C for 3 min. To eliminate the effects of adiabatic heating, sample temperatures were adjusted prior to pressure treatment. The impact of HHP was dependent on strain and fat content in ground beef. When samples were cooled prior to treatment to account for adiabatic temperature changes, *E. coli* was more resistant to HPP in low fat (3%) ground beef compared to resistance in high fat (35.7%) ground beef. In yogurt, the impact of the fat content on treatment lethality was limited. The reduction of cell counts for different strains ranged from less than 2 to more than 8 log CFU/g, indicating that lethality of HHP is highly strain dependent.

To reduce the prevalence of pathogenic microorganisms such as *L. monocytogenes* and pathogenic *E. coli* in food processing facilities, extensive cleaning and sanitation practices are used. Unfortunately, bacteria form biofilms, a complex network for cells surrounded by extracellular components that not only allows for sessile cells to attach to surfaces but also increases resistance to sanitizers. The aim of this research was to investigate if sessile cells of *L. monocytogenes* in single-, dual- and multi-species biofilms on RTE chicken meat and in broth solutions are more

resistant to HPP compared to planktonic cells. Sessile cells were transferred onto RTE chicken meat using methods that mimic the transfer from a meat slicer onto the product. The RTE chicken meat and broth samples were treated at 600 MPa for 3 mins at 20 °C. *L. monocytogenes* cells in broth were more sensitive to high pressure compared to planktonic cells on meat. When using the method that mimics the transfer from slicer to meat, there was no difference in resistance between sessile and planktonic cells. This is the first study to investigate the resistance of *L. monocytogenes* cells recovered from biofilms to HPP.

L. monocytogenes biofilms are believed to contribute to its long-term persistence in food processing facilities. Once the organism establishes itself in the food processing facility it can contaminate foods products through cross contamination from the environment. The final aim of this research was to use comparative genomics with total of 398 *Listeria* genomes to determine if there was a correlation between source of isolation and the ecology of different strains of *L. monocytogenes*. *L. monocytogenes* is a diverse species with 13 serotypes and is grouped into four genetically diverse lineages (I-IV). Strains in lineage I are more frequently associated with human clinical cases and are more virulent than strains in lineage II. Strains of *L. monocytogenes* belonging to phylogenic clade lineage II are more likely to persist in food processing facilities compared to strains from lineage I. Furthermore, an overall trend was observed in which a higher proportion of lineage II strains were isolated from food processing facilities and meat. The pressure resistance strains isolated from meat and dairy processing facilities were evaluated and resistance between lineage I and II was compared. At 400 MPa, lineage I strains were more resistant to HPP compared to lineage II strains but there was no difference when treated at 500 MPa or greater. The different contamination routes and phenotypic response to intervention methods observed by *L. monocytogenes* from lineage I and II demonstrate the need for different control measures to

minimize the spread and contamination of this pathogen in food processing facilities. With optimization, HHP could be a potential post processing intervention to inactive *L. monocytogenes* and pathogenic *E. coli*.

Preface

Strains of *Listeria monocytogenes* were provided by Ministère de l'Agriculture, des Pêcheries et de l'Alimentation du Québec (MAPAQ) and HPB *L. monocytogenes* strains were provided by Franco Pagotto at Health Canada. A portion of DNA sequence libraries in Chapter 4 were done by Oregon State University Centre for Quantitative Life Science in collaboration with Dr. J Kovacevic. I conducted the data analysis and comparative genomes with assistance of Dr. D Simpson and Dr. M Gänzle. Dr. S Otto completed the logistic regression analysis model.

A version of Chapter 2 has been submitted to European Food Research and Technology.

Dedication

TO MY LOVING PARENTS

Thank you for your unconditional love and support. Your hard work, dedication and sacrifices made to move to this country have given me the opportunity of an education and I will forever be grateful.

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APT- All purpose tween agar
a_w-water activity
BC- benzalkonium chloride
BHI-brain heart infusion broth
CC-clonal complex
CFU/ colony forming units
CHO- carbohydrates
dTSB-diluted tryptic soy broth (10%)
EMC-extracellular components
EPS-extracellular polymeric substance
FCS-food contact surfaces
FDA- food and drug administration
gDNA-genomic DNA
HPP-high pressure processing
LLO-listeriolysin O
LB- Luria-Bertani
MEE-multilocus enzyme electrophoresis
MES- 2-(N-morpholino)ethanesulfonic acid
MLST-multilocus sequence typing
MPa-Megapascal
NFCS-non-food contact surfaces
PALCAM- Polymyxin Acriflavin Lithium-chloride Ceftazidime Esculin Mannitol
PBS-phosphate buffered saline
PFGE- pulse field electrophoresis
QAC-quaternary ammonium compound
RPM- revolutions per min
RTE-Ready to eat
SDS-sodium dodecyl sulfate
SNP-single-nucleotide polymorphism
SS-stainless steel
TSA- tryptic soy agar
TSB-tryptic soy broth
TTSS- type III secretion system
UHPH- ultra-high pressure homogenization

1.0 Introduction and Literature Review

1.1 Introduction

Each year in Canada, *Listeria monocytogenes* and *Escherichia coli* O157:H7 are estimated to cause 178 and 12,800 cases of foodborne illness, respectively, (Thomas et al., 2013). *E. coli* is among the top three pathogens to cause illness *L. monocytogenes* does not have the highest case frequency but does have the highest fatality rate of 20-30% Thomas et al., 2013, 2015). Outbreaks of listeriosis are often associated with post processing contamination of ready-to-eat meat products (RTE) (Kurpas et al., 2018). Beef cattle are typically the reservoir for *E. coli* O157:H7 and contamination may occur at any stage of processing (Bell, 1997; McEvoy et al., 2000). In 2008, the largest listeriosis outbreak occurred in Canada, resulting in 57 illnesses and 22 deaths (Weatherill, 2009). The outbreak was linked to improper cleaning and sanitization of slicing equipment, which allowed the transfer of *L. monocytogenes* onto RTE deli meat during slicing and packaging (Weatherill, 2009). In 2012, the largest beef recall in Canadian history occurred after detection of *E. coli* O157:H7 (Currie et al., 2019). These outbreaks along with others have prompted revisions and increased controls to increase food safety in Canada. Although improved control measures have been put in place to reduce the prevalence of *L. monocytogenes* and *E. coli* O157:H7, outbreaks still occur (Buchanan et al., 2017; Chen et al., 2017; Coulombe et al., 2020; Omer et al., 2018).

1.2 High pressure processing

High pressure processing (HPP) is a non-thermal technology aimed to inactivate microorganisms and extend shelf life. Applied treatment pressure varies depending on the aim of the treatment, but

generally range from 100-900 MPa (Campus, 2010). Adiabatic temperature changes within a sample occur when there is an increase in pressure. During pressurization the temperature of the product increases but returns to initial temperature during depressurization (Landfeld et al., 2011; Patazca et al., 2007). Rasanayagam et al., (2003), found that there is an increase of 3 °C/100 MPa for water and up to 9 °C/100 MPa for oils and fats. The relationship between adiabatic temperature changes and fat content has yet to be studied in foods. An advantage of HPP compared to conventional thermal treatments is that the pressure is applied isostatically and uniformly regardless of packaging or volume of food product (Yordanov & Angelova, 2010). Depending on what product is treated by HPP, changes to nutritional and sensory characteristics are less pronounced when compared to thermal treatments. Many studies have found that treatment of fresh juices by HPP, little changes to sensory characteristics were observed (Aaby et al., 2018; Chang et al., 2017; Song et al., 2022; Zhang et al., 2019). However, in raw meat products, HPP changes the color and texture of the product which can be challenging as consumer perception is an important driving force for processors (Carlez et al., 1995; De Alba et al., 2012; Schilling et al., 2009). The change in meat color from bright red to brown is a result of oxidation of oxymyoglobin to metmyoglobin (Bak et al., 2019). Current application of HPP is being used to treat food products such as guacamole, juices, seafood and meat and continues to grow worldwide (Rastogi et al., 2007).

1.2.1 Mechanism of inactivation

The degree of inactivation by HPP is varies among organisms. Gram-positive bacteria are generally more resistant to pressure compared to gram-negative; however, this is dependent on processing parameters (Smelt, 1998). The cell membrane is the primary target of pressure induced

damage in vegetative bacteria (Cheftel, 1995; Smelt, 1998). Applied pressure permeabilizes the outer membrane causing deformation and disruption of ion exchange in the cell. An increased uptake of fluorescent dye demonstrated membrane damage by *E. coli* because of applied pressure as low as 100 MPa (Pagán & Mackey, 2000). In addition to membrane damage, loss of function associated with the cytoplasmic membrane proteins and enzyme as also been described (Mañas & Mackey, 2004; Molina-Höppner et al., 2004; Simpson & Gilmour, 1997).

While HPP causes damages to the cell membrane, this does not always result in cell death. Sublethal injury caused by HPP has been described in the literature (Kimura et al., 2017; Ma et al., 2019; Nasiłowska et al., 2021). In low pH foods such as fruit juices, a decrease in bacterial counts was observed when stored at refrigeration temperatures (Garcia-Graells et al., 1998; Linton et al., 1999). Damage to cell membranes and cell repair after HPP is believed to explain the increased bactericidal effect observed during storage in low acid foods (Jordan et al., 2001). In higher pH foods such as milk, *L. monocytogenes* and *E. coli* were recovered at 4 °C after pressure treatment (Bozoglu et al., 2004). The recovery of sublethal injured cells after HPP poses challenges for application as this has the potential to lead to foodborne outbreaks.

1.2.2 Factors affecting pressure inactivation

Although HPP has proven to be an effective method for inactivation of bacteria in many food products, resistance to applied pressure has been observed. Many factors influence the magnitude of sensitivity to pressure, these include bacterial growth phase, parameters of treatment, and food matrix.

Bacterial response to HHP is dependent on the physiological state of the cell prior to exposure to high pressure. *E. coli* grown to stationary phase are more resistant to pressure compared to cells in the exponential phase (Benito et al., 1999; Pagán & Mackey, 2000). The increase in pressure resistance observed in the different physiological states are proposed to be a result of increased membrane fluidity (Casadei et al., 2002). Not only was the same effect observed for *L. monocytogenes* in UHT milk when treated with HPP, the authors noted a difference in the inactivation kinetics of the strain when grown in the different phases. A log-linear inactivation for mid-exponential phase cells was noted whereas for mid-stationary phase a tailing effect was observed (Hayman et al., 2007). The tailing effect is a phenomenon that occurs when a small portion of the bacterial population survives the HHP treatment. The tailing effect can be problematic for processors and should be considered when optimizing parameters for inactivation as recovery of cells during storage cause a public health concern.

An increase in microbial inactivation is observed when pressure and or the time of treatment is increased. O' Reilly et al., (2000), observed a higher degree of inactivation for *E. coli* and *Staphylococcus aureus* in a cheese slurry when applied pressure was increased from 300 to 400 MPa. The authors also concluded that while a longer process time would be sufficient to inactivate bacteria at the same pressure, an increase in pressure alone has a greater antimicrobial impact. Similar results were observed when black tiger shrimp inoculated with *E. coli* were treated with various pressure and holding times. The increased pressure and holding time increased the inactivation; however, there was increased lipid oxidation, and significant changes to color and texture of the shrimp (Kaur et al., 2016). Finally, increasing or decreasing process temperature affects the lethality of the treatment. Teixeira et al., (2016), evaluated the impact of process

temperature on inactivation of *L. monocytogenes* in broth, as treatment temperature increased the inactivation increased. Interestingly, highest resistance was observed at a process treatment of -5 °C compared to other temperatures evaluated and was thought to be a result of rapid adjustment of membrane fluidity.

The food matrix plays a significant role in bacterial inactivation. In liquid products such as juices, milk, or broth, bacteria are more sensitive to HPP compared to when they are on meat. The D-value is time required to achieve a 1 $\log_{10}$ bacterial reduction when a treatment is applied. The D-value for *L. monocytogenes* at 600 MPa in food and broth varies depending on the matrix. Dogan & Erkmen, (2004) observed the highest D-value in milk (2.43 mins) and the lowest value in orange juice (0.87 mins). The acidity of the juice could increase the inactivation. *E. coli* O157:H7 is more resistant to pressure in milk than it is in poultry meat or in phosphate buffered saline (PBS) when treated at 600 MPa for 15 mins (Patterson et al., 1995). Li et al., (2016) and Garcia-Hernandez et al., (2015) found the highest resistance in beef, followed by tzatziki (dairy based sauce) and tomato juice when treated at 600 MPa for 5 mins (beef) and 3 mins (tzatziki and tomato juice). The increased resistance of bacteria in meat has been reported in multiple meat products (dry cured ham, Bover-Cid et al., 2015; RTE cooked meat, Hereu et al., 2012; beef carpaccio, De Alba et al., 2012; and (beef steak, Li & Gänzle, 2016), but the actual mechanism of resistance remains unclear.

1.3 Shiga toxin producing *Escherichia coli*

Escherichia coli strains are gram-negative microorganisms that are mostly commensal but various pathogenic strains have been described. *E. coli* are a diverse species that is characterized by shared O (lipopolysaccharide, LPS) and H (flagellar) and K (capsular) surface antigens (Chart et al.,

1991). If an isolate only contains the O antigen it is defined as a serogroup, while combination of O and H antigens is defined as a serotype (Nataro & Kaper, 1998). Pathogenic *E. coli* O157:H7 was first described as a human pathogen after causing two foodborne outbreaks in 1982 linked to undercooked beef patties (Riley et al., 1983). *E. coli* O157:H7 and other enterohemorrhagic *E. coli* are highly virulent and have a low infectious dose (<10) cells to cause illness (Kaper et al., 2004). The mechanism of infection is highly complex, in summary, the bacterium will attach to epithelium cells by formation of attaching and effacing (A/E) lesions that require proteins that are encoded in locus of enterocyte effacement (LEE) pathogenicity island (PAI) (Jerse et al., 1990). Once attached, an actin-rich pedestal will form from the host cell to underneath the bacteria which is hypothesized to keep bacteria in place (Celli et al., 2000). A needle like structure is formed by the bacterial cell using type III secretion system (TTSS) between the bacteria and the host cell in which virulence proteins such as toxins (Shiga toxin) are passed into the host cell (Hueck, 1998). The Shiga toxin causes damage to the intestinal endothelium, hemorrhaging, bloody diarrhea in severe cases kidney damage or death can occur (Chan et al., 2016).

1.3.1 Ecology of *E. coli*

A major reservoir for *E. coli* O157:H7 are ruminant animals (Borczyk et al., 1987; Wells et al., 1991; Zhao et al., 1995). *E. coli* O157:H7 was isolated from fecal samples after an outbreak linked to a visit to a dairy farm and the consumption of unpasteurised milk (Borczyk et al., 1987). Chapman et al. (1993), isolated *E. coli* O157:H7 from bovine rectal swabs and meat carcass after slaughter. Not only is *E. coli* O157:H7 found in cattle, it has been isolated in sheep and pig (Chapman et al., 1997). Kudva et al., (1996), isolated *E. coli* O157:H7 in fecal samples of asymptomatic sheep, 31% of the samples tested positive and interestingly the prevalence of the

pathogen was higher in June but in November, *E. coli* O157:H7 was not detected. The seasonal variation in *E. coli* O157:H7 has also been observed in cattle (Hancock et al., 1994). Finally, *E. coli* O157:H7 has also been isolated from deer and other animals (Heuvelink et al., 1999; Renter et al., 2001; Rice et al., 2003).

1.3.2 Routes of contamination for shiga-toxin producing *E. coli*

Transmission of shiga toxin producing *E. coli* O occurs after the consumption of contaminated meat and dairy products that have not been treated with sufficient kill step to inactivate the microorganism. Consumption of undercooked meat has caused multiple outbreaks. In 1993, a large *E. coli* O157:H7 outbreak occurred linked to undercooked beef burgers killing 4 children (CDC, 1993). As a result of this outbreak major improvements were made in meat safety (Rangel et al., 2005). Although changes have been made, *E. coli* O157:H7 outbreaks linked to undercooked meat continue to occur (CDC, 2014b; Currie et al., 2019; Watahiki et al., 2014). Foodborne outbreaks linked to shiga toxin producing *E. coli* have been linked to other food products such as dairy (Gaulin et al., 2012; Jenkins et al., 2022; Keene et al., 1997), produce (Buchholz et al., 2011; CDC, 2012; Coulombe et al., 2020; Hilborn et al., 1999; Michino et al., 1999), and cookie dough (Neil et al., 2012). In 2000, 7 people died in Ontario after consumption of improperly treated water contaminated with *E. coli* O157:H7 (Holme, 2003). Manure was the source of contamination. Cattle can shed shiga-toxin producing *E. coli* in feces, which can result in contamination of feed, soil and water or humans through direct contact (Heron et al., 1993; Salvadori et al., 2009; Trevena, 1999). Once shed, shiga toxin producing *E. coli* can contaminate food products and cause outbreaks.

1.4 Listeria monocytogenes

L. monocytogenes is a gram-positive pathogenic bacterium that causes foodborne outbreaks and is particularly a concern for the elderly, immune compromised and pregnant women. There are 13 serotypes of *L. monocytogenes* that are grouped into four genetically diverse lineages (I-IV) (Nadon et al., 2001; Orsi et al., 2011; Salcedo et al., 2003; Ward et al., 2010). Serotyping differentiates *L. monocytogenes* based on the expression of somatic (O) and flagellar (H) antigens on the cell surface. Somatic antigens divide serogroups into 1/2 and 4 and flagellar antigens subdivide serogroups into serovars 1/2a, -b, -c and 4b (Seeliger & Hohne, 1979). Strains of serotypes 1/2b, 4b, 3b and 3c are grouped into lineage I, while 1/2a 1/2c and 3a serotypes are grouped into lineage II. Lineage III and IV predominately include 4a, 4b and 4c serotypes (Doumith et al., 2004; Nadon et al., 2001). Using multiplex PCR, marker genes are used to differentiate serotypes (Doumith et al., 2004). Although serotype 4b is found in both lineage I and III, district molecular features were discovered while using the proposed marker gene ORF 2110 (Liu et al., 2006). In addition to lineage and serotype characterization, multilocus sequence typing (MLST) analysis is another characterization method that amplifies seven housekeeping by PCR. A number is given for each allele and a sequence type is assigned to each isolate that matched the known allele (Ragon et al., 2008; Sabat et al., 2013). These approaches are often used for epidemiological surveillance and source tracing of *L. monocytogenes* strains that cause foodborne outbreaks. Within lineage I and II, serovar 1/2b, 4b and 1/2a are responsible for the majority of human listeriosis cases. Serovar 1/2a and 1/2b are frequently isolated in food products but serovar 4b strains cause over 50% of listeriosis worldwide despite being less common in food products (Cartwright et al., 2013; Maury et al., 2016). The pathogenicity of *L. monocytogenes* is mediated

by various virulence factors found within the genome or clustered in pathogenicity islands (LIPI-1,-3, and -4). Internalin locus *inlAB* and LIPI-1 allows for the internalization of the pathogen into non-phagocytic cells (epithelial and endothelial cells) (Gaillard et al., 1991; Quereda et al., 2021). Once in the host cell, *L. monocytogenes* escapes vacuoles using listeriolysin O (LLO) and two phopholipases then replicating and spreading cell-to-cell by means of actin-assembly-inducing protein (ActA) (Radoshevich & Cossart, 2018; Wagner et al., 2021). While most *L. monocytogenes* strains contain the internalin locus and LIPI-1, other pathogenicity islands are only found in some lineages. LIPI-3 is conserved in a subset of lineage I strains and LIPI-4 in CC4 strains, approximately 30% lineage II isolates have a truncation in the *inlA* gene and LIPI-4 has been identified in some lineage III and IV isolates (Cotter et al., 2008; Jacquet et al., 2004; Maury et al., 2016; Moura et al., 2016; Orsi et al., 2011).

1.4.1 Ecology of *L. monocytogenes*

L. monocytogenes is ubiquitous in nature and has been isolated from environmental sources such as water, soil, manure and produce (Farber & Peterkin, 1991). Not only are ruminant animals a reservoir for *L. monocytogenes*, but it has also been isolated in other wildlife animal species such as deer, bear, and birds (Hurtado et al., 2017; Iida et al., 1991; Weindl et al., 2016). The growth condition of *L. monocytogenes* is influenced by various factors, including temperature, pH, water activity and salt content. The temperature for growth ranges from 0-45 °C with an optimal temperature of 30-37 °C (Farber & Peterkin, 1991). Growth and survival of *L. monocytogenes* in harsh environments with a broad pH range of 4.3-9.6, high salt concentration (>10%) and low water activity (a_w) (0.90) has been documented (Nolan et al., 1992; Patchett et al., 1992).

1.4.2 Routes of contamination

L. monocytogenes has been isolated from various sources and is one of the few foodborne pathogens that can contaminate food products multiple ways. At the farm level, *L. monocytogenes* can be shed in fecal matter of infected symptomatic and asymptomatic ruminant animals (Bandelj et al., 2018; Hurtado et al., 2017). Once shed, the organism can contaminate irrigation water or manure used in produce production. With minimal processing after harvest, if *L. monocytogenes* is present on produce it can be transmitted to humans during consumption and cause illness. In 1981, untreated manure from sheep with mastitis from an udder infection with *L. monocytogenes* was used to fertilize cabbage. The cabbage was used in coleslaw and caused a foodborne outbreak resulting in 42 cases of listeriosis (Schlech et al., 1983). Outbreaks linked to contaminated irrigation water have not been reported; however, *L. monocytogenes* has been isolated from irrigation water (MacGowan et al., 1994; Stea et al., 2015). While contamination on produce can occur from contaminated water or manure, in 2014 *L. monocytogenes,* was isolated from food contact surfaces after killing seven people in an outbreak linked to apples (Angelo et al., 2017).

L. monocytogenes contamination of ready-to-eat (RTE) foods occurs through cross contamination from the processing facility onto the finished product (Lin et al., 2006; Muhterem-Uyar et al., 2015; Nesbakken et al., 1996). *L. monocytogenes* has been isolated from food contact surfaces (FCS) such as cutting boards, knives, mincers, and non-food contact surfaces (NFCS) such as conveyor belts, floors, and drains (Kushwaha & Muriana, 2009; Lin et al., 2006). Cross contamination from any of these sources can introduce the bacterium to the food product or onto another surface and can result in an outbreak or spread of *L. monocytogenes* from one spot to another. In 2008, a listeriosis outbreak occurred in Canada resulting in 22 deaths. Cross-

contamination from meat slicers onto the sliced deli meats prior to packaging was implicated as the source of the organism (Weatherill, 2009). In the second deadliest USA *L. monocytogenes* outbreak in 2011, *L. monocytogenes* was isolated from the cooler and carrier truck where the pathogen was transferred onto cantaloupe and subsequently resulted in 30 deaths from listeriosis (McCollum et al., 2013). Recently, *L. monocytogenes* was isolated in the floor drains after a multistate outbreak of listeriosis was linked to ice cream killing 3 people (Conrad et al., 2023). To reduce the spread of bacteria throughout food processing facilities and onto food products, cleaning and sanitization programs aim to prevent contamination of NFCS and FCS and minimize spread throughout the facility.

1.5 Pathogen control in food processing facilities

To reduce the prevalence of pathogenic microorganisms such as *L. monocytogenes* and *E. coli* O157:H7 in food processing facilities, extensive cleaning and sanitation practices are in place. Cleaning refers to the physically and chemically remove food and debris from the equipment (Schmidt, 1997). During the cleaning step, if machinery can be disassembled, it is taken apart and each part is individually cleaned. Sanitization is a process is that reduces bacterial contamination by 5-logs in 30 seconds (Schmidt, 1997). In food processing facilities, the three major groups of sanitizers used include halogen-based compounds (eg. hypochlorite), peroxides (eg. peracetic acid) and quaternary ammonium compounds (Quats) (eg. benzalkonium chloride (BC)) (Aryal & Muriana, 2019; Donaghy et al., 2019; Schmidt, 1997).

1.5.1 Effect of sanitizers

The effects of commercial sanitizers on *L. monocytogenes* and *E. coli* O157:H7 have been investigated on planktonic and sessile cells. Antimicrobial activity of sanitizers is more effective in inactivation of bacterial cells in suspension (planktonic) compared to sessile cells (Cruz & Fletcher, 2012).

Bang et al., (2014), investigated the effects of two halogen-based sanitizers, *E. coli* O157:H7 biofilms reduced below detection limited when treated with sodium hypochlorite (NaOCl) for 15 minutes. Furthermore, the same level of inactivation was achieved when treated with chlorine dioxide (ClO_2) for a shorter time. Interestingly, Aryal & Muriana, (2019), found *L. monocytogenes* biofilms were more sensitive to NaOCl compared to *E. coli* O157:H7 biofilms when treated with the same concentration. The authors concluded a shorter treatment time at a higher sanitizer concentration resulted in reduction below detection level for *L. monocytogenes* biofilms compared to *E. coli* O157:H7 biofilms.

Peroxyacetic acid is an oxidizing agent that has a wide scope of action against both gram-positive and gram-negative bacteria (Ding & Yang, 2013). A 9.2 log reduction in planktonic cells was achieved when cells were treated for 5 min at 4 °C with 200 ppm peroxyacetic acid-hydrogen peroxide sanitizer (Svoboda et al., 2016). After exposure to 1% hydrogen peroxide for 30 mins *L. monocytogenes* was inactivated by the treatment when grown under planktonic conditions (Yun et al., 2012). Peroxyacetic acid was the most effective sanitizer reducing *L. monocytogenes* and *E. coli* O157:H7 biofilms below detection level within 5 mins (Aryal & Muriana, 2019).

Quaternary ammonium compounds are less effective on gram-negative bacteria compared to gram-positive (Gerba, 2015). At the manufacturer's recommended concentration, a 5-log reduction of planktonic *L. monocytogenes* can be achieved after 15 min of treatment with a quaternary ammonium compound (QAC) (Carballo & Araújo, 2012). Hua et al., (2019), determined that an increase in sanitizer concentration or extended treatment time increased the lethality of QAC on *L. monocytogenes* biofilms but a 5-log reduction was not attainable. Planktonic and younger sessile cells *L. monocytogenes* (6 h and 1 day) are more sensitive to QAC compared to cells in matured biofilms grown for 7 days (Chavant et al., 2004). Aryal & Muriana, (2019), found that *E. coli* O157:H7 biofilms are more resistant to QAC sanitizers compared to *L. monocytogenes* biofilms. A treatment of 15 minutes resulted in > 7 log reductions of *L. monocytogenes* after 2 hours, whereas *E. coli* O157:H7 was only reduced by 4 logs.

The effects of commercial sanitizers on *L. monocytogenes* have been investigated on planktonic and sessile cells. Antimicrobial activity of sanitizers is more effective in inactivation of cells of *L. monocytogenes* in suspension compared to sessile cells (Cruz & Fletcher, 2012). At the manufacturer's recommended concentration, a 5-log reduction of planktonic *L. monocytogenes* can be achieved after treatment of cells with a quaternary ammonium compound (QAC) for 15 mins (Carballo & Araújo, 2012). Hua et al., (2019), determined an increase in sanitizer concentration or extended treatment time increased the lethality of QAC on *L. monocytogenes* biofilms but a 5-log reduction was not attainable. Planktonic and younger sessile cells *L. monocytogenes* (6 h and 1 day) are more sensitive to QAC compared to matured biofilms grown for 7 days (Chavant et al., 2004). A 9.2 log reduction in planktonic cells was achieved when treated for 5 min at 4 °C with 200 ppm peroxyacetic acid-hydrogen peroxide sanitizer (Svoboda et al.,

2016). After exposure to 1% hydrogen peroxide for 30 mins *L. monocytogenes* was inactivated by the treatment when grown under planktonic conditions (Yun et al., 2012). Although sanitizers are effective on planktonic cells, the increased resistance of sessile cells has been hypothesized to contribute to its persistence in food processing facilities (Pan et al., 2006).

1.6 Biofilms

In food processing facilities, food contact surfaces are often grades 304 and 316 stainless steel due to its high thermal conductivity and resistance to corrosion (Gedge, 2008). Bacteria can attach to food contact surfaces and grow into microcolonies and produce biofilms (Costeton et al., 1995). There are four stages of biofilm formation; (1) initial attachment, (2) microcolony formation, (3) biofilm maturation and (4) detachment or dispersal (4) (Davey & O'toole, 2000; Landini et al., 2010). Bacterial cells within the biofilm are called sessile cells and are phenotypically different from their planktonic state. Although some strains of bacterial species can produce biofilms, single species biofilms are rare in nature (Giaouris & Simões, 2018). The microbial diversity in food processing facilities is vast and includes *Pseudomonas, Enterobacteriaceae*, *Acinetobacter* and *Bacillus* (Fagerlund et al., 2021). In multispecies biofilms, a synergetic interaction between species can increase their resistance to sanitizers and contribute to their persistence in the food processing facility(Burmølle et al., 2006; Simões et al., 2009). Sessile cells of *L. monocytogenes* and *E. coli* O157:H7 have displayed higher tolerance and resistance to environmental stressors, such as acid and sanitizers (Lundén et al., 2008; Ryu & Beuchat, 2005; Uhlich et al., 2010). If the steps implemented to eradicate the contamination of bacteria from contact surface to food product are ineffective the risk of outbreak increases, and persistence remains.

1.6.1 *L. monocytogenes* biofilms

Biofilm formation by *L. monocytogenes* is hypothesized as one reason for its persistence in food processing facilities (Moretro & Langsrud, 2004). *L. monocytogenes* can form biofilms on materials, such as stainless steel, commonly found in food processing facilities (Blackman & Frank, 1996; Bonsaglia et al., 2014). Multiple structures of *L. monocytogenes* biofilms have been described including monolayers, or two layers of cells with different density on stainless steel (Chae & Schraft, 2000; Chavant et al., 2002) and a honeycomb-like structure (Guilbaud et al., 2015; Marsh et al., 2003). In addition to the network of cell within the biofilm, *L. monocytogenes* produces extracellular components (EMC) during the maturation step in biofilm formation. The nature of the *L. monocytogenes* EMC material remains unknown; however, it has been proposed to consist of polysaccharides, proteins and extracellular DNA (Colagiorgi et al., 2016).

1.6.2 Factors contributing to biofilm formation

The ability of *L. monocytogenes* to form biofilms is dependent on several factors including the strain. The relationship between lineage and biofilm formation has been investigated but remains unclear. Borucki et al., (2003), assessed the biofilm capability of 80 strains and determined that strains in lineage II were better biofilms formers compared to lineage I strains. When assessing the effect of various growth media and temperature, Lee et al., (2019), determined that strains from lineage II are more efficient biofilms formers at 37 °C in brain heart infusion broth (BHI), BHI+NaCl and diluted BHI+NaCl compared to strains that belong to lineage I. However, Djordjevic et al., (2002), concluded that lineage I strains are better biofilm formers than lineage II and III strains. Finally, Weiler et al., (2013), concluded that biofilm formation was not lineage or

serotype specific but rather strain specific. While the relationship between lineage and biofilm formation remains inconclusive, it is clear intraspecies variability exists.

Within food processing facilities, the resident microbiota is dependent on what food product is produced. Raw meat and fish processing facilities are predominately dominated by *Pseudomonas, Acinetobacter* and *Enterobacteriaceae* and in dairy facilities the prevalent organisms are lactic acid bacteria, *Staphylococcus* and *Bacillus* (Maes et al., 2019; Møretrø & Langsrud, 2017). Interactions of *L. monocytogenes* and *Pseudomonas* show increased attachment and colonisation onto food contact surface (Puga et al., 2018; Sasahara & Zottola, 1993). In a dual species biofilm with *Bacillus,* a cooperative and competitive interaction with *L. monocytogenes* was observed depending on the strain on *Bacillus* used (Alonso et al., 2020).

Nutrient availability plays a significant role in biofilm formation of *L. monocytogenes*, as higher NaCl concentration increases biomass production in a biofilm (Lee et al., 2019; Pan et al., 2010). In low nutrient conditions or starved conditions, the reports on biofilm formation of *L. monocytogenes* are inconclusive. Some researchers have found that in a nutrient dense medium, *L. monocytogenes* Scott A produces more biofilm compared to that in a diluted medium. Differences in biofilm formation were also reported by Folsom et al., (2006). When Cherifi et al., (2017), evaluated the biomass of two strains of *L. monocytogenes* grown in microfluidic conditions, they found that the biomass and the number of dead cells was larger in nutrient poor media compared to that in nutrient rich media; however, in static conditions, biofilm biomass and the number of live cells were higher in nutrient rich media. Better biofilm formation in nutrient poor media compared to that in nutrient rich media has been reported by others (Kadam et al.,

2013). Adhesion of the *L. monocytogenes* to food contact surfaces can occur in the presence of limited nutrients and can result in contamination on food.

Growth temperature is an important factor to consider when assessing biofilm formation of *L. monocytogenes* as the temperature in a food processing facility could vary depending on the product produced. During production, the temperature in production facilities for perishable foods, including RTE meats is typically <10 °C. This is intended to reduce the growth of bacteria; however, it would only slow the growth of *L. monocytogenes*.

Several studies have investigated the effect of temperature on biofilm production by *L. monocytogenes.* At higher temperatures, *L. monocytogenes* produces a higher biomass of biofilm compared to that at lower temperatures (Bonsaglia et al., 2014; Di Bonaventura et al., 2008; Kadam et al., 2013; B. H. Lee et al., 2019). Cold stress tolerance of *L. monocytogenes* contributes to its ability to survive and grow at cooler temperatures used in food processing facilities. Cold-adapted conditions are conditions in which cells have been grown at temperatures below ideal growth temperature (typically refrigeration) (Tasara & Stephan, 2006). Cold shock is a physiological response of a cell when subjected to a sudden downshift in temperature (Thieringer et al., 1998). Although *L. monocytogenes* can form biofilms at refrigeration temperatures, the studies noted above grew the inoculum at 30-37 °C prior to biofilm formation. When strains of *L. monocytogenes* are cold adapted (grown at 4 °C), strains prefer growth in a planktonic state rather than as sessile cells on surfaces; however, when subjected to cold stress (pre-cultured at 37 °C followed by biofilm growth at 4 °C) better adhesion to surfaces occurs (Lee et al., 2017). Once *L. monocytogenes* is introduced to a food processing facility from the contamination source, it can

adhere to surfaces, contaminate food products, adapt to cold temperatures, and grow on foods during storage (Lee et al., 2017).

1.6.3 Genes involved in biofilm formation

Multiple genes have been identified to play a role in biofilm formation by *L. monocytogenes,* but the exact genetic factors involved is unclear. Using a genome-wide mutagenesis approach flagellar associated genes and quorum sensing genes were identified as important to biofilm formation by *L. monocytogenes* (Alonso et al., 2014; Chang et al., 2012). Studies have suggested that virulence genes are closely related to biofilm formation by *L. monocytogenes.* Lemon et al., 2007, concluded motility of *L. monocytogenes* during initial stages of biofilm formation is important for attachment. Quorum sensing is a communication system in which cells within the biofilm can communicate by producing signal molecules (Waters & Bassler, 2005). Deletion of *argD* a gene encoding the precursor of putative quorum sensing peptide resulted in decreased biofilm formation (Riedel et al., 2009). Finally, a virulence transcription factor PrfA plays a role in biofilm formation, deletion in *prfA* (Δ*prfA*) resulted in defective biofilm formation for several strains (Lemon et al., 2010).

1.7 Persistence of *L. monocytogenes* in food processing facilities

As mentioned above, *L. monocytogenes* can contaminate foods products through cross contamination from the environment. The organism can establish itself in the processing facility and persist over long periods of time. Persistence is defined as the long-term survival of a pathogen in a food processing plant (Ferreira et al., 2014). A strain is considered persistent when and isolate of the strain is repeatedly found over a minimum of 3 months or more (Cherifi et al., 2018; Ortiz et al., 2016). To determine if isolates when isolated at different times are the same strain, single-

nucleotide polymorphism (SNP) analysis is used. SNP analysis involves counting the number of nucleotide difference between two or more sequences to measure genetic differences. If the SNP ≤ 20 the isolates in which the genetic material was extracted from are considered identical (Pightling et al., 2018). Food processors use cleaning and sanitation practices to control for contamination of pathogens and reduce the spread within the facility. Phenotypic and genotypic serotype characterization methods are used to identify persistent strains. The persistence of *L. monocytogenes* in food processing facilities was first described by (Harvey & Gilmour, 1994). The authors described using multilocus enzyme electrophoresis (MEE) and restriction fragment length polymorphism analysis to identify a 1/2 serotype *L. monocytogenes* strains that frequently reoccurred over a year in raw milk and non-dairy food from different producers. Furthermore, the study suggests the possibility of *L. monocytogenes* establishment in food products and processing facilities which can result in contamination. MEE is a subtyping method that characterizes organisms using enzyme polymorphism and pulsed-field gel electrophoresis (Selander et al., 1986). Since the first report on persistence of *L. monocytogenes* may studies have identified persistent isolates in other food processing facilities such as meat (Berzins et al., 2009; Cherifi et al., 2020; J. Lundén, Autio, Sjoberg, et al., 2003; Pasquali et al., 2018; Rodríguez-Campos et al., 2019), fish (Holch et al., 2013; Leong et al., 2015; Mędrala et al., 2003; Wiktorczyk-Kapischke et al., 2022), dairy (Chen et al., 2017; Kabuki et al., 2004; Varsaki et al., 2022) and produce facilities (Sullivan et al., 2022).

Reasons for *L. monocytogenes* persistence is unknown but occurrence of common subtypes has been observed in food processing facilities and in clinical samples. In food processing facilities, strains in lineage II and serotype 1/2a are more frequently isolated than lineage I (Orsi et al., 2011).

Gray et al., (2004), demonstrated that *L. monocytogenes* strains isolated from RTE food differed from strains associated with human clinical cases. The authors found that lineage II isolates were more commonly isolated from RTE food while lineage I isolates were clinical (Gray et al., 2004a). Maury et al., (2016), not only confirmed the above finding but further characterized *L. monocytogenes* clones in each lineage and described the differences in virulence. Within lineage II, hypovirulent clones CC9 and CC121 are the most prevalent and commonly isolated from foods hypervirulent clones CC1, CC2, CC4, and CC6 in lineage I, are most prevalent and more common in clinical infections (Maury et al., 2016). Hypovirulent clones CC9 and CC121 are the most prevalent clones isolated from meat products and hypervirulent CC1 is the most frequent clone isolated from dairy products (Maury et al., 2019). Not only do CC9 and CC121 isolates produce more biofilms in the presences of sanitizers, at refrigeration temperatures the presence of sanitizers favor biofilm formation (Maury et al., 2019). Persistent strains of *L. monocytogenes* have increased resistance to benzalkonium chloride (BC) a QAC used for sanitation (Fox et al., 2011; Lundén et al., 2003; Müller et al., 2013; Ortiz et al., 2014a). Cherifi et al., (2018), found that the presence of the *bcrABC* cassette (associated with resistance to BC) in group of persistent strains isolated from a pork slaughterhouse. The increased resistance to sanitizers and the presence of this cassette offers explanation for persistence of *L. monocytogenes* in food processing facilities.

In addition to increased sanitizer resistance, biofilm formation has also been linked to the persistence of *L. monocytogenes*. Borucki et al., (2003), determined persistent strains of *L. monocytogenes* are better biofilm formers compared to non-persistent strains. Ochiai et al., (2014), also concluded persistent strains formed a greater biomass of biofilms at 37 ºC and the variance in biofilm formation was lower than non-persistent stains (persistent strains are more consistent at

forming biofilms than non-persistent stains). *L. monocytogenes* cells in biofilms are more resistant to sanitizers (Chavant et al., 2004; Cruz & Fletcher, 2012; Hua et al., 2019). Increased resistance to sanitizers when exposed to a low dose of sanitizer has resulted in increased tolerance to sanitizers (Bland et al., 2022; Møretrø & Langsrud, 2017). Ortiz et al. (2014b) determined that in the presence of sub-inhibitory concentrations persistent strains of BC resistant strains of *L. monocytogenes* were able to grow to higher numbers than without the presence of BC. While hypervirulent strains are less virulent, they are commonly found in meat processing facilities and may have resistance genes that allow for survival of anti-*Listeria* controls and increasing the risk of foodborne illnesses.

1.8 Research and objectives

Although HHP has been proven to reduce numbers of pathogens, there is wide variability in the inactivation of *E. coli* and *L. monocytogenes*. The inactivation is dependent on several factors including the food matrix, physiological conditions (growth temperature and phase) and process parameters. Within the food matrix many factors influence *E. coli* inactivation; however, the effects of pressure on inactivation in complex matrices needs to be investigated for both *E. coli* and *L. monocytogenes*. Deconstructing the food matrix into its basic macronutrients would provide insight on how the constituents aid in pressure resistance. Furthermore, while physiological conditions of the bacterial cell influence the efficacy of HPP the impact of HPP on sessile cells is limited. Sessile cells from contaminated surfaces can transfer on to ready to eat meat products during slicing and if HPP is a proposed post processing step, understanding how the state impacts resistance will provide information suggested parameters for inactivation. The route of contamination of *L. monocytogenes* on food products and in the food processing facility varies.

The ecology of *L. monocytogenes* is complex but understanding if there is a link between the ecology of *L. monocytogenes* and source of isolation can help optimize process controls that aim to reduce contamination onto food products.

This research tested the following hypotheses:

1. High fat content and changes in carbohydrate composition increases the high pressure resistance of *E. coli* in ground meat and yogurt;
2. Sessile cells are more resistant to pressure compared to planktonic cell in broth and RTE chicken meat;
3. There is a higher prevalence of *L. monocytogenes* lineage II strains isolated from meat products compared to lineage I strains. Furthermore, there are more clinical isolates in lineage I clade compared to lineage II;
4. Lineage II strains of *L. monocytogenes* are better biofilms formers than strains from lineage I.

To test these hypotheses, the objectives of this research are to:

1. determine the effects of carbohydrates (CHO) and fat content on survival of different strains of *E. coli*;
2. determine the effect of adiabatic temperature change during HHP on the survival of *E. coli* in ground beef and yogurt;
3. using a yogurt model system compare survival of *E. coli* in a dairy matrix with survival in meat with different percentages of fat;

4. compare the pressure resistance of sessile cells of *L. monocytogenes* in single-, dual- and multi-species biofilms in broth and on RTE chicken meat to planktonic cells;
5. determine if there are lineage-specific preferences for L. monocytogenes persistence in food processing facilities, providing more understanding on possible routes of contamination;
6. determine differences in pressure resistance and biofilm formation of various *L. monocytogenes* dairy and meat isolates from lineage I and II;
7. using a metadata of *L. monocytogenes* and comparative genomics to determine a correlation between the ecology of phylogenetic clades and isolation sources.
8. determine if there are lineage-specific preferences for L. monocytogenes persistence in food processing facilities, providing more understanding on possible routes of contamination;

1.9 References

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2.0 Food composition and adiabatic heating affect the survival of *Escherichia coli* during high hydrostatic pressure processing

2.1 Introduction

High hydrostatic pressure processing (HHP) is a non-thermal technology that aims to reduce or eliminate foodborne pathogens to maintain the safety of ready-to-eat (RTE) meats, seafood, juices, and vegetable products (Hsu et al., 2015; Syed et al., 2016) When compared to thermal processing, minimal nutritional and sensory changes are observed in food products treated with high pressure (San Martín et al., 2002); however, the inactivation of bacteria in food can be variable depending on the food matrix and the organism (Alpas & Bozoglu, 2000; Gänzle & Liu, 2015; Li et al., 2016; Martín et al., 2021).

The inactivation of *Escherichia coli* by HHP depends on the intrinsic properties of a food including pH and water activity (a_w), the physiological conditions of the culture, including the growth temperature and phase, and process parameters (Alpas et al., 2000; Kilimann et al., 2005; Li et al., 2016; Y. Liu et al., 2015; Ulmer et al., 2002). Li et al., (2016) reported the greatest reduction of *E. coli* in bruschetta, followed by the reduction in tzatziki; however, in ground beef inoculated with the same strains, little to no inactivation occurred (Garcia-Hernandez et al., 2015). Li et al., (2016) attributed post-pressurization survival of *E. coli* in ground beef and tzatziki (a yogurt-based product) to a combination of pH and presence of divalent cations; however, this effect was only observed during storage and does not account for the differences in survival of *E. coli* to pressure treatment in different foods. Thus, the effects of pressure on inactivation in complex food matrices requires further investigation to provide insight on the interaction of the food matrix and survival of bacteria.

Fat content may play a role in resistance of organisms to HHP, similar to what is observed in studies on thermal processing. The reports on the effects of fat on heat resistance in meat products is inconsistent. Some studies on resistance of *E. coli* in ground beef reported a direct positive correlation between heat resistance of *E. coli* and fat content (Ahmed et al., 1995; Brar et al., 2018; Byrne et al., 2002; Smith et al., 2001). However, this is not always the case and effects can depend on the temperature of heating. Brar et al., (2018), reported a positive correlation between fat content and resistance of *E. coli* in ground beef but at temperatures greater than 60°C, there was no impact of fat on thermal resistance. Others have reported that the heat resistance of *E. coli* is higher in low fat beef patties (Liu et al., 2015). The effects of fat on pressure resistance is not as well documented as the effects of fat on heat resistance and what has been reported is inconsistent. Researchers have reported that higher fat protects to *E. coli* exposed to 400 MPa but effects are strain dependent (Jiang et al., 2015), but others have reported that fat has no effect on the high pressure resistance of *E. coli* (Sota et al., 2021).

In high pressure processing of foods, the effect of fat on pressure resistance may depend on the type of fat. The resistance of *Listeria innocua* to HHP in ground chicken increased in the presence of tallow as compared to the resistance when olive oil was used (Escriu & Mor-Mur, 2009). The percentage of fat in the range of 0 to 50% did not affect the pressure resistance of a variety of bacterial genera subjected to 100-500 MPa in ovine milk (Gervilla et al., 2000); however, greater than 30% fat in dry cured ham increased the pressure resistance of *Listeria monocytogenes* (Bover-Cid et al., 2015). There have been no reports in the literature on the effects of different fat types on the pressure resistance of *E. coli*. and there are few reports on the effects of fat on pressure resistance of *E. coli* in meat and other foods.

Carbohydrates (CHO) can influence the resistance of bacteria to HHP. Increasing the sucrose concentration from 10% to 50% increases the pressure resistance of *Lactococcus lactis* and *E. coli* (Molina-Höppner et al., 2004; Van Opstal et al., 2003). Sucrose at 18% protected against inactivation of *Lactococcus lactis* at 200 MPa; however, it had little protective effect at 600 MPa (Van Opstal et al., 2003). When 0.5% galactose was added to a mixture of ripe cheese and water the survival of *L. monocytogenes* subjected to 400 MPa for 10 min increased; however, addition of glucose and lactose had no protective effect (Morales et al., 2006). It is possible that disaccharides protect the integrity of the cytoplasmic membrane of some gram-positive bacteria exposed to HHP (Molina-Höppner et al., 2004; Morales et al., 2006). The carbohydrate profile of yogurt is largely composed of lactose, galactose and glucose (Ohlsson et al., 2017). Although Li et al., (2016), attributed the increased survival of *E. coli* in yogurt to divalent bonds and pH perhaps in the comparison between the pressure resistance of *E. coli* in yogurt and meat, the CHO present in yogurt play a role in the response to HPP.

It is well established that adiabatic heating and cooling occurs during pressurization and depressurization of foods. The extent of adiabatic heating depends on the initial temperature and the composition of the food materials, as different food components will experience different temperature changes (Knoerzer et al., 2010). Adiabatic heating accounts for an increase of about 3 °C/100 MPa in water, 8.3 °C/100 MPa in extracted beef fat and 3.8 °C/100 MPa for whole milk (Buzrul et al., 2008; Rasanayagam et al., 2003). Patazca et al., (2007), determined the change in temperature of various foods when different pressures were applied. As the pressure increased, the quasi-adiabatic temperature of mayonnaise and vegetable decreased from 7.0 and 9.2/100 MPa to 5.3 and 6.6/100 MPa respectively. In cream cheese and Hass avocados with the same fat content

as mayonnaise and vegetable oil (~35%) the quasi-adiabatic temperature change was less pronounced (Patazca et al., 2007). Modification of the fat content will impact the extent of adiabatic heating during compression and hence the treatment temperature. Few studies account for the effect of the fat content on the adiabatic temperature changes during pressure processing and the resulting impact on the survival of *E. coli* during HHP.

Therefore, the objectives of this study were to determine: i) the effects of CHO and fat content on survival of different strains of *E. coli*; and ii) the effect of adiabatic temperature change during HHP on the survival of *E. coli* in ground beef and yogurt. A yogurt model system was used to compare survival of *E. coli* in a dairy matrix with survival in meat with different percentages of fat.

2.2 Methods and Materials:

2.2.1 Bacterial strains and culture conditions

Four strains of *E. coli* with varying pressure resistance were obtained from the University of Alberta Food Microbiology Culture Collection: *E. coli* AW 1.7, MG 1655, AW 1.3, and DM 18.3. *E. coli* MG 1655 is sensitive to high pressure and AW 1.7, AW 1.3 and DM 18.3 are pressure resistant and all four strains are validated as surrogates for verotoxigenic *E. coli* (Garcia-Hernandez et al., 2015). Strains were streaked from frozen (-80 °C) stock cultures onto Luria-Bertani (LB) agar (Difco, Beckton Dickinson, Sparks, MD, USA) and incubated for 24 h at 37 °C. Individual colonies were picked and inoculated into LB broth, which was incubated at 37 °C and 200 rpm for 16-18 h followed by a second subculture grown under the same conditions. The second subculture was used to inoculate food samples.

2.2.2 Pressure treatment of food

Samples were treated in a Multivessel Apparatus U111 (Unipress Equipment, Warsaw, Poland) at 600 MPa and 20 °C or 30 °C for 3 min, depending on the experiment. Pressure vessels were 12 X 58 mm and temperature of the unit was maintained by a thermostat jacket coupled to an external glycol bath (LAUDA Proline, Delran, NJ). Polyethylene glycol was used as pressure transferring fluid. The temperature of the vessel was monitored with an internal thermal couple and temperature changes during compression and decompression stayed within 2 °C. The vessel compression rate to 600 MPa was ~ 1 min and decompressed in ~ 30 s. After the treatment, samples were placed on ice and cell counts of treated and untreated samples were determined by surface plating on LB agar that was incubated at 37 °C for 24 h. All experiments were performed in triplicate.

2.2.3 Effect of carbohydrate on pressure resistance of *E. coli*

The CHOs used in this experiment were glucose, fructose, galactose, lactose and a mixture of the four CHOs with a final concentration of 1%. The sugars were added to a 100 mmol/L 2-(N-morpholino)ethanesulfonic acid (MES) (Fisher, Ottawa, Canada) buffer with a pH of 5.5. Overnight cultures of *E. coli* were centrifuged and rinsed twice with sterile 0.85 % saline solution. The pellets were held on ice for no more than 30 min until added to 10 mL of fresh MES buffer+CHO. The initial cell counts in the CHO and MES buffer were 10^7-10^8 CFU/mL. MES buffer without CHO was used as a control for the experiment. Once the pellets were added to the buffer and CHO solution, 0.30 mL of culture and CHO buffer solution was added to 4 cm R3603 tygon tubing (Akron, PA, USA). The tubing was heat sealed and held on ice for no more than 60 min before pressure treatment.

2.2.4 Effect of fat on the pressure resistance of *E. coli*

Three ground beef samples were obtained from a federally inspected meat processing facility with a fat content of 15.5, 24.4 or 35.7%. For ground beef samples with 3% fat, beef striploin obtained from a federally inspected beef processing facility was ground using a food processor (Kitchenaid®, Mississauga, Canada). Prior to grinding, the visible fat was removed from the exterior. The crude fat content was measured with the Soxtec™ 2050 fat extraction apparatus (Foss® Analytical, Hilleroed, Denmark) following the method of Roy et al. (2018), according to the Association of Official Analytical Chemists International, 991.36 (Thiex et al., 2003). Plain yogurt with 0, 2, 3 and 6% fat (Astro, Canada) were purchased from a local grocery store. Yogurt samples were formulated with 15 and 35% fat using crème fraiche (40% fat; Liberté, Canada) to adjust the fat content. Sample preparation for HHP treatment followed the protocol of Li et al., (2016). Each strain of *E. coli* (1.0 mL) was inoculated into 10 mL of yogurt or 10 g of ground beef with an initial inoculum of 10^8-10^9 CFU/mL or g. The inoculated food product was massaged manually for 2 min. Subsamples (400 µL or µg) were placed into individual 4 cm R3603 tygon tubes (Akron, PA, USA), heat-sealed and treated with high pressure as described above. For ground beef, samples were placed in the vessel conditioned to 20 °C and for yogurt samples, the vessel was conditioned to 20 or 30 °C.

2.2.5 Effect of adiabatic heating on survival of *E. coli* in ground beef and yogurt

The ground beef and yogurt used had 3, 15.7, 24.4 and 35.6% fat or 0, 2, 3, 6, 15 and 35% fat, respectively. To compare the effect of adiabatic heating in a dairy and meat matrix the pH of the yogurt samples was adjusted to 5.5 with 10 M NaOH, using a pH meter (Fisher, Ottawa, Canada).

To eliminate the effect of adiabatic heating, samples were cooled in an ice bath for 5 min prior to processing. Adiabatic compression heating profiles were calculated using 3 °C/100 MPa for aqueous phase and 9 °C/100 MPa for lipid phase (Rasanayagam et al., 2003). Equation 1 was used determine the temperature to cool samples to eliminate the effect of adiabatic compression heating. Ground beef samples with 3, 15.7, 24.4 and 35.6% fat were adjusted to -0.2, -3.6, -6.8 and -10.8 °C, respectively. Initially, samples were subjected to high pressure at 20°C. To eliminate the possibility of subjecting frozen samples to pressure treatment, the temperature for the ground beef was adjusted to 9.1, 6.4, 3.2 and 0.8°C for samples with 3, 15.7, 24.4 and 35.6% fat, respectively, and samples were processed at 30°C. For yogurt samples with 0, 2, 3, 6, 15 and 35% fat, the sample temperatures were adjusted to 12, 11.3, 10.9, 9.8, 6.6 and -0.6 °C, respectively, and samples were processed at 30°C.

$$Eq1.\ Sample\ temperature = Tt - \big(6 * (3 * 1 - \%Sw)\big) + (6 * (9 * 1 - \%Sf))$$

Where *Tt*= treatment temperature, *Sw* amount of water in sample and *Sf* is the amount of fat in sample

2.2.6 Enumeration after pressure treatment

Cells were enumerated prior to HPP treatment and after treatment by surface plating. For samples in solution ie. in MES buffer, and in yogurt, appropriate dilutions were spread onto the surface of LB agar to determine cell counts of *E. coli*. Plates were incubated at 37°C for 24 h. To enumerate the bacterial load in the ground beef before and after treatment, ground beef was placed into 1 mL peptone water, vortexed for 2 min and enumerated as described above.

2.2.7 Statistical analysis

All bacterial counts were converted to log (CFU)/mL or g prior to statistical analysis. Significant differences among cell counts were determined by Analysis of Variance using the PROC GLM procedure of the University Edition of SAS (SAS Institute Inc., Cary, NC). Tukey's posthoc test was used to determine differences among means at $P<0.05$. Each experiment was replicated 3 times.

2.3 Results:

2.3.1 Effect of carbohydrate on the pressure resistance of *E. coli*

To determine the effect of the type of CHO on the pressure resistance of *E. coli*, 100 mmol/L MES buffer with 1% glucose, fructose, galactose, lactose and a mixture of all CHOs was treated at 600 MPa at 20°C for 3 min. The presence or type of CHO did not affect the pressure resistance of *E. coli* ($P>0.05$) for all strains except *E. coli* AW 1.7 (Figure 1.1). The greatest reduction of *E. coli* AW 1.7 was observed in the absence of CHO.

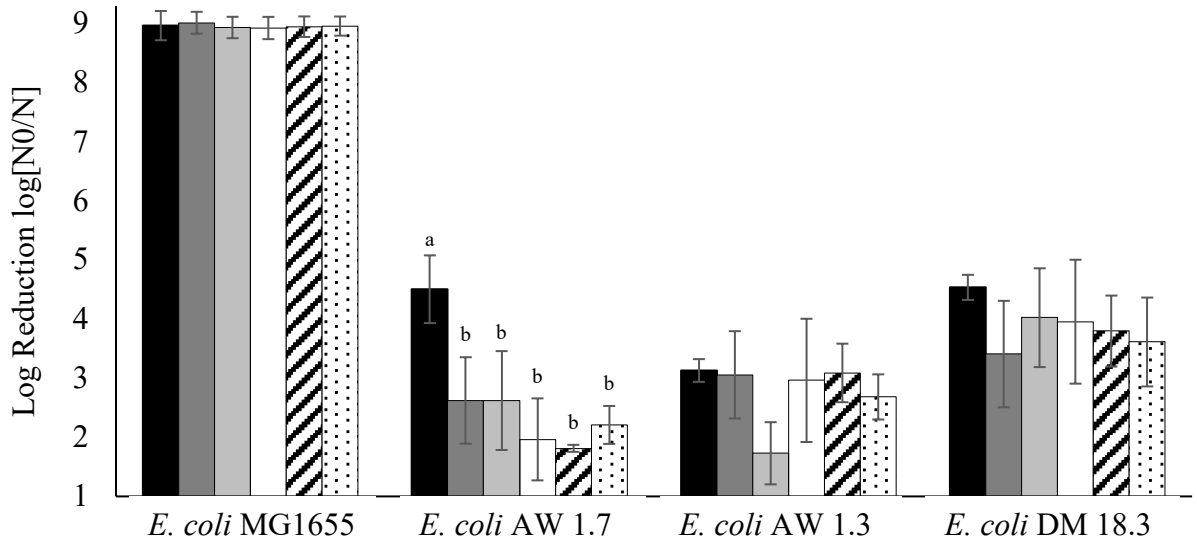

Figure 2.1 Reduction of cell counts (log[N0/N]) of different strains of *E. coli* inoculated into MES buffer supplemented with 1% carbohydrate. Control sample is MES buffer (pH 5.5) without carbohydrate. Samples labelled "All" contained a mixture of all carbohydrates with a final concentration of 1%. Each bar indicates the carbohydrate used: control■, glucose■, fructose■, lactose□, galactose (black diagonal), all (black polka dots). Letters indicate differences in log reduction within each strain among samples with different carbohydrates using one-way ANOVA ($P<0.05$) and Tukey's posthoc test. Data are means ± standard deviations of three independent replicates.

2.3.2 Effect of fat on pressure resistance of *E. coli*

To examine the effect of fat content on pressure resistance of *E. coli,* samples of ground beef with 3, 15.5, 24.4 and 35.7% fat were treated at 600 MPa and 20°C for 3 min. No difference ($p>0.05$) in pressure resistance was observed for the pressure resistant strains *E. coli* AW 1.7, AW 1.3 and DM 18.3 (Figure 2.2). In ground beef with 3%, pressure sensitive *E. coli* MG 1655 had a similar log reduction to that of the pressure resistant *E. coli* AW 1.7; however, when the fat content was increased to 15.5% or higher *E. coli* MG 1655 was sensitive to high pressure. Yogurt (pH 4.1-4.4) with 0, 2, 3, and 6% fat was subjected to the same pressure treatments as used for the ground beef. All strains were sensitive to the treatment (Figure 2.3) regardless of the fat content.

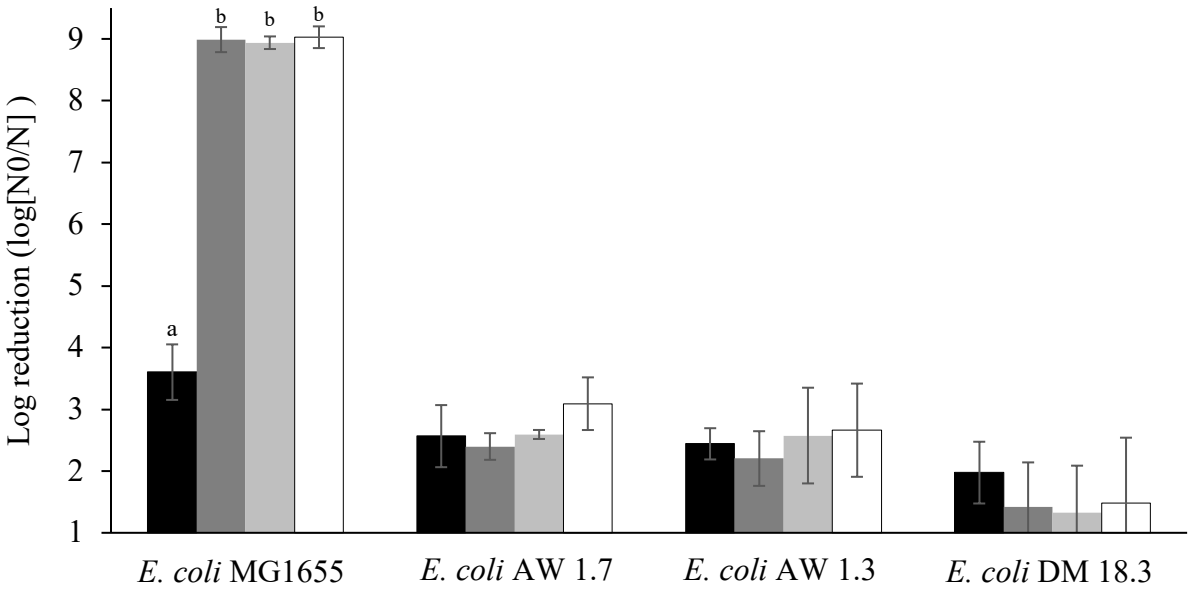

Figure 2.2 Reduction of cell counts (log[N0/N]) of different strains of *E. coli* inoculated in ground beef with 3 ■, 15.5 ■, 24.4 ■ or 35.7 □% fat treated at 600 Mpa and 20°C for 3 min. Letters indicate differences in log reduction within each strain among samples with different fat content using one-way ANOVA ($P<0.05$) and Tukey's posthoc test. Data are means ± standard deviations of three independent replicates.

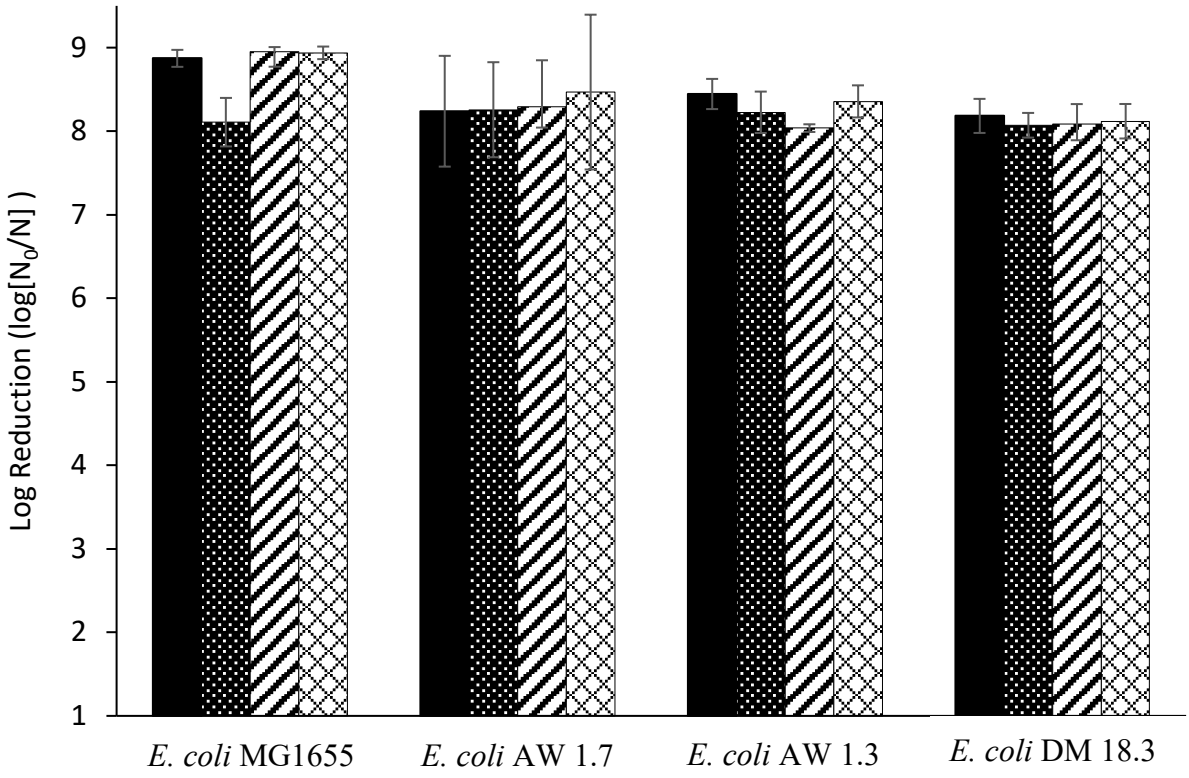

Figure 2.3 Reduction of cell counts (log[N0/N]) of different strains of *E. coli* inoculated in yogurt (without pH adjustment) subjected to high pressure treatment of 600 MPa for 3 mins at 20°C. Each bar is yogurt with a different percentage of fat: solid bars 0%, dotted 2%, diagonal strips 3% and hatched bars 6%. Data are means ± standard deviations of three independent replicates.

2.3.3 Effect of adiabatic heating on survival of *E. coli* in ground beef and yogurt

To determine if adiabatic temperature changes influence the survival of *E. coli* subjected to high pressure in beef, sample temperatures were adjusted to eliminate the effect of adiabatic heating. When adiabatic temperature change was accounted for, the pressure sensitive strain, *E. coli* MG 1655 was resistant to pressure in ground beef with 3 and 15.5% fat when pressure treated at 20°C (Figure 2.4, solid bars); however, in ground beef with 24.4% fat or higher the strain was sensitive to high pressure, regardless of process temperature. The percentage of fat in ground beef had

limited impact on the pressure resistance of *E. coli* AW 1.7 when processed at 20°C but when processed at 30°C (dotted bars), *E. coli* AW 1.7 was more resistant to pressure in ground beef with 3% fat than in samples with a higher fat content. Similar results were observed for *E. coli* AW 1.3 processed at 20 or 30°C. Pressure resistant *E. coli* DM 18.3 was significantly ($P<0.05$) more resistant to pressure at 20°C in ground beef with 3% fat compared to cells treated in ground beef with 24.4 or 35% fat. For the pressure sensitive strain *E. coli* MG 1655, in ground beef with 15.5% fat, a change in process temperature from 20 to 30°C significantly ($P<0.0001$) reduced pressure resistance. A change in process temperature also reduced the pressure resistance of the pressure resistant strain *E. coli* DM 18.3 in ground beef with 35.7% fat.

The heat map in Figure 2.5 compares the difference in process lethality in ground beef samples treated at 20°C that had no temperature adjustment (Figure 2.2) to samples where the temperature was reduced prior to pressure treatment to account for adiabatic heating (Figure 2.4). For *E. coli* MG 1655, AW 1.7 and AW 1.3, reducing the temperature prior to treatment did not alter the treatment lethality or increased the resistance (Figure 2.4), which is consistent with the known effects of temperature on the lethality of pressure treatment of *E. coli* and other organisms. For *E. coli* DM 18.3, reducing the temperature prior to treatment increased the process lethality in beef with 15.5, 24.4 and 35.7% fat (Figure 2.4).

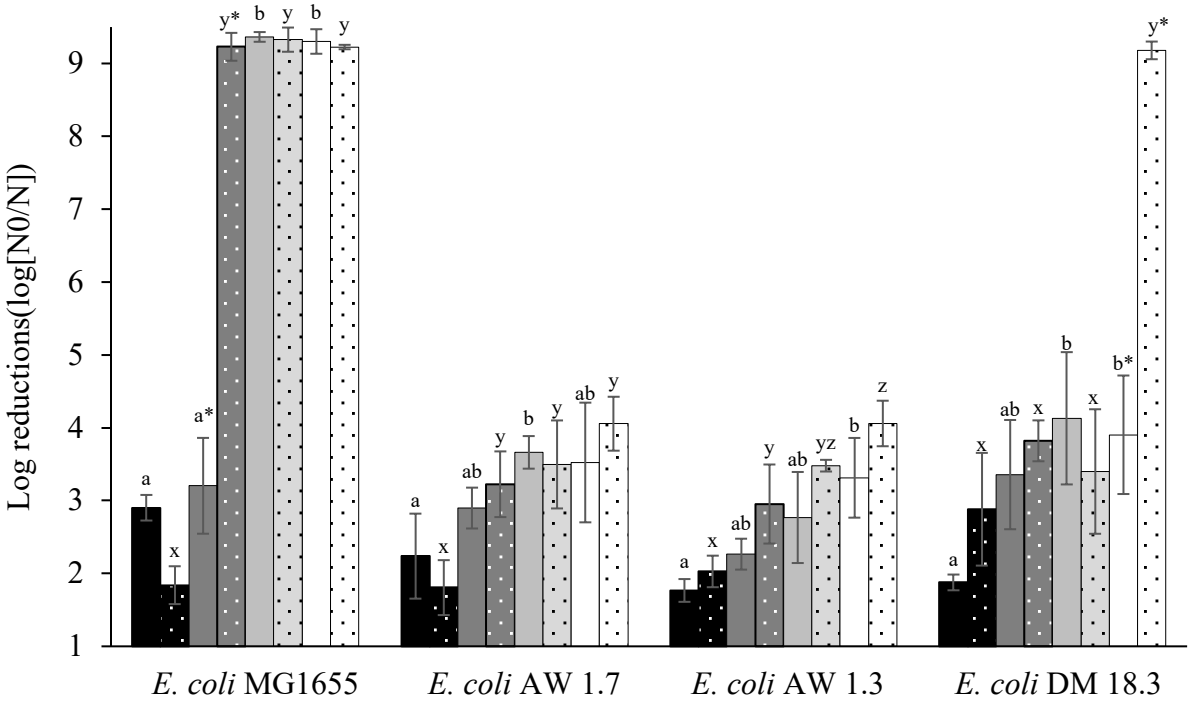

Figure 2.4 Reduction of cell counts (log[N0/N]) of different strains of *E. coli* inoculated in ground beef with 3 ■, 15.5 ■, 24.4 ■ and 35.7 □% fat treated at 600 MPa and 20°C (solid bars) and 30°C (dotted bars) for 3 min after sample temperature was adjusted to eliminate the effect of adiabatic temperature change during pressurization. Superscripts a, b, c indicate differences (P<0.05) among means within strain for each percentage of fat for samples processed at 20°C; x, y, z indicate differences (P<0.05) within a strain for samples processed at 30°C using one-way ANOVA. Asterisks indicate differences (P<0.05) between samples processed at either 20 or 30°C using a two-way ANOVA. Data are means of three independent experiments ± standard deviations.

The effect of fat content and adiabatic temperature change was also evaluated in yogurt. The experiments were performed after adjustment of the pH to 5.5 to match the pH of ground beef. Differences in pressure resistance in yogurt with different fat content and adjustment for adiabatic heating were observed for all strains except for *E. coli* MG 1655 which remained pressure sensitive regardless of fat content or adjustment for adiabatic heating (Figure 2.6). With exception of *E. coli* DM 18.3, the reduction of the treatment temperature in low fat yogurt to account for adiabatic heating change did not impact treatment lethality (*E. coli* MG1655) or reduced treatment lethality

(*E. coli* AW 1.7 and AW 1.3). In yogurt with 6, 15 and 35% fat, reducing the temperature did not impact treatment lethality or increased treatment lethality in the strains *E. coli* AW 1.7, AW 1.3 and DM 18.3. For *E. coli* AW 1.7, the treatment lethality after temperature adjustment was higher in yogurt with 15% fat when compared to samples with either a higher or lower fat content (Figure 2.6). Without temperature adjustment, *E. coli* AW 1.7 was more sensitive to high pressure in yogurt with 0% fat than in yogurt with 6% fat or higher.

	Log reduction (CFU/g)			
% Fat	3%	15.5%	24.4%	35.7%
ΔT (°C)	20.2	23.6	26.8	30.8
Strain				
E. coli MG 1655				
E. coli AW 1.7				
E. coli AW 1.3				
E. coli DM 18.3				

Figure 2.5 Heat map to depict the impact of temperature adjustment prior to treatment at 600 MPa at 20 °C for 3 min in ground beef with different percentages of fat. The calculation of the temperature differential in the two treatments (higher without adjustment) was based on compression heating of about 3 °C / 100 MPa in the aqueous phase and of 9 °C in the lipid phase. The original data for the reduction of cell counts is shown in Figure 2.1 (no temperature adjustment) and Figure 2.4 (adjustment of temperature prior to treatment). Cells are not shaded if reducing the treatment temperature did not change process lethality, shaded in blue if reducing the temperature reduced ($P < 0.05$) process lethality and shaded in red if reducing the temperature increased ($P < 0.05$) treatment lethality.

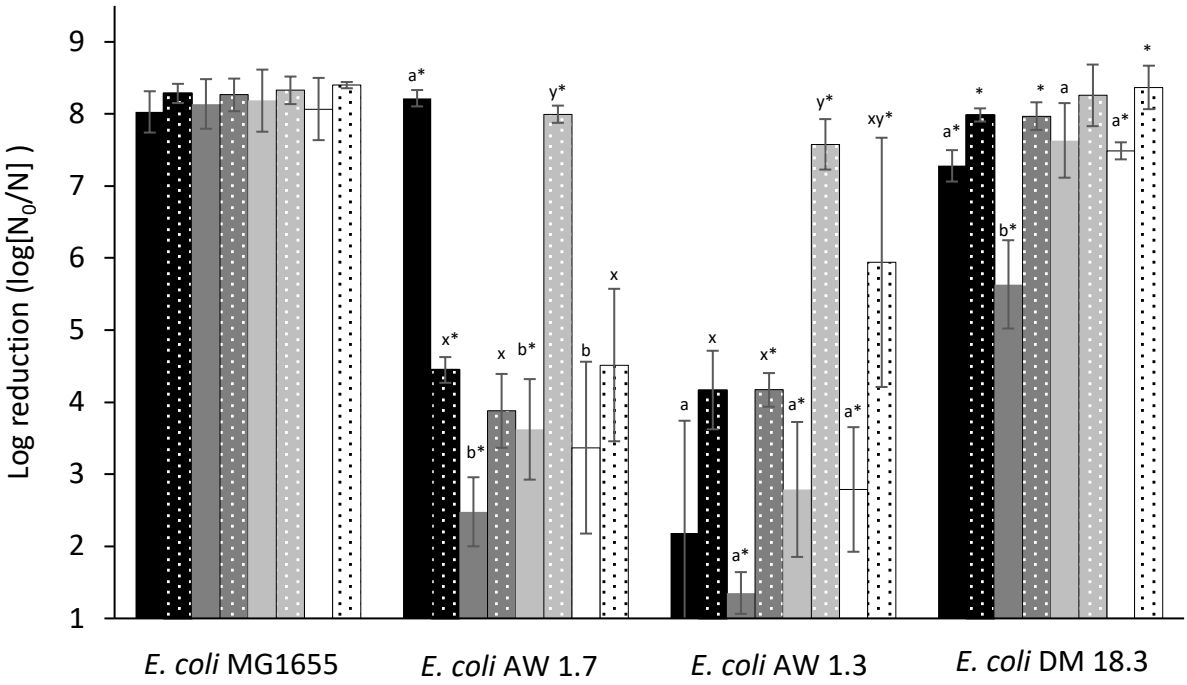

Figure 2.6 Reduction of cell counts (log[N0/N]) of different strains of *E. coli* inoculated in yogurt at pH 5.5 with 0 ■, 6 ■, 15 ■ and 35 □% fat treated at 600 MPa and 30°C for 3 min without (solid bars) and with (doted bars) temperature adjustment to account for adiabatic temperature change during pressurization. Superscripts a, b, c indicate differences (P<0.05) among means within a strain for each percentage of fat for samples without temperature adjustment; x, y indicate differences (P<0.05) within a strain for samples with temperature adjustment using one-way ANOVA and Tukey's posthoc test. Asterisks indicate differences (P<0.05) among yogurt samples when sample temperature was or was not adjusted using two-way ANOVA. Data are means of three independent experiments ± standard deviations.

2.4 Discussion:

The impact of different constituents of yogurt and ground beef on the pressure resistance of *E. coli* was evaluated. The specific type of CHO had no influence on the pressure resistance for all strains of *E. coli* used in this study. Comparisons of the effect of the type of CHO has not previously been reported. The carbohydrates selected in this study reflected the CHOs found in yogurt naturally. The pressure resistance of *E. coli, Lactococcus lactis*, *L. monocytogenes* and *Cronobacter*

sakazakii can increase in the presence of high concentrations of sugars (Arroyo et al., 2011; Molina-Höppner et al., 2004; Morales et al., 2006; Yuan et al., 2017). In the current study, the presence of sugars slightly increased the pressure resistance of *E. coli* AW 1.7; however, they did not affect pressure resistance of the other strains of *E. coli*. Simpson & Gilmour, (1997), reported a variability in pressure resistance among strains of *L. monocytogenes* in the presence of varying glucose concentrations. The increase in pressure resistance of *E. coli* AW 1.7 in this study could be a result of increased solute concentration. Van Opstal et al., (2003), observed that the presence of sucrose in concentrations of more than 10% increased pressure resistance of *E. coli* when compared to control samples without sucrose. An increased solute concentration decreases the water activity (a_w) and prompts the accumulation of compatible solutes in bacterial cells, resulting in higher resistance to pressure (Hayman, Anantheswaran, et al., 2008). However, in the current study, the concentration of carbohydrates added to the buffer was much lower than that used in previous studies. The currently study investigated the influence of CHO presence and did not investigate the impact of concentration. While studies have determined increased CHO concentrations increases bacterial resistance this study investigated the influence of type of CHO. Although the exact mechanism of this specific protective agent is remains unknown, the pressure resistance of *E. coli* to different types of carbohydrates is strains specific.

The effect of fat content on the pressure resistance of bacteria has been investigated in various food matrices; however, results are inconsistent. A high fat content in a food increased the pressure resistance of *E. coli, S. aureus* and *L. innocua* (Carballo et al., 1997; García-Graells et al., 1999; Gervilla et al., 2000). However, *L. monocytogenes* was less resistant to high pressure (up to 400 MPa) in milk with 10 and 15% fat as compared to milk with a fat content of less than 3.6% (Roig-

Sagués et al., 2009). In the current study, the fat content of ground beef did not influence the pressure resistance of strains of *E. coli* that are known to be pressure resistant. This contradicts the expectation that adiabatic heating in samples with a higher fat content results in a higher treatment temperature and a higher lethality (Ahmed et al., 1995; Chhabra et al., 1999; Juneja et al., 2001). In contrast, when the same strain was treated at 600 MPa for 15 min in low fat milk it was less resistant than when treated in milk with 3.6% fat (García-Graells et al., 1999). This illustrates that the relationship between fat content and pressure resistance is dependent on the food matrix. The fat content of yogurt had no effect on the pressure resistance of all strains, which was likely due to the low pH of the yogurt (ranging between 4.1-4.4). Under high pressure conditions, damage to the cell membrane and dissipation of the proton motor force eliminates acid resistance of *E. coli* (Gänzle & Vogel, 2001; Kilimann et al., 2005). When cells are subjected to pressure in yogurt, the cell membrane of *E. coli* would be damaged, and the low pH would result in cell death. Fat may have an impact on pressure resistance, but effects are dependent on the food matrix and the strain. Resistance to high pressure is likely due to intrinsic factors within the food, a conclusion that others have also come to (Molina-Gutierrez et al., 2002).

In fats and oils the adiabatic temperature change is as high as 9 °C/100 MPa, resulting in a higher process temperature when compared to the corresponding low fat samples. Most bacteria including *E. coli* are most resistant to high pressure if treated at a temperature which is 10 – 20 °C below the bacterial growth temperature (Buckow & Heinz, 2008; Sonoike et al., 1992). Compression to 600 MPa at an initial temperature of 30 °C temporarily increases the treatment temperature by up to 30 °C, well into the temperature range where a higher treatment temperature increases treatment

lethality. Reducing the initial sample temperature by the predicted increase during adiabatic compression eliminates this differential effect but introduces two confounding factors.

First, the temperature of some of the high fat samples was reduced to -1.5 °C (freezing point of meat) and thus induced a phase change prior to compression, which is reversed in the initial stages of compression. Pressure treatments that included a phase change from Ice I to Ice III increase the treatment lethality against *L. innocua* (Luscher et al., 2004). Others have documented a greater resistance of *Listeria* and *E. coli* to pressure at subzero processing temperatures that included an ice to liquid phase transition (Moussa et al., 2006; Teixeira et al., 2016). While literature data is insufficient to predict how phase changes during sample preparation or compression impact survival of *E. coli* in meat, it is plausible to assume that they may impact survival.

Second, cooling of inoculated samples induces a cold shock response that impact resistance to pressure. The bacterial cold shock response includes the production of cold shock proteins and the accumulation of compatible solutes (Hoffmann & Bremer, 2011; Najjar et al., 2007; Smiddy et al., 2004), which increase pressure resistance (Molina-Höppner et al., 2004; Welch et al., 1993; Wemekamp-Kamphuis et al., 2002), but also the modification of membrane lipids, which decreases pressure resistance in *E. coli* (Chen & Gänzle, 2016). In yogurt but not in meat, the effect of temperature reduction prior to compression was highest in yogurt with 15% fat (Figure 2.6). This sample was cooled to 6.6 °C, i.e. a temperature that is just below the minimum growth temperature of *E. coli* (Shaw et al., 1971) and most likely to induce a cold shock response and changes in the composition of the cytoplasmic membrane (Álvarez-Ordóñez et al., 2009; Annous et al., 1997). In particular, the composition of the cytoplasmic membrane closely links to resistance to high pressure processing (Chen & Gänzle, 2016; Rowlett et al., 2017). *E. coli* AW 1.7 has a

higher proportion of cyclopropane fatty acids when compared to other strains of *E. coli* (Chen & Gänzle, 2016; Ruan et al., 2011) and deletion of the cyclopropane fatty acid synthase reduced resistance to pressure (Chen & Gänzle, 2016). The strain-to-strain variation in the response to temperature adjustment (Figures 2.4 and 2.6) also conforms with the hypothesis that cold adaptation confounds the results of temperature adjustment. The adjustment of the initial sample temperature has commonly been used to account for compression heating (Barbosa-Cánovas et al., 2014; Lau & Turek, 2007) but results of the present study should be confirmed by using equipment that allows either isothermal or adiabatic pressure treatments to minimize confounding factors.

The impact of fat on the lethality of HHP differed substantially between yogurt and meat even though the levels of fat and the pH were comparable; in meat but not in yogurt, an increase of the fat content consistently decreased the pressure resistance of *E. coli* (Figures 2.4 and 2.6). This may relate to other components of the food matrix, e.g. divalent cations but may also reflect the different types of fat (Garcia-Hernandez et al., 2015). The fat content of yogurt was adjusted with pasteurized crème fraiche, which lacks any enzyme activity and only adds triglycerides to the matrix. Rodríguez-Alcalá et al., (2015) observed no change in the triglyceride concentration of milk when treated up to 900 MPa. When yogurt was treated with ultra-high pressure homogenization (UHPH) a process using high pressure and high temperature, little to no lipid oxidation was observed (Serra et al., 2008) In this experiment fat content of meat was adjusted with adipose tissue, which also adds hemoproteins and phospholipids. Oxidative stress may contribute to the increased sensitivity to pressure in meats with high fat for *E. coli.* Studies have shown that HPP induces lipid oxidation in various meat products (Cava et al., 2009; Dissing et al., 1997; Kantono et al., 2020; Wiggers et al., 2004). When ground beef is subjected to higher

pressures, either the release of iron from myoglobin or the iron-catalyzed oxidation of unsaturated fatty acids in the meat matrix (Cheah & Ledward, 1995, 1997). Haem-bound or non-heme iron released during HPP become available to generate free radicals which can strongly favors lipid oxidation in meat (autoxidation) (Carlez et al., 1995). The increase in secondary by-products generated by lipid oxidation could increase oxidative stress for *E. coli,* ultimately resulting in a higher reduction in beef with high fat.

The relationship among adiabatic heating, fat content and pressure resistance of *E. coli* is not clear. It is apparent that the effect of adiabatic heating and fat content on the pressure resistance of *E. coli* is strain specific for both yogurt and ground beef. Others have come to the same conclusion regarding pressure resistance and fat content in different food matrices but have not accounted for adiabatic heating. To our knowledge, this is the first study that has reported the impact of adiabatic temperatures on pressure resistance in food matrices with different fat content. The results from this study illustrate the importance of the food matrix on the pressure resistance of *E. coli.* The relationship between pressure resistance and fat content in the food matrix is strain specific for *E. coli*. More specifically, when investigating the effect of fat content on the pressure resistance of *E. coli*, adiabatic temperature change is an important factor to consider.

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3.0 Pressure resistance of planktonic and sessile cells of *Listeria monocytogenes* in mono-, dual- and multi- species biofilms

3.1 Introduction

Listeria monocytogenes is a pathogenic bacterium that causes illness in high-risk groups particularly in elderly, pregnant women, and immunocompromised individuals (Farber & Peterkin, 1991). Although listeriosis is not common, the mortality rate is the highest compared to other foodborne pathogens (Thomas et al., 2013). In 2008 the largest *L. monocytogenes* outbreak occurred in Canada resulting in changes to Health Canada's policy on *L. monocytogenes* in ready-to-eat (RTE) foods. The changes implemented outlined new categories of RTE food in effort to control and protect the health and safety of Canadians (Government of Canada, 2011). The definition of RTE food was one of the major changes to the policy resulting in two categories: Category 1 RTE foods are classified as foods that support the growth of *L. monocytogenes* throughout the shelf life of the product; and Category 2 which has two subgroups, and validation to demonstrate control of the growth of *L. monocytogenes* may be required. RTE deli meats are classified as category 1 under the 2011 and the new 2023 policy as *L. monocytogenes* can grow throughout the stated shelf life (Government of Canada, 2011, 2023). RTE deli meats have a high water activity (a_w) and pH and are stored at refrigeration temperatures, which are conditions that facilitate the growth of *L. monocytogenes* (Barbosa et al., 1994; Farber & Peterkin, 1991; Ingham et al., 2004).

L. monocytogenes has been the causative agent for multiple foodborne outbreaks linked to RTE deli meats through contamination from food contact surfaces and meat slicers (Thomas et al., 2020; Weatherill, 2009). Contamination of bacteria on food contact surfaces have also been linked to

biofilm formation, and biofilms have been found on drains, cutters and conveyors (Wagner et al., 2020) .

Biofilms are a network of microbial cells that are embedded in an extracellular polymeric matrix and adhere to surfaces allowing the spread of bacteria from surface onto food product. *L. monocytogenes* is not a strong biofilm former compared to other microorganisms and the density of the biofilms is dependant of many factors including, strain, growth temperature and surface material (Borucki et al., 2003; Djordjevic et al., 2002; Doijad et al., 2015). *L. monocytogenes* can attach to food contact surfaces and some strains are able to produce biofilms contributing to its persistence in food processing facilities (Renier et al., 2011). Although some strains of *L. monocytogenes* can produce biofilms, single species biofilms are rare in nature (Giaouris & Simões, 2018). The microbial diversity in food processing facilities is vast and includes *Pseudomonas, Enterobacteriaceae*, *Acinetobacter* and *Bacillus* (Fagerlund et al., 2021). Currently, cleaning and sanitation practices are in place to control for the contamination of pathogenic microorganism on food products. However, studies have found that sessile cells of *L. monocytogenes* and other pathogenic microorganisms are resistant to sanitizers currently used in industry (Pan et al., 2006; Pang et al., 2019; Stopforth et al., 2003; Xu et al., 2021; Yang et al., 2016).

High pressure processing (HPP) is a non-thermal processing technology that can be used to reduce the prevalence of pathogenic bacteria in food products. The effect of HPP on planktonic *L. monocytogenes* has been investigated with conflicting results. Some studies have found that HPP is an effective method to extend shelf life and reduce *L. monocytogenes* numbers (Cava et al., 2021; Hayman et al., 2004; Pérez-Baltar et al., 2021) however, other demonstrate that HPP alone

is not sufficient to eliminate *L. monocytogenes* on RTE meat products (Morales et al., 2006; Teixeira et al., 2018). The impact of HPP on sessile cells of *L. monocytogenes* on RTE meat has yet to be investigated. The objective of this study was to compare the pressure resistance of sessile and planktonic cells of *L. monocytogenes* in single-, dual- and multi-species biofilms on RTE chicken meats and in broth solutions.

3.2 Materials and Methods

3.2.1 Bacterial strains and growth conditions

Details of bacterial isolates used in the experiments are outlined in Table 3.1 along with the composition of the biofilms and cultures used in the study. All strains were stored at -80°C in media with 25% glycerol. Prior to experiments, cultures of *L. monocytogenes* were streaked onto tryptic soy agar (TSA; Difco, Becton Dickinson, Sparks, MD, USA), and *Escherichia coli* and *Aeromonas australiensis* were streaked onto Lennox broth agar (LB; Difco) and incubated at 37 °C for 24 h. *Carnobacterium maltaromaticum* UAL307 was streaked onto All Purpose Tween agar (APT; Difco) and incubated at 30 °C for 48 h. A single colony was picked and inoculated into the corresponding broth media. Prior to use in experiments, subcultures of *L. monocytogenes, E. coli* and *A. australiensis* were incubated at 37 °C at 200 rpm for 16-18 h and *C. maltaromaticum* was incubated at 30 °C at 200 rpm for 16-18 h. After overnight incubation, the average cell count of *L. monocytogenes* was ~8.9 CFU (colony forming units)/mL, *E. coli* 8-77 was ~7.8 CFU/mL, *A. australiensis* ~7.9 CFU/mL and *C. maltaromaticum* was ~7.8 CFU/mL

3.2.2 Preparation of stainless steel coupons

Prior to experiments, stainless steel (SS) coupons (type 304, SS-8, No. 4 finish, 50 mm^2; Stanfos, Edmonton, AB, Canada) were cleaned and sterilized. To clean the coupons, coupons were manually scrubbed with a soft brush and dish soap (Dawn, Procter & Gamble, Cincinnati, OH, USA), and soaked in 95% ethanol overnight. After manually cleaning and soaking, coupons were immersed in 95% ethanol and sonicated (Bransonic 52, Branson Ultrasonics Corporation, Danbury, CT, USA) for 15 mins. Coupons were rinsed with deionized water, air-dried and autoclaved at 121 °C for 20 min.

Table 3.1 Bacterial strains used in this study and composition of biofilms

Biofilm	Strains	Abbreviation	Reference
Mono-species			
	L. monocytogenes cocktail:	LM cocktail	
	FSL R2-499		(Fugett et al., 2006)
	FS 15		(Bohaychuk et al., 2005)
	2P-263SS		(Kovacevic, 2007)
	NP-221SS		(Kovacevic, 2007)
Dual-species			
	L. monocytogenes cocktail + *E. coli* 8-77	L+E	(Visvalingam et al., 2019)
	L. monocytogenes cocktail + *A. australiensis* 03-09	L+A	(Visvalingam et al., 2019)
	L. monocytogenes cocktail + *C. maltaromaticum* UAL 307	L+C	(Martin-Visscher et al., 2008)
Multi-species			
	L. monocytogenes cocktail + *E. coli* 8-77 + *A. australiensis* 03-09 + *C. maltaromaticum* UAL 307	All	
	L. monocytogenes cocktail + *E. coli* 8-77 + *A. australiensis* 03-09	All-C	

3.2.3 Growth of individual strain *L. monocytogenes* biofilms on SS coupons (method 1)

Biofilm growth on SS followed the protocol of Xu et al., (2021) with minor modifications. Biofilms of four individual strains of *L. monocytogenes* were grown on the surface of 50 mm^2 SS coupons. Coupons were placed in sterile 100 mm X 15 mm round petri dishes (Fisherbrand, Fisher Scientific, Ottawa, Canada) containing 25 mL tryptic soy broth diluted to 10% (dTSB; Difco). Each strain of *L. monocytogenes* (250 µL) was added to individual petri dishes containing the coupon and dishes were incubated at 22 °C for 14 days.

3.2.4 Growth of mono-, dual- and multispecies biofilms on SS coupons (method 1)

Mono-, dual- and multi- species biofilms were grown on the surface of SS coupons that were placed in round petri dishes as described above. For mono-species biofilms, a cocktail of equal volumes of each of the four strains of *L. monocytogenes* were combined, and 250 µL of the cocktail was added to the petri dish containing dTSB. For dual-species inoculum, 125 µL of the cocktail of *L. monocytogenes* and 125 µL of the other species were mixed and added to the 25 mL dTSB to achieve a 100-fold dilution of the bacterial suspension. Cultures used for dual-species biofilms are outlined in Table 3.1. For multi-species biofilms two inoculum were prepared, one contained equal volumes (62.5 µL) of the cocktail of *L. monocytogenes* and each of the other species (All), and one contained equal volumes (83.3 µL) of the cocktail of *L. monocytogenes*, of *E. coli* 8-77 and of *A. australiensis* (All-C) were mixed to achieve a 100-fold dilution.

3.2.5 Growth of mono-, dual- and multispecies biofilms on SS coupons (method 2)

To mimic cell transfer conditions observed in during slicing a second method of biofilm transfer to meat was explored (Method 2). To grow biofilms on SS coupons, four 50 mm^2 coupons were placed into individual glass-bottomed food storage containers containing 250 mL dTSB solution. The bacterial inoculum was prepared as described above, a total of 2.5 ml of prepared inoculum was added to the dTSB to achieve a 100-fold dilution. Each of the containers with the coupons and bacterial suspensions were covered with sterile aluminum foil and containers were incubated at 22 °C for 14 days.

3.2.6 Sample preparation of sessile cells

To determine the pressure resistance of cells detached from the SS coupons, after incubation of SS coupons for 14 days, coupons washed twice with sterile deionized H_2O to remove planktonic cells. The coupons were air dried for approximately 30 mins. Sessile cells were removed from the dried coupons using a sterile cotton swap (Puritan Medical Products Company LLC, Guilford, Maine, USA) that had been dipped into 0.1% (w/v) sterile peptone water (Becton Dickinson) and the tip of the cotton swab was rubbed against the surface of the coupon vertically and horizontally for 2 min. The cotton swab was placed into 2 mL of 0.1% peptone water, and vortexed for 2 min. 500 µL of peptone water containing single strain *L. monocytogenes* or mono-, dual or multi- species detached sessile cells were transferred to 4 cm R3603 tygon tubing (Saint-Gobain, Courbevoie, France) and heat sealed with a hair-straightener (TONI&GUY®, Model TGST2976F, London, UK). All samples were held at room temperature until pressure treatment (~30 mins).

To determine the pressure resistance of cells in the spent media (the suspension which the coupons had been incubated in for 14 days), 500 µL suspension was transferred into 4 cm tygon tubing and sealed. Samples were held at room temperature until pressure treatment.

Finally, the pressure resistance of sessile cells transferred onto RTE cooked chicken meat was determined. Jumbo chicken hot dogs (organic chicken, sea salt, white pepper, onion powder, cardamom, mace) were obtained from Sunworks Farms (Edmonton, Alberta). Cylindrical meat samples were cut from the hot dogs using a sterile cork borer (Cole-Palmer, Montreal, QC, Canada). Each sample was 0.5 cm diameter by ~4.25 cm in length. SS coupons were washed and dried as described above prior to inoculating RTE chicken cylinders. To transfer cells onto the cylinders two methods were used (method 1 and method 2). For method 1, a single coupon with attached sessile cells was used along with a second sterile coupon to transfer the biofilm onto the meat. The meat was sandwiched between the two coupons and rolled back and forth for 2 min as shown in Figure 3.1. For method 2, cylinders were sandwiched between two coupons both with sessile cells, the SS coupons were rolled back and forth for 2 mins (Figure 3.1). Cylinders were transferred into tygon tubing as described above for pressure treatment.

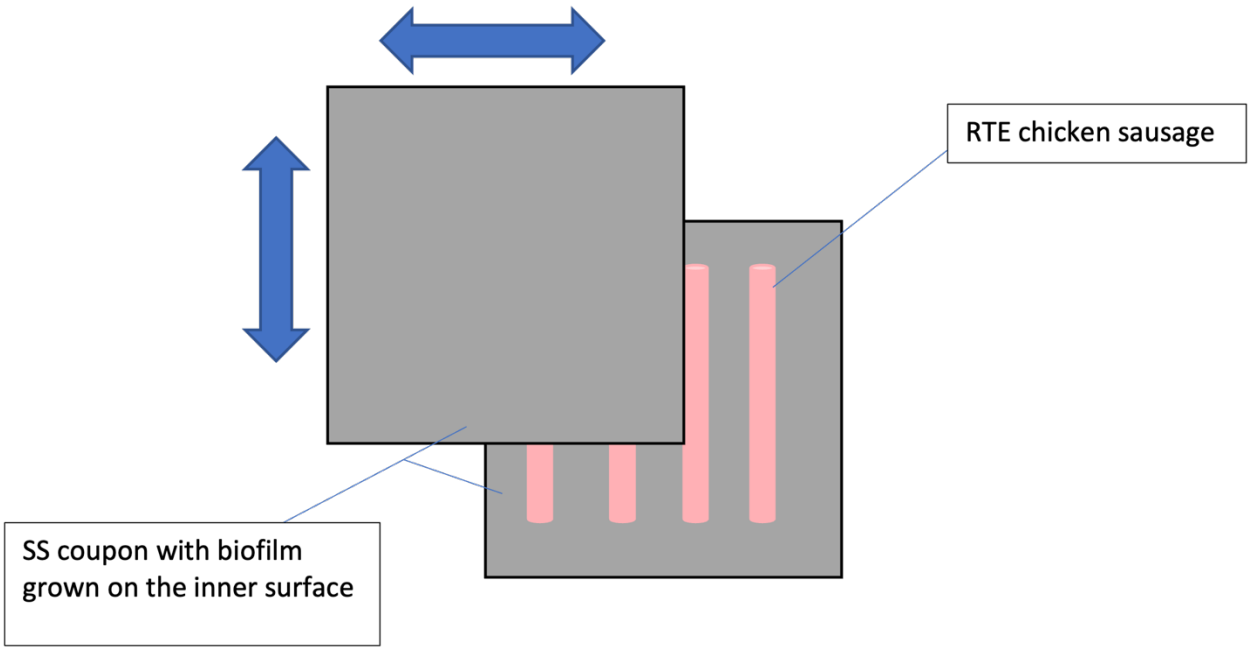

Figure 3.1 Method of transferring biofilms from the inner surface of stainless steel coupons onto the surface of RTE chicken meat (pink cylinders). The top coupon was moved vertically and horizontally (blue arrows) over the cylinders for 2 min. For method 1, the top coupon was sterile and for Method 2, a biofilm was on the surface of the coupon that was next to the meat cylinders.

3.2.7 Sample preparation of planktonic cells

To determine the pressure resistance of planktonic cells in broth, equal volumes of each individual strain of *L. monocytogenes* was mixed to form a cocktail. Once the *L. monocytogenes* cocktail was obtained, mono-, dual- or multi-species cultures as outlined in Table 1 were prepared. For sample preparation, 1 mL of each culture was prepared prior to HPP treatment. For dual-species cultures, 500 µL *L. monocytogenes* cocktail and 500 µL of the other species were mixed. For multi-species biofilms two inocula were prepared, one contained equal volumes (250 µL) of the *L. monocytogenes* cocktail and each of the other species (All), and one contained equal volumes

(333.3 µL) of the *L. monocytogenes* cocktail, of *E. coli* 8-77 and of *A. australiensis* (All-C). Single strains of *L. monocytogenes* or mono-, dual- or multi-species cultures (500 µL) were transferred into tygon tubing as described above for pressure treatment.

To transfer planktonic cells on meat, RTE meat samples were cut in cylinders as described above and cultures were prepared in 0.85% saline solution. Cultures were prepared in saline solution by taking the cultures prepared above, centrifuging (7000 X g for 5 mins) and resuspending into 1 mL 0.85 % NaCl. Inoculation of meat samples followed the procedure described by Teixeira et al., (2016), with one modification. To achieve a ~$10^{8.5-9.0}$ CFU/ mL, cylinders were placed into cultures prepared in saline solution for 1 min instead of dipping three times. Inoculated RTE meat cylinders were transferred into tygon tubing and sealed as outlined above.

3.2.8 Pressure inactivation of planktonic and sessile cells of *L. monocytogenes* in solution and on RTE chicken meat.

Prepared samples were treated in a Multivessel Apparatus U111 (Unipress Equipment, Warsaw, Poland) at 600 MPa at 20 °C for 3 min as described in Teixeira et al., 2016. The temperature of the unit was maintained by a thermostat jacket coupled to an external water bath. Polyethylene glycol was used as pressure transferring fluid. The vessel compression rate to 600 MPa was ~ 1 min and decompressed in ~ 30 s.

3.2.9 Enumeration and sample preparation

Cells were enumerated prior to HPP treatment and after treatment by surface plating. For samples in solution ie. in broth (planktonic), spent media or cells recovered from SS coupons, appropriate

dilutions were spread onto the surface of PALCAM agar and TSA to determine cell counts of *L. monocytogenes*. Plates were incubated at 37°C for 48 h. To enumerate the bacterial load on the meat before and after treatment, meat cylinders were placed into 1 mL peptone water, vortexed for 2 min and enumerated as described above for both methods. In addition to surface plating on PALCAM and TSA, for method 2, the cell counts of *E. coli* 8-77 and *A. australiensis* was determined by surface plating on McConkey Agar and TSA (Table 3.4). All plates were incubated for 48 h at 37 °C, cell counts for *E. coli* 8-77 and *A. australiensis* were enumerated after 24 h of incubation to avoid overgrowth of each culture.

3.2.10 Recovery of *L. monocytogenes* sessile cells during post pressure storage

To assess the recovery of sessile cells of *L. monocytogenes* during refrigerated storage of meat, after high pressure treatment RTE chicken meat samples were stored at 4 °C sealed in the tygon tubing until plating. Samples were plated after 45 and 90 days of storage. At each sampling day, the RTE chicken meat cylinders were removed from tygon tubing, and enumeration was completed by surface plating as described above.

3.2.11 Statistical analysis

All experiments were replicated three times. For HPP treatment experiments, data were converted to log reductions [$\log(N/N_0)$ where N represents the cell count of treated samples and N_0 represents the cell count of untreated samples. The model included log reductions for each culture and the state bacterial cells (planktonic broth, sessile broth, spent media, planktonic ham, and sessile ham). The experimental data was analyzed by one-way Analysis of Variance (ANOVA) using the PROC

GLM procedure of SAS University Edition (Version 3.4; SAS Institute. Inc., Cary, NC, USA). Tukey's posthoc test was used to determine differences among means ($P < 0.05$).

3.3 Results

3.3.1 Composition of biofilms

For each of the individual strains of *L. monocytogenes* grown on stainless steel coupons, cell counts recovered from the stainless steel coupons were all approximately 6-7 log CFU/mL (Table 3.2). When cells were transferred onto the RTE chicken meat, a higher concentration of cells was recovered from meat as compared to cells detached from the coupons. For all strains including the cocktail close to a one log increase was observed with the highest difference between detached cells and cells recovered from meat observed for *L. monocytogenes* FSL 270-SS (1.79 log increase). The number of cells in the spent media was comparable to the concentration of cells transferred onto the RTE chicken.

The concentration of *L. monocytogenes* recovered from coupons inoculated with dual- or multi-species biofilms determined on TSA was approximately 7 log CFU/mL for all cultures except for L+A (Table 3.3), which had a 1-log CFU/mL lower concentration than other biofilms. When enumerated on PALCAM agar, cell concentrations were comparable to cell counts on TSA. Using method 1 for inoculation of meat cylinders resulted in the transfer of approximately 7.3 log CFU/cm^2 of *L. monocytogenes*. The highest concentration of *L. monocytogenes* was recovered from the multi-species biofilm without *Carnobacterium* (All-C) and lowest was recovered from the meat that had been inoculated with the dual-species biofilm containing *Aeromonas* (L+A). Cell counts on TSA and PALCAM were and comparable for all cultures.

Table 3.2 Initial cell counts of biofilms of the *L. monocytogenes* cocktail and single species recovered from the spent media, detached from the stainless steel coupons and recovered from the RTE chicken meat (method 1). Counts were determined on TSA and data are means ± standard deviations of three independent replicates.

Strain	Matrix	Cell Counts (log CFU/mL)
LM Cocktail	Spent media	7.55±0.30
	Coupon	5.94±0.33
	RTE chicken meat	7.37±0.49
FSL R2-499	Spent media	7.28±0.53
	Coupon	5.89±0.11
	RTE chicken meat	6.82±0.59
FS-15	Spent media	7.77±0.65
	Coupon	6.30±0.82
	RTE chicken meat	7.35±0.61
270-SS	Spent media	7.26±0.53
	Coupon	5.63±0.17
	RTE chicken meat	7.42±0.14
263-SS	Spent media	7.43±0.23
	Coupon	6.40±0.58
	RTE chicken meat	7.03±0.63

Using method 2 the concentration of *L. monocytogenes* recovered from the coupons was comparable to concentrations in method 1 on TSA and PALCAM (Table 3.4). However, the for dual-species biofilms containing *Aeromonas* (L+A) and the biofilms containing all of the cultures, a higher concentration of cells was transferred onto meat using method 2. For multi-species biofilm without *C. maltaromaticum* (All-C), the opposite was observed. The concentration of *E. coli* recovered from the coupons and meat was consistent for all samples in broth or on meat. The

recovery of *A. australiensis* for all samples recovered from biofilms was low when enumerated on TSA and was extremely low when enumerated on McConkey agar.

Table 3.3 Average cell counts (log CFU/mL) of sessile cells of *L. monocytogenes* retrieved from stainless steel coupons with different biofilms and RTE chicken meat inoculated using Method 1. Counts were determined on TSA and PALCAM agar and data are means ± standard deviations of three independent replicates.

Composition of Biofilm	Matrix	Media	Average
L+C	Coupon	TSA	6.98±0.90
		PALCAM	6.18±0.27
	RTE chicken meat	TSA	7.74±0.27
		PALCAM	7.33±0.37
L+A	Coupon	TSA	5.98±0.45
		PALCAM	6.24±0.82
	RTE chicken meat	TSA	6.11±1.99
		PALCAM	6.74±0.11
L+E	Coupon	TSA	6.83±0.60
		PALCAM	6.81±0.58
	RTE chicken meat	TSA	8.45±0.49
		PALCAM	7.53±-.91
All	Coupon	TSA	7.54±0.56
		PALCAM	6.60±0.28
	RTE chicken meat	TSA	7.23±0.80
		PALCAM	6.90±0.72
All-C	Coupon	TSA	7.65±0.69
		PALCAM	6.09±0.69
	RTE chicken meat	TSA	8.36±0.21
		PALCAM	6.94±0.90

Table 3.4 Mean counts (Log CFU/mL) of sessile cells detached from stainless steel coupons and from RTE chicken meat inoculated using Method 2. Counts of *Listeria* (L), *E. coli* (E) and *Aeromonas* (A) were determined on Trypic Soy (TSA) and McConkey agars. Additionally, *Listeria* was enumerated on PALCAM agar. Data are means ± standard deviations of three independent replicates.

Biofilm	Matrix	Species	Media	Average
LM cocktail	Coupon	L	TSA	6.28±0.27
		L	PALCAM	6.34±0.08
	RTE meat	L	TSA	8.01±0.62
		L	PALCAM	7.47±0.25
L+C	Coupon	L	TSA	7.84±0.14
		L	PALCAM	7.18±0.63
	RTE meat	L	TSA	8.41±0.09
		L	PALCAM	7.55±0.15
L+A	Coupon	L	TSA	5.70±0.16
		L	PALCAM	5.61±0.04
		A	TSA	4.65±0.42
		A	McConkey	3.60±0.67
	RTE meat	L	TSA	7.32±0.09
		L	PALCAM	7.21±0.36
		A	TSA	4.37±0.08
		A	McConkey	3.55±0.25
L+E	Coupon	L	TSA	6.85±0.51
		L	PALCAM	6.44±0.20
		E	TSA	6.14±0.43
		E	McConkey	5.58±0.78
	RTE meat	L	TSA	7.95±0.47
		L	PALCAM	7.57±0.26
		E	TSA	6.39±1.01
		E	McConkey	5.99±0.60
All	Coupon	L	TSA	6.98±0.59
		L	PALCAM	5.77±0.17
		E	TSA	5.89±0.10
		E	McConkey	5.24±0.65
		A	TSA	5.54±0.08
		A	McConkey	3.84±0.40
	RTE meat	L	TSA	8.29±0.17
		L	PALCAM	7.10±0.35
		E	TSA	6.57±0.31
		E	McConkey	5.33±0.87
		A	TSA	5.55±0.76
		A	McConkey	1.43±2.48
All-C	Coupon	L	TSA	6.49±0.61
		L	PALCAM	5.22±1.09
		E	TSA	5.66±0.34
		E	McConkey	5.35±0.93
		A	TSA	5.59±0.11
		A	McConkey	3.86±0.76
	RTE meat	L	TSA	6.75±0.78
		L	PALCAM	7.05±0.44
		E	TSA	6.00±0.34
		E	McConkey	4.73±0.83
		A	TSA	5.18±1.50
		A	McConkey	2.70±2.46

3.3.2 Pressure resistance of single- dual, -multispecies cultures of *L. monocytogenes* in broth and RTE chicken meat.

To assess the effect of high pressure on sessile and planktonic cells of *L. monocytogenes*, 4 individual cultures and a 4-strain cocktail of *L. monocytogenes* were treated with high pressure at 600 MPa for 3 mins at 20 °C either in broth and on RTE chicken meat. Planktonic cells of *L. monocytogenes* in broth were significantly more sensitive to pressure compared to planktonic and sessile cells on chicken meat for all strains and the cocktail except for *L. monocytogenes* FSL R2-499 (Figure 3.2). There was no difference in pressure resistance between planktonic cells in broth and sessile cells on meat for *L. monocytogenes* FSL R2-499. Planktonic cells in broth of *L. monocytogenes* 263-SS in broth were the most resistant to HPP compared to planktonic cells in broth for other strains ($P<0.05$); however, the high pressure resistance of sessile cells in broth were similar for all strains. There was no difference ($P>0.05$) in the pressure resistance of planktonic and sessile cells on meat for the cocktail and *L. monocytogenes* FS-15 and 270-SS. Sessile cells of *L. monocytogenes* 263-SS and FSL R2-499 recovered from meat were more pressure resistant than planktonic cells recovered from meat. The pressure resistance of the cells in the spent media were comparable to the resistance of sessile cells in broth.

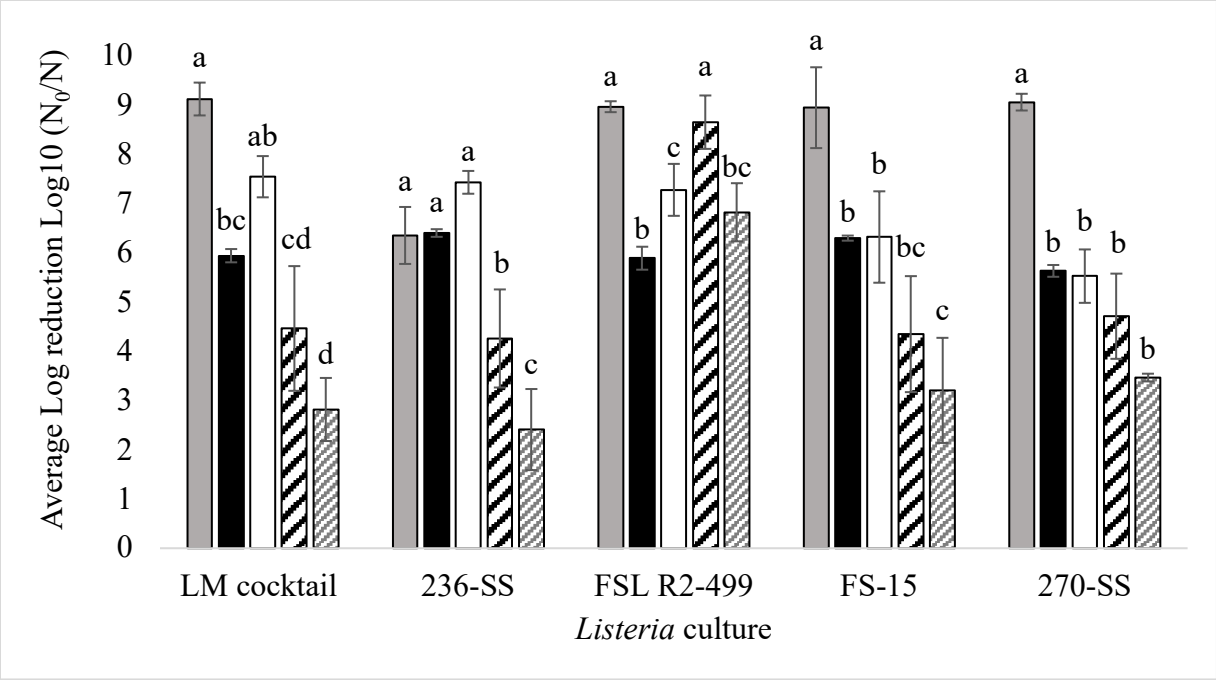

Figure 3.2: Average log reduction (Log [N0/N]) of *Listeria monocytogenes* cocktail and single strains of planktonic cells recovered from biofilms on stainless steel and sessile cell recovered from RTE chicken meat in broth and RTE chicken meat treated at 600 MPa at 20 °C for 3 mins. Cells were transferred to meat with Method 1. Cells were enumerated on TSA. Planktonic cells in 10% TS broth (grey bars), sessile cells in 10% TS broth (black bars), cells in spent media (white bars); planktonic cells on RTE chicken meat (black diagonal stripes) and sessile cells on RTE chicken meat (horizontal stripes). Letters indicate differences in log reduction within each strain among different cell types ($P<0.05$). Data are means ± standard deviations of three independent replicates.

Generally, when cells recovered from mono-, dual- and multi species biofilms containing *L. monocytogenes* were pressure treated, planktonic cells of *L. monocytogenes* in broth were more sensitive to pressure compared to sessile cells transferred onto RTE chicken meat using method 1 (Figure 3.3). However, sessile cells recovered from the dual species biofilm containing *A. australiensis* 03-09 (L+A) and pressure treated on meat were more pressure sensitive than planktonic cells or sessile cells pressure treated in broth. When treated in multi-species biofilms, the presence of *A. australiensis* 03-09, *E. coli* 8-77 and *C. maltaromaticum* UAL 307 (All), sessile

and planktonic cells pressure treated on RTE chicken meat were more pressure resistant than both planktonic and sessile cells pressure treated in broth. The presence of *C. maltaromaticum* UAL 307 increased the pressure resistance of sessile cells pressure treated on meat when compared to the pressure resistance of cells in the multi-species biofilm without *C. maltaromaticum* UAL307 (ALL-C). Enumeration of *A. australiensis* 03-09, *E. coli* 8-77 and *C. maltaromaticum* UAL 307 in all samples after pressure treatment resulted in counts below the detection level (data not shown).

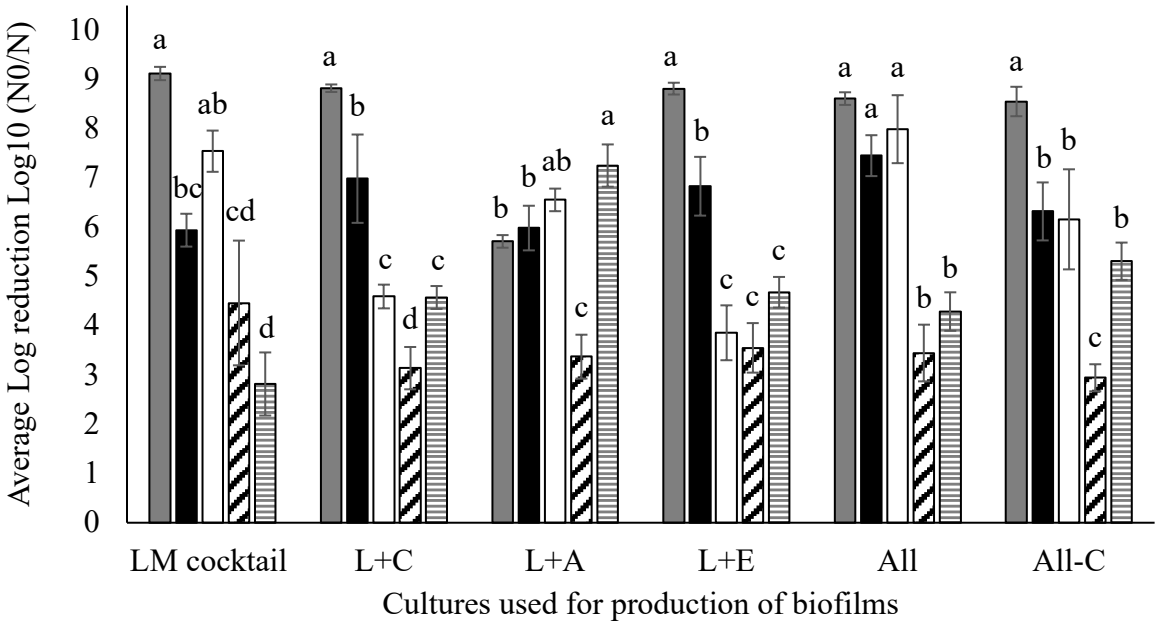

Figure 3.3 Average log reduction (log [N0/N]) of planktonic cells of *Listeria monocytogenes* in broth or sessile cells recovered from mono-, dual- and multi-species biofilms on stainless steel coupons and from RTE chicken meat treated at 600 MPa at 20 °C for 3 mins. Cells were transferred to meat with Method 1. Planktonic cells in 10% TS broth (grey bars), sessile cells in 10% TS broth (black bars), cells in spent media (white bars); planktonic cells on RTE chicken meat (black diagonal stripes) and sessile cells on RTE chicken meat (horizontal stripes). Data are from cells enumerated on TSA. Letters indicate differences in log reduction within each strain among different cell types ($P<0.05$). Data are means ± standard deviations of three independent replicates.

3.3.3 Pressure resistance of single- dual, -multispecies *L. monocytogenes* cultures in broth and RTE chicken meat transferred using Method 2.

To determine the pressure resistance of sessile *L. monocytogenes* cells transferred onto RTE chicken meat using method 2, cells or meat cylinders were treated at 600 MPa for 3 mins at 20 °C (Figure 3.4). Cells recovered in broth, regardless of state (planktonic or sessile) were more sensitive to pressure treatment compared to cells that were pressure treated on meat ($P>0.05$). There was no difference in the pressure resistance of sessile and planktonic cells on RTE chicken meat in all cultures except for those that had been grown in a multi species biofilm (ALL). The presence of *C. maltaromaticum* UAL 307 in dual species biofilms made no difference to the pressure resistance of cells but when it was present in a multi-species biofilm, the pressure resistance of cells treated on meat was reduced when compared to the resistance of cells in a multi-species biofilm without *C. maltaromaticum* UAL 307.

Table 3.5 Recovery of sessile cells of *Listeria monocytogenes* (Log_{10} CFU/mL) transferred from mono-, dual- and multi-species biofilms onto RTE chicken meat which were subjected to 600 MPa at 20 °C for 3 mins and stored at 4 °C for 0, 45 and 60 days. Letters indicate differences in cell numbers within each storage time ($P<0.05$). Data are means ± standard deviations of three independent replicates.

	Cultures used for production of biofilms					
Storage time (d)	LM cocktail	L+C	L+A	L+E	All	All-C
0	3.20 ± 0.35^{ab}	3.14 ± 0.87^{b}	3.10 ± 0.35^{b}	3.88 ± 0.86^{ab}	4.18 ± 0.33^{a}	3.26 ± 0.61^{ab}
45	9.42±0.31	6.72±0.52	7.94±1.58	9.04±0.49	8.69±1.12	8.20±1.46
90	9.63±0.10	9.21±0.22	9.46±0.14	8.824±1.33	9.11±0.38	9.35±0.32

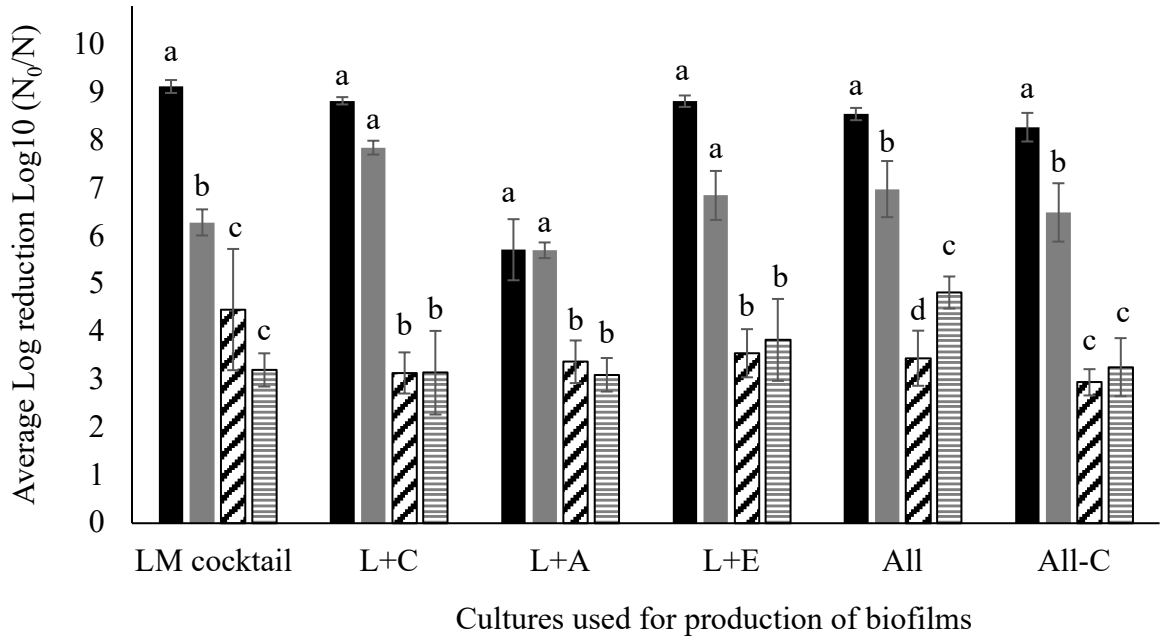

Figure 3.4 Average log reduction (log [N0/N]) of planktonic cells of *Listeria monocytogenes* in broth or sessile cells recovered from mono-, dual- and multi-species biofilms on stainless steel coupons and from RTE chicken meat treated at 600 MPa at 20 °C for 3 mins. Cells were transferred to meat with Method 2. Planktonic cells in 10% TS broth (grey bars), sessile cells in 10% TS broth (black bars), planktonic cells treated on RTE chicken meat (black diagonal stripes) and sessile cells treated on RTE chicken meat (horizontal stripes). Data are from cells enumerated on TSA. Letters indicate differences in log reduction within each strain among different cell types ($P<0.05$). Data are means ± standard deviations of three independent replicates.

3.3.4 Recovery of *L. monocytogenes* during storage of meat samples after pressure treatment

After high pressure treatment, RTE meat samples were stored at 4°C and the recovery of *L. monocytogenes* cells was determined after 0, 45 and 90 days of storage (Table 3.5). Immediately after HPP treatment, the highest cell counts of *L. monocytogenes* were observed on samples that had been inoculated with multi-species biofilms with all four species of bacteria (ALL). These counts were significantly higher ($P<0.05$) than counts recovered from samples that had been

inoculated with dual-species biofilms containing *L. monocytogenes* and *C. maltaromaticum* UAL 307 (L+C) and dual-species biofilms containing *L. monocytogenes* and *A. australiensis* 03-09 (L+A), which had the lowest cell counts and therefore were the most sensitive to HPP. However, by day 45, there was no difference in cell counts for all samples.

3.4 Discussion

L. monocytogenes poses a serious threat to public health and with its increased resistance to commercial sanitizers, the use of other processing technologies post process could increase food safety and reduce the prevalence of foodborne outbreaks. Two methods to transfer sessile cells of *L. monocytogenes* onto RTE chicken were compared in this study. Method 2 was optimized to mimic transfer to meat from a meat slicer where cells could be attached to both sides of the blade. The cell transfer was very comparable for both methods, in some instances one method transferred more cells depending on the biofilm but overall, both methods transferred more cells onto meat than were recovered from the coupon. The lower recovery of cells from the SS coupons could be a result of the swabbing method used. Vorst et al., (2004), investigated the recovery using various sampling methods, and were able to recover *L. monocytogenes* using a cotton swab; however, when the stainless steel plates were viewed with a scanning electron microscope (SEM), cells of *L. monocytogenes* were still present. Studies have determined that recovery of bacteria on dry surfaces is lower when compared to wet surfaces (Lahou & Uyttendaele, 2014; Moore & Griffith, 2002). Although the surface of the coupons used in this study were dry, the tip of the swab was hydrated prior to swabbing. Although the recovery of sessile *L. monocytogenes* cells was lower than on meat, it is important to note that regardless of method used and composition of the biofilm, the recovery of *L. monocytogenes* was consistent when using the cotton swab.

While the focus of this study was to evaluate the pressure resistance of *L. monocytogenes*, the interaction of *L. monocytogenes* with other organisms was also evaluated. When assessing the recovery of *L. monocytogenes* from biofilms grown with mono-, dual-, and multispecies, the concentration of recovered *L. monocytogenes* was consistent regardless of culture and method except for dual-species cultures containing *A. australiensis*. When *A. australiensis* was grown with *L. monocytogenes*, the concentration of recovered *L. monocytogenes* lower than when grown with other species or alone. Initially, the lower concentration was thought to be a result of bacteriocin-like substances (BLS) produced by some *Aeromonas* spp. that have antagonistic activity against *Staphylococcus aureus* (Moro et al., 1997). However, Messi et al., (2003), showed that BLS produced by *A. hydrophila* had no antimicrobial activity against *L. monocytogenes* but some *Listeria* spp. were sensitive. In biofilms containing *Aeromonas* spp. and *Bacillus* spp., the concentration of *Aeromonas* biomass was enhanced when *Bacillus* was present compared to when *Aeromonas* was grown alone (Cheng et al., 2014). In this study, when *Aeromonas* was grown in cultures with *E. coli* and *C. maltaromaticum* a higher concentration was observed; however, in a co-culture, *L. monocytogenes* limits the growth or adhesion of *Aeromonas*. It is unclear why there is an antagonistic relationship between *L. monocytogenes* and *A. australiensis* in this study but there is an interaction occurring which is impacting the growth of both organism in a co-culture.

The effect of high pressure processing on sessile cell was investigated in this study. There were differences in the pressure sensitivity of the different strains of *L. monocytogenes*. Sessile cells of *L. monocytogenes* FSL R2-499 were more sensitive to pressure when subjected to pressure treatment on RTE chicken meat compared to the *L. monocytogenes* cocktail or other strains of *L. monocytogenes*. High pressure treatment completely inactivated the cells in the spent media and

sessile cells on the meat (Table 3.2). The concept of strain variability and pressure resistance is not new as studies have concluded that the pressure resistance of *L. monocytogenes* is dependant on several factors, including strain (Alpas et al., 2000; Van Boeijen et al., 2008; van Boeijen et al., 2010). In food systems, bacteria are more resistant to pressure compared to that in broth. The lethality of the pressure treatment to bacteria in food systems is linked to several factors including the state of matter of the food product (liquid vs. solid) (Erkmen & Dogan, 2004; Garcia-Hernandez et al., 2015; Li et al., 2016; Simpson & Gilmour, 1997), the fat content of the product (Bover-Cid et al., 2015; Escriu & Mor-Mur, 2009; Ramaswamy et al., 2009), the intrinsic factors of the food (Gouvea et al., 2020; Teixeira et al., 2016) and the microorganism treated with pressure (Whitney et al., 2007; Zagorska et al., 2021). Biofilm forming capabilities by *L. monocytogenes* varies based on strain and serotype. Folsom et al., 2006 found that strains of *L. monocytogenes* serotype 4b were better biofilm formers compared to *L. monocytogenes* serotype 1/2a when grown in TSB but when grown in a 10% TSB solution (dTSB), *L. monocytogenes* serotype1/2a was a better biofilm former than *L. monocytogenes* serotype 4b. *L. monocytogenes* FSL R2-499 is 1/2a serotype and FS 15 is serotype 4b but this did not affect their ability for form biofilms as the initial cell counts for both strains prior to HPP was comparable at ~6 log CFU/mL (Table 3.2). This study confirms that in a food system, some strains of *L. monocytogenes* are more resistant to pressure when treated on RTE chicken meat when compared to pressure treatment in broth, which is consistent with previous reports (Simpson & Gilmour, 1997).

The effect of HPP on sessile cells of *L. monocytogenes* in single-, dual- and multi species biofilms was investigated using two methods to transfer sessile cell onto RTE chicken meat. Sessile cells are often more resistant to stressors compared to planktonic cells. For example, sessile cells of *S.*

aureus and *Salmonella* are more resistant to sodium hypochlorite than planktonic cells (Joseph et al., 2001; Luppens et al., 2002). When sessile cells were grown on to the surface of a single coupon and transferred onto the RTE chicken meat, sessile cells of *L. monocytogenes* in a single species biofilm containing the four-strain cocktail of *L. monocytogenes* had the same pressure resistance as planktonic cells inoculated onto the same meat product (Figure 3.3). However, this was not always the case, especially for the biofilm containing *A. australiensis*. The lethality of HPP on sessile cell populations has not been widely investigated; however, sessile cells of *Staphylococcus aureus* were more resistant to ultra-high pressure homogenization (275 MPa) than planktonic cells. This contrasts with the results in the current study where planktonic cells of *L. monocytogenes* were more sensitive to HPP compared to the detached sessile cells in broth for all cultures except cells recovered from a dual culture with *Aeromonas* and from a multi-species biofilm with all species (Figure 3.3). On RTE chicken meat the pressure resistance of sessile cells were generally similar to that of planktonic cells except for biofilms containing *A. australiensis* that were transferred using method 1. In a dual-species biofilm with *Pseudomonas fluorescens* or *Lactobacillus plantarum, L. monocytogenes*is more resistant to benzalkonium chloride compared to the resistance in a single-species biofilm (Haddad et al., 2021). Others have also concluded that dual-species biofilms are more resistant to sanitizers and that extracellular polymeric substance (EPS) plays a role in protection of cells against the sanitizer (Liu et al., 2017). In the current study, the presence of *C. maltaromaticum* UAL307 in the biofilms did not impact the growth of *L. monocytogenes*. This is surprising as this organism produces three bacteriocins that are known to kill *L. monocytogenes* on RTE meats (Blaine et al., 2009). Bacteriocins are known to inhibit the growth of *L. monocytogenes* during post-pressure storage (Castro et al., 2017; Dallagnol et al., 2017; Garriga et al., 2002; Teixeira et al., 2018) but this was not the case in this study. It is entirely

possible that this organism did not survive the pressure treatment. Transfer of sessile cells to RTE chicken meat using two coupons both with biofilms resulted in more consistent results among the types of cells and across the different biofilms. The initial cell concentration of cells that transferred with both methods was between 6-8 log for all biofilms (Table 3.3 and 3.4).

During storage, by 45 d of storage at 4 °C numbers of *L. monocytogenes* increased to that which was similar or higher than the initial inoculum prior to HPP treatment. Others have found that counts of *L. monocytogenes* remained below the detection level after storage at 4 °C (Garriga et al., 2004; Hayman et al., 2004). The higher recovery of *L. monocytogenes* in this study on meat compared to other studies could be a result of lack of nitrites in the RTE chicken used in this study and a higher initial inoculum., allowing cells to survive the pressure treatment. Nitrite has a synergetic effect and increases inactivation of *L. monocytogenes* when coupled with a mild pressure treatment (300 and 225 MPa) (De Alba et al., 2013). The sea salt used in the formulation of the RTE chicken sausages used in this study would provide a small amount of nitrite to the product but the levels of nitrite in sea salt are very low (Herrador et al., 2005) and not likely to contribute to any synergistic effects with HPP.

3.5 Conclusion

To our knowledge the lethality of HPP on sessile cells of *L. monocytogenes* recovered from mono-, dual, and multi-species biofilms and detached in broth and transferred to RTE meat products has not been investigated. *L. monocytogenes* poses a threat to public health as it typically transferred to food products from food contact surfaces post-processing and without further treatment to the product can cause illness when given ideal conditions for *L. monocytogenes* to grow. The pressure

resistance of sessile cells transferred onto meat was comparable to planktonic cells inoculated onto meat but the pressure resistance of either planktonic or sessile cells in broth was generally much lower than that on meat. When researchers are screening strains of *L. monocytogenes* for pressure resistance to determine likelihood of survival on meat, they should be aware that screening in broth will underestimate the resistance of *L. monocytogenes* to high pressure. Using stainless steel coupons with biofilms on both coupons can be used as a method to mimic the transfer of cells from surfaces to food products. This study confirms that bacteria are more resistant to HPP in food systems, and this is important to consider when determining parameters for inactivation by HPP.

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4.0 Phylogenetic analysis of *Listeria monocytogenes* with respect to source of isolation and its persistence in food processing facilities

4.1 Introduction

Listeria monocytogenes is a gram positive bacterium that causes food-borne disease after growth in food with a long refrigerated shelf life (Walker et al., 1990). Listeriosis is relatively infrequent, e.g. in Canada, it causes less than 200 cases responsible for over 178 foodborne illnesses annually in Canada (Thomas et al., 2013), but has a high mortality and thus substantially contributes to foodborne mortality (Behravesh et al., 2011). *L. monocytogenes* is a diverse bacterial species that is categorized into 13 serotypes and consists of four phylogenetic lineages (Ward et al., 2008; Wiedmann et al., 1997). *L. monocytogenes* is additionally divided in multiple clonal complexes or sequence types. Of the 13 serotypes, approximately 95% of strains isolated from food and clinical patients are of serovar 1/2a, 1/2b, 1/2c and 4b (Tappero et al., 1995). Serotype 1/2b and 4b strains in lineage I are more frequently associated with human clinical cases and are more virulent than lineage II strains (Orsi et al., 2011). Lineage II strains are more commonly isolated from food and food processing facilities (Gray et al., 2004b). Lineage III and IV strains are predominately isolated from animal sources and are rarely associated with human disease and foodborne outbreaks (Ragon et al., 2008; Ward et al., 2008). All lineage I and II strains are pathogenic and have caused outbreaks; however, the relative virulence of the lineages can differ substantially. Internalin A, encoded by *inlA*, is a bacterial cell well-anchored protein that plays a role in virulence of *L. monocytogenes* (Gaillard et al., 1991). Several studies have identified a truncation in the *inlA* gene of lineage II strains, which reduces virulence (Jacquet et al., 2004; Nightingale et al., 2008; Severino et al., 2007). Globally, *L. monocytogenes* serotype 4b isolates belonging to lineage I clade

have been responsible for the majority of listeriosis outbreaks (CDC, 2014a; Jennison et al., 2017; Mammina et al., 2009; Orsi et al., 2011) but strains of lineage II also have been consistently linked to outbreaks (Pirone-Davies et al., 2018).

L. monocytogenes can persist in food processing facilities and this long-term persistence was linked to multiple foodborne outbreaks (Lachmann et al., 2021; Miettinen et al., 1999). Cherifi et al., 2018, defined persistence of *L. monocytogenes* by isolating genetically similar isolates in the same plant over a period of longer than 3 months. Chemical disinfectants, such as peracetic acid and benzalkonium chloride, are widely used in food processing facilities to eliminate foodborne pathogens. While chemical disinfectants are effective on planktonic cells, bacteria can form biofilms that have increased resistance to the disinfectants compared to cells in the planktonic state (El-Azizi et al., 2016). Persistent strains of *L. monocytogenes* were suggested to differ genetically from transient strains and contain genes that confer resistance to sanitizers (Daeschel et al., 2022; Müller et al., 2014). The control measures to prevent the transient presence or persistence of *L. monocytogenes* in food processing faculties differs depending on the food product in question. In produce, fresh meat processing facilities, and in facilities that produce raw milk cheese, *L. monocytogenes* in the production facility and on the final product originate from the raw food product materials, from the environment, or from strains that persist in the production facility (Fox et al., 2009; Kim et al., 2018). In contrast, in ready-to-eat (RTE) meat and seafood processing facilities as well as in dairy processing plants that use pasteurized milk, environmental contamination or persistent strains of *L. monocytogenes* are the primary routes of transmission of the bacterium to the final product (López et al., 2008; Lundé N et al., 2003; Mędrala et al., 2003; Wiktorczyk-Kapischke et al., 2022). Traditional characterization of persistent isolates of *L.*

monocytogenes in food processing facilities focuses on serotyping and pulse field gel electrophoresis (PFGE) (Chasseignaux et al., 2001; Cruz & Fletcher, 2011; Ferreira et al., 2011; J. Lundén, Autio, Sjoberg, et al., 2003; J. M. Lundén et al., 2002; Miettinen et al., 1999). Subtyping provides information on specific subtypes that are associated with outbreaks and are persistent in the facility. Pulse field gel electrophoresis (PFGE) characterizes *L. monocytogenes* from different sources within the facility and can determine if isolates from different isolation sources are identical. While serotyping and PFGE are quick and easy methods for isolate characterization, little information is known about the route of contamination. The objective of this study is to determine if there are lineage-specific preferences for persistence of *L. monocytogenes* in food processing facilities, providing more understanding on possible routes of contamination.

In most jurisdictions that regulate the presence of *L. monocytogenes* in food, its detection on ready to eat (RTE) food products that support the growth of this organism will result in a recall (Weatherill, 2009). High pressure processing is a non-thermal processing technology that is used as post-processing technology to inactivate *Listeria* on RTE products with a long refrigerated storage life (Simonin et al., 2012). In high acid foods and liquid products, HPP treatment at 600 MPa can inactivate *L. monocytogenes* and achieve a 5-log reduction (Dogan & Erkmen, 2004; O'Neill et al., 2019; Stratakos et al., 2019). However, the treatment is insufficient to consistently achieve a 5-log reduction of *L. monocytogenes* in RTE meat products, a vehicle for this pathogen (Pérez-Baltar et al., 2020; Teixeira et al., 2016). The aim of this study is to determine if there is a difference in pressure resistance and biofilm formation of *L. monocytogenes* isolates from lineage I and II. Furthermore, a metadata of *L. monocytogenes* and comparative genomics was used to determine a correlation between the ecology of phylogenetic clades and source of isolation.

4.2 Methods and Materials

4.2.1 Bacterial strains and growth conditions

Bacterial strains and sources are listed in Table 4.1. All strains were stored at -80 ºC in tryptic soy broth (TSB; Difco, Becton-Dickinson, Sparks, MD, USA) with 25% glycerol. Prior to experiments, strains of *L. monocytogenes* were streaked onto tryptic soy agar (TSA; Difco, Becton-Dickinson, Sparks, MD, USA), incubated at 37 °C for 24 h. A single colony was picked and inoculated into TSB and grown aerobically at 37 °C at 200 rpm for 16-18 h.

4.2.2 High pressure treatment

Inocula for HPP treatment were prepared by inoculation of 10 mL TSB with a single colony of *L. monocytogenes* (Table 4.1) and incubation at 37 °C at 200 rpm for 16-18 h. Aliquots of 750 µL of each isolate of *L. monocytogenes* was transferred into a 4 cm piece of tygon tubing (R3603; Saint-Gobain, Courbevoie, France) and heat sealed with a heat source on both ends. Samples were held at room temperature until pressure treatment (~30 mins). Samples were treated in a Multivessel Apparatus U111 (Unipress Equipment, Warsaw, Poland) at 400, 500 and 600 MPa at 20 °C for 3 min. A thermostat jacket coupled to an external water bath maintained the temperature of the unit. Polyethylene glycol was used as pressure transferring fluid. The compression rate to 400, 500 and 600 MPa ranged from 45 s - ~1 min with a decompression rate of 30 s. To enumerate surviving cells, samples were plated onto the surface of PALCAM agar (Oxoid, Nepean, ON, Canada) and onto TSA to determine counts of sublethally injured cells. Plates were incubated at 37 ºC for 48 h prior to enumeration. Prior to data analysis, results were expressed as log-transformed ratios of cell counts before treatment to cell counts after treatment [$\log(N_0/N)$].

Table 4.1 *Listeria monocytogenes* isolates used in this study for high pressure inactivation and biofilm formation.

Strain designation	Source	Sero-group	CC	Line-age	Reference
1NP-221SS	Non persistent strain from drain	IIb	CC5	I	Kovacevic, 2007
2NP-270SS	Non persistent strain from meat rack	IIb	CC5	I	Kovacevic, 2007
2P-263SS	Grinding machine persistent	IIb	CC224	I	Kovacevic, 2007
717 555 06	Raw milk	IVb	CC6	I	MAPAQ[a]
726 317 01	Raw milk	IVb	CC6	I	MAPAQ
992 506 01	Raw milk	IIb	CC5	I	MAPAQ
FS 15	Hot dogs	IVb	CC2	I	Bohaychuk et al., 2006
HPB 1047	Cheese	IIb	CC3	I	Health Canada
HPB 1111	Clinical	IIb	CC3	I	Health Canada
HPB 1883	Soft unpasteurized cheese (food)	IVb	CC1	I	Health Canada
HPB 2130	Chocolate milk	IIb	CC3	I	Health Canada
HPB 394	Cheese	IIb	CC3	I	Health Canada
ILSI (IAFNS) 28	Human isolate from coleslaw outbreak	IVb	CC1	I	Bille & Rocourtb, 1996
ILSI (IAFNS) 39	Chocolate milk	IIb	CC3	I	Bille & Rocourtb, 1996
ILSI (IAFNS) 4	Mexican style cheese	IVb	CC1	I	Bille & Rocourtb, 1996
ILSI (IAFNS) 40	Human isolate from chocolate milk outbreak	IIb	CC3	I	Bille & Rocourtb, 1996
IP-101SS	Drain in meat processing facility	IIb	CC5	I	Kovacevic, 2007
FSL J1-177	Human (Sporadic)	IIb	CC288	I	Fugett et al., 2006
FSL N1-227	Food (epidemic)-orginally frankfurters	IVb	CC6	I	Fugett et al., 2006
FSL N3-013	Pate	IVb	CC2	I	Fugett et al., 2006
08-5578	Human blood 2008 outbreak	IIa	CC8	II	Gilmour et al., 2010
08-5923	Human blood	IIa	CC8	II	Gilmour et al., 2010
16PF0833	Chocolate milk	IIa	CC155	II	Hanson et al., 2019
1903319-03	Meat processing plant	IIa	CC321	II	This study
1903503-05	RTE whole muscle meat	IIa	CC321	II	This study
2002579-04	Meat processing plant	IIa	CC321	II	This study
2002581-01	RTE whole muscle pork	IIa	CC321	II	This study
2002658-01	RTE whole muscle beef	IIa	CC321	II	This study
20028600-6	Meat processing plant	IIa	CC321	II	This study

Table 4.1 cont.

Strain designation	Source	Sero-group	CC	Line-age	Reference
2002997-02	Meat processing plant (NFCS)	IIa	CC321	II	This study
2003014-01	RTE whole muscle pork	IIa	CC321	II	This study
2003634-11	Meat processing plant	IIa	CC321	II	This study
454 046 01	Raw cheese milk	IIa	CC11	II	MAPAQ
473 799 02	Cheese curds	IIa	CC37	II	MAPAQ
473 800 01	Raw milk	IIa	CC37	II	MAPAQ
723 341 01	Raw milk	IIa	CC8	II	MAPAQ
726 759 01	Raw milk	IIa	CC8	II	MAPAQ
763 910 04	Raw cheese milk	IIa	CC14	II	MAPAQ
811 618 01	Goat cheese	IIa	CC200	II	MAPAQ
899 494 01	Raw milk	IIa	CC226	II	MAPAQ
902 722 01	Raw milk	IIa	CC8	II	MAPAQ
922 472 01	Raw milk	IIa	CC199	II	MAPAQ
934 294 02	Cheese	IIa		II	MAPAQ
950 008 01	Raw milk	IIa	CC89	II	MAPAQ
ATCC 15313	Rabbit	IIa	CC7	II	Murray et al., 1926
FSL C1-056	Human (Sporadic)	IIa	CC7	II	Fugett et al., 2006
FS 12	Fermented sausage	IIa	CC8	II	Bohaychuk et al., 2006
HPB 1698	Semi-soft pasteurized cheese	IIa	CC14	II	Health Canada
HPB 1707	Semi-soft pasteurized cheese	IIa	CC14	II	Health Canada
HPB 2355	Dairy (environmental)	IIa	CC8	II	Health Canada
HPB 2522	Goats milk (animal)	IIa	CC7	II	Health Canada
HPB 2523	Sheep's milk (animal)	IIa	CC415	II	Health Canada
HPB 2921	Environmental	IIc	CC9	II	Health Canada
HPB 2980	Clinical	IIa	CC7	II	Health Canada
HPB 318	Dairy plant (environmental)	IIa	CC8	II	Health Canada
HPB 4715	Refrigerator door, restaurant (environmental)	IIa	CC8	II	Health Canada
HPB 5176	Milking cow (animal)	IIa	CC207	II	Health Canada
HPB 6632	Milk sample from reservoir of milking cow (animal)	IIa	CC570	II	Health Canada
FSL R2-499	Sliced Turkey	IIa	CC11	II	Fugett et al., 2006
2002997-04	Meat processing plant		ST168	L. welsh imeri	This study

a) MAPAQ, Ministère de l'Agriculture, des Pêcheries et de l'Alimentation du Québec

4.2.3 Biofilm formation on stainless steel coupons and biomass quantification

For growth of biofilms on stainless steel, 20 µL of individual overnight cultures were added to 2 mL of 10% TSB (TSB; Difco, Becton-Dickinson, Sparks, MD, USA) to achieve 100-fold dilution as described in Xu et al., (2021). To grow the biofilms, stainless steel (SS) coupons (type 304, SS-8, No. 4 finish, 12 mm diameter; Stanfos, Edmonton, AB, Canada) were placed in the bottom of 24-well flat bottom cell culture plates (Corning, Glendale, AZ, USA) and 2 mL of bacterial culture and diluted TSB was added to each well. The plates were incubated at 37 °C for 48 h. The crystal violet method was used to quantify biomass of the *L. monocytogenes* biofilms as described by (Xu et al., 2021). In summary, after 48 hours of growth, coupons were rinsed three times with sterile water and air dried for 25 mins. Coupons were placed in 300 µL of 1% crystal violet (Sigma-Aldrich, St. Louis, MO, USA) and incubated for 30 mins. Excess stain was removed by rinsing the coupons with sterile water. To release the dye, coupons were added to 1% sodium dodecyl sulfate (SDS; Sigma-Aldrich) and incubated at ambient temperature with agitation for 30 mins. The absorbance of the resulting supernatant was measured at 570 nm by spectrophotometer (Beckman Coulter, Brea, CA, USA).

4.2.4 DNA extraction

Genomic DNA (gDNA) was extracted using a Wizard Genomic DNA Purification Kit (Promega Corporations, Madison, WI, USA) with minor modifications. To obtain gDNA, strains of *L. monocytogenes* were grown overnight at 37 °C. DNA was isolated following the protocol 'Isolating Genomic DNA from Gram Positive and Gram Negative Bacteria', with two modifications: Protein precipitation (step 10) was repeated three times. Additionally, 750 µL of

room temperature isopropanol was added to 2 mL microcentrifuge tube (step 12) instead of 600 µL in a 1.5 mL microcentrifuge tube. The quality and quantity of each sample was assessed using a spectrophotometer (NanoDrop® ND 1000; Thermo Fisher Scientific, Wilmington, DE, USA) to ensure the ratio of the absorbance at 260 nm to the absorbance at 280 nm was greater than 1.75.

4.2.5 Whole genome sequencing, assembly, and data analysis

The DNA libraries of 41 *L. monocytogenes* and 1 *L. welshimeri* strains were prepared at Oregon State University Centre for Quantitative Life Sciences (Corvallis, OR, USA) using the PlexWell kit (seqWell; Beverly, MA, USA) according to the manufacturer's instructions. Prior to sequencing, quality of was checked again using dsDNA broad range assay kit for DeNovix DS-11 Spectrophotometer/Fluorometer (DeNovix; Wilmington, DE, USA). Libraries were sequenced using 2 × 150-bp paired-end sequencing on the Illumina NextSeq 2000 platform.

Whole genome sequencing of the 10 *Listeria* isolates obtained from RTE meat processing plants in Alberta (Table 4.1) was done by Microbes NG (University of Birmingham, UK) using Illumina with 2 x 250-bp paired-end reads.

Reads obtained through either sequencing platform were uploaded to BV-BRC (https://www.bv-brc.org/) and trimmed using TrimGalore and CutAdap to eliminate adaptor sequences. The assembly on trimmed reads was performed using SPAdes and annotation was done by RAST tool kit (RASTtk) (Olson et al., 2022; Wattam et al., 2017). Core genome MLST (cgMLST) was done using the BIGSdb-*Lm* platform, where isolates were compared to cgMLST profiles found in the database (Moura et al., 2016).

4.2.6 Phylogenetic analysis of strains of *L. monocytogenes* differing with respect to their source of isolation

The selection of *L. monocytogenes* strains for phytogenic analysis was done using the metadata available from GenBank. Isolates were categorized into seven ecological niches: dairy, meat, produce, seafood, clinical, environmental, and other (Table S4.1) and selection was based on rarefaction (Sanders, 1968). The isolate thresholds were ~40 within each category because produce and seafood contained the lowest number of isolates. Within each category, the selection criteria were date, country of origin and isolation source. To ensure that selection was representative of as many countries within the threshold, if there were multiple strains isolated from the same source in the same country in the same year, only one isolate was selected. Within the meat category, *L. monocytogenes* strains isolated from meat products aimed for human consumption or from the animal carcass were selected. The clinical isolates selected for this study were based on human listeriosis outbreaks or strains isolated from human hosts. Isolates were categorized as "other" if they did not clearly fit into any of the 6 categories, e.g., isolates from breast milk, pet food and eggs.

A total of 398 *Listeria* spp. genomes including strains in Table 4.1 and S4.1 were annotated using Prokka 1.14.6 with a blastP identity cut off of 95% (Seemann, 2014). The pangenome was assessed using Roary to generate the core genome alignment (Page et al., 2015) and a phylogenetic tree was constructed using FastTree2 using minimum-evolution subtree-pruning-regrafting (SPRs) and maximum-likelihood (ML) nearest-neighbor interchanges (NNIs) (Price et al., 2010). A generalized time-reversible (GTR) model was also used to account for nucleotide evolution and rates of evolution. The average nucleotide identity (ANI) of one strain each of the four lineages of

L. monocytogenes and closely related species (Table S3) was calculated with the OAT software (Lee et al., 2016).

4.2.7 Whole genome SNP analysis

Three prior publications report the number of SNPs that differentiate *Listeria* isolates obtained over a period of 3 months to 17 years (Alvarez-Molina et al., 2021; Daeschel et al., 2022; Harrand et al., 2020) A literature search identified 5 additional publications that report isolation of strains of *L. monocytogenes* from food processing plants that were sampled with an interval of 1-5 years but did not provide a SNP analysis. These assembled genomes were downloaded from NCBI (https://www.ncbi.nlm.nih.gov/assembly). In addition, 9 isolates of *L. monocytogenes* and one isolate of *L. welshimeri* that were obtained between October 2019 to August 2020 from a RTE processing plant in Alberta, or from products obtained in that facility were included (Table 4.1 and Table S4.1). A list of *L. monocytogenes* and their accession is provided in Table S4.2. Single nucleotide polymorphisms (SNPs) analysis of these strains was performed using Snippy v2.6 with the default settings (https://github.com/tseemann/snippy). Reference genomes for SNP analysis are bolded and underlined in Table S4.2. An average nucleotide identity was done to determine the genomic similarity of 10 *Listeria* spp. Strains used for ANI are shown in Table S4.3. *L. monocytogenes* EGD-e was used as a reference organism.

4.2.8 Statistical analysis

High pressure processing and biofilm formation experiments were replicated three times. For HPP experiments, data were converted to log reductions [$\log(N/N_0)$ where N represents the cell count of treated samples and N_0 represents the cell count of untreated samples. The model included log

reductions of HPP for each strain and pressure (400 500 and 600 MPa). The experimental data was analyzed by one-way Analysis of Variance (ANOVA) using the PROC GLM model with Tukey's post hoc pairwise test on SAS University Edition (Version 3.4; SAS Institute. Inc., Cary, NC, USA). Logistic regression model was used to determine if the probability of lineage I and II is dependent on the source of isolation using odds ratios (OR; STATA/IC v15.1, College Station, TX, USA). The logistic regression model included all isolates in the study (Table 4.1, S4.1 and S4.2). If the OR was >1.0 the proportion of lineage I was increased compared to lineage II in the category, if the OR was <1.0, the proportion of lineage II was increased. Isolates in the "other" category and isolates in lineages III and IV were excluded from the analysis. A likelihood ratio test was used to determine if the proportion of Lineage I vs II varies within the different source isolation categories.

4.3 Results

4.3.1 Phylogenetic analysis of strains of *L. monocytogenes* differing with respect to their source of isolation

A dataset of 389 genomes including 60 genomes sequenced in the present study that allows comparative genomics by lineage was compiled and is depicted in a phylogenetic tree (Figure 4.1). The tree includes *L. innocua* and *L. welshimeri* as outgroups. Strains that are displayed on the tree are color coded by the four major clades, lineage IV, III, II and I, and by source of isolation. Strains of lineage II accounted for the highest proportion of isolates (59.4%) compared to lineage I (40.4%) (Table 4.2). Within lineage II clade, the proportion of isolates from environmental, meat

and seafood were twice as high compared to lineage I. Lineages III and IV are represented only by 9 each.

The average nucleotide identity of *Listeria* spp. is shown in Table 4.3. The heatmap shows that lineages I and II share an ANI of 94.85%. Comparing lineage III or lineage IV to lineage I and II also revealed ANI values below the species threshold of 95%. An inter-lineage ANI of more than 95% was obtained only for strains representing lineages III and IV. The ANI between all strains of *L. monocytogenes* and all other species of *Listeria* were below 90%.

To determine whether the proportion of isolates from environmental sources, meat products or meat processing facilities and seafood is significant, the logistic regression model was performed on 369 genomes of lineages I and II. Assembly statistics shown in Table 4.2. A logistic regression was used to determine if the proportion of lineage I and II was dependant on source, the clinical category was used as reference group. The odds ratio was less than one for the categories of environment, meat and produce. This low odds ratio was not significant ($P > 0.05$) but was identified as trend ($0.1 > P > 0.05$) for a higher proportion of lineage II strains in the categories of environment and meat.

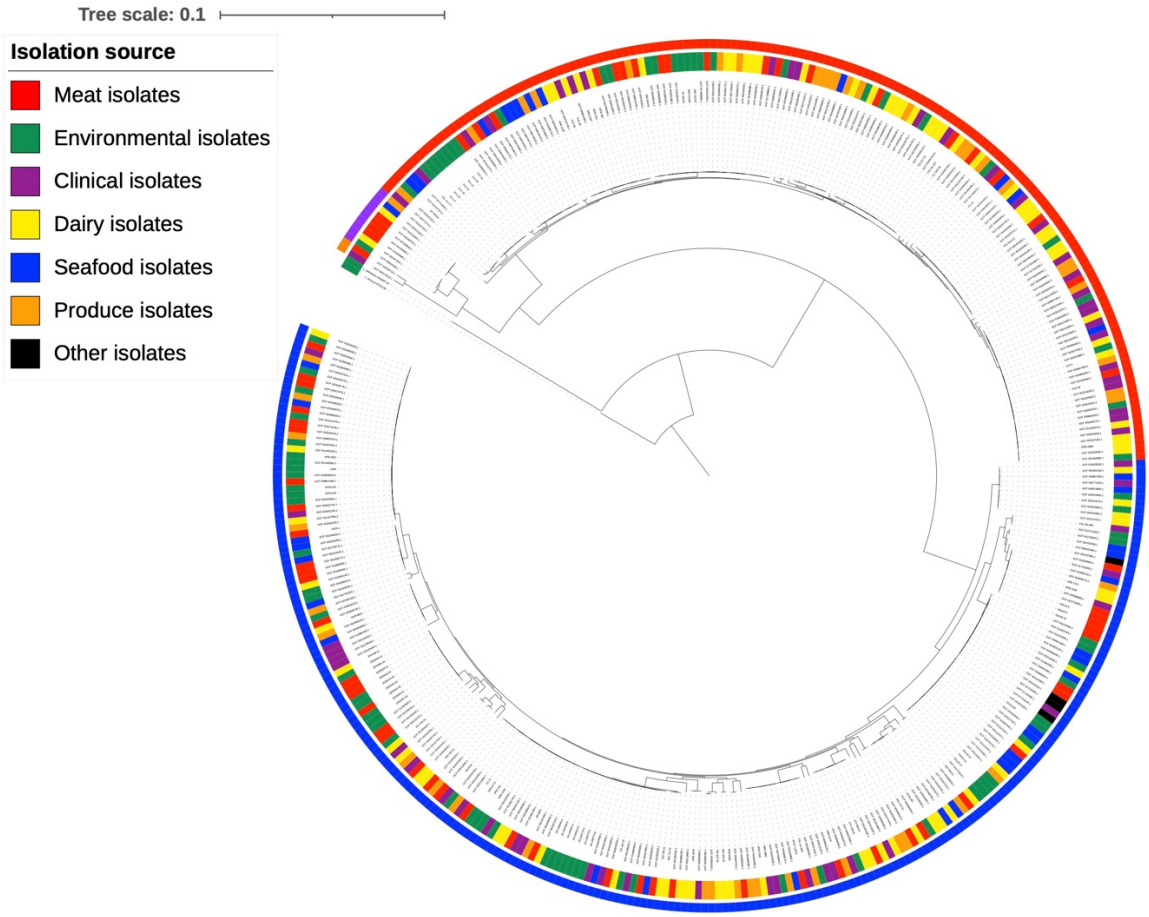

Figure 4.1 Core genome phylogenetic tree of 389 *Listeria monocytogenes* strains isolated from 6 ecological source and visualized on iTOL (**https://itol.embl.de/**). The colour strip on the outside of the tree represents the ecological isolation source. The colour strip on the inside indicates the lineage each isolate belongs to shown on the legend on the left. The orange color strip represents isolates belonging to lineage IV, purple lineage III, red lineage I and lastly, blue lineage II. The tree was rooted by *Listeria innocua.*

Table 4.2 Logistic regression analysis of 369 strains of *Listeria monocytogenes* isolated from different sources. Clinical category was used as a reference group.

Lineage I (yes) vs Lineage II (no)	Isolate Source Category						
	Clinical	**Dairy**	**Environ-mental**	**Meat**	**Produce**	**Seafood**	**Number of genomes, proportion (%) or P-value for the group**
Total no. of isolates per category	**58**	**79**	**85**	**64**	**45**	**38**	**369**
Proportion of Lineage II	51.7	51.9	65.9	68.8	48.9	68.4	**59.4**
Proportion of Lineage I	48.3	48.1	34.1	31.2	51.1	31.6	**40.6**
Odds Ratio	Reference group	0.993	0.555	0.487	1.11	0.495	
P-value	-	0.984	0.091	0.056	0.776	0.107	**0.062**

Table 4.3 Average nucleotide identity (ANI) analysis of nine isolates of *Listeria* spp. Boxes shaded in yellow show strains of *L. monocytogenes*.

Strain	*L. cossartiae*	*L. marthii*	*Lm*[1] 850658	*Lm*[2] FSL J1-208	*Lm*[3] 02-6680	*Lm*[4] 1998	*L. farberi*	*L. innocua*	*L. welshimeri*
L. cossartiae									
L. marthii	93.70								
Lm 850658	89.66	89.52							
Lm FSL J1-208	89.19	89.20	95.34						
Lm 02-6680	89.39	89.15	94.73	93.20					
Lm 1998	89.66	89.85	93.11	92.36	94.85				
L. farberi	87.31	87.37	88.50	88.10	88.78	88.56			
L. innocua	86.56	87.56	88.31	87.62	88.33	88.11	92.12		
L. welshimeri	86.28	86.29	86.63	86.63	86.67	86.72	86.74	87.02	

1. *L. monocytogenes* Lineage III
2. *L. monocytogenes* Lineage IV
3. *L. monocytogenes* Lineage I
4. *L. monocytogenes* Lineage II

4.3.2 Whole genome SNP analysis

Strain level persistence in food processing plants was based on SNP analysis of isolates obtained with a distance of 3 or more months. We used a SNP count of 20 to delineate the same strain; this conforms to outbreak investigations which use a SNP count of 10 – 20 to identify outbreak strains and prior analyses of isolates from food processing plants (Alvarez-Molina et al., 2021; Daeschel et al., 2022; Harrand et al., 2020; Lachmann, et al., 2021). The SNP analysis is shown in Table 4.4. The yellow highlighted boxes indicate a SNP ≤ 20 and grey illustrate SNP > 20. A total of 33 isolates were analyzed to determine the SNPs. Of these 34 isolates, 26 were assigned to lineage II and 8 were assigned to lineage I and one to *L. welshimeri* (Figure 4.1). All 9 isolates of *L. monocytogenes* obtained in this study over a period of 11 months differed by fewer than 20 SNPs, indicating these represent the same strain. The highest number of SNPs was 12, differentiating strains 2002658-01 and 2002581-01 (Table S4.4). Two isolates from a Denmark rabbit processing facility within a 2-month period were identical. *L. monocytogenes* ISALM 27 and 31 from the processing facility contained only 18 SNPs while *L. monocytogenes* ISALM 8 and 10 isolated from the rabbit carcass contained only 0 and 6 SNPs when compared to ISALM 10. This indicated that *L. monocytogenes* ISALM 27 and 31 and 8, 9 and 10 are identical and likely the same strain however ISALM 10 and 31 have a SNP count of 22561. Five of the 7 isolates from a German outbreak investigation were the same strain. All 7 isolates from the Jamaican meat processing facility were isolates of the same strain. Of the persisting strains identified in this study, 7 were lineage II while one was a lineage I strain.

Table 4.4 SNP analysis of strains of *Listeria monocytogenes* isolated from food processing plants. Yellow boxes indicate 20 SNPs or fewer. Grey boxes illustrate > 20 SNPs.

	Strain									
	ISALM									
Isolate	10	9	8	27	29	31				
10	-									
9		-								
8			-							
27				-						
29					-					
31						-				
	JLm									
Isolate	14	15	16	17	19	20	36			
14	-									
15		-								
16			-							
17				-						
19					-					
20						-				
36							-			
	LI00									
Isolate	373-0	545-0	773-0	774-0	785-0	913-0	913-1			
373-0	-									
545-0		-								
773-0			-							
774-0				-						
785-0					-					
913-0						-				
913-1							-			
	Alberta processing facility									
Isolate	4-11	3-05	9-04	0-06	7-02	4-01	1-01	8-01	9-03	7-04
4-11	-									
3-05		-								
9-04			-							
0-06				-						
7-02					-					
4-01						-				
1-01							-			
8-01								-		
9-03									-	
7-04										-

4.3.3 High pressure treatment and biofilm formation

To assess the difference in pressure resistance and biofilm formation of strains of *L. monocytogenes* of lineage I and II isolated from clinical, environmental, dairy and meat sources, 59 strains were treated with HPP at 400, 500 and 600 MPa, and the density of the biofilm formed on stainless steel was measured. Pressure resistance and biofilm formation are depicted adjacent to the phylogenetic tree of the isolates in Figure 4.2. Biofilm formation did not differ between strains of lineage I and II ($P>0.05$). Treatment with 500 MPa reduced cell counts of 19 of 23 lineage I isolates, and of 32 of 36 lineage II isolates to below the detection limit; treatment with 600 MPa reduced cell counts of all isolates to below the detection limit. Treatment at 400 MPa reduced cell counts of 35% of lineage I isolates by more than 2 log (CFU/mL) but only 6% of lineage II isolates displayed a comparable resistance (Figure 4.2). None of the lineage I isolates but 4 of 39 of lineage II isolates were reduced by more than 6.7 log (CFU/mL), i.e. to cell counts below the detection limit. Overall, strains belonging to lineage I were significantly more resistant to the treatment at 400 MPa compared to strains from lineage II ($P<0.001$).

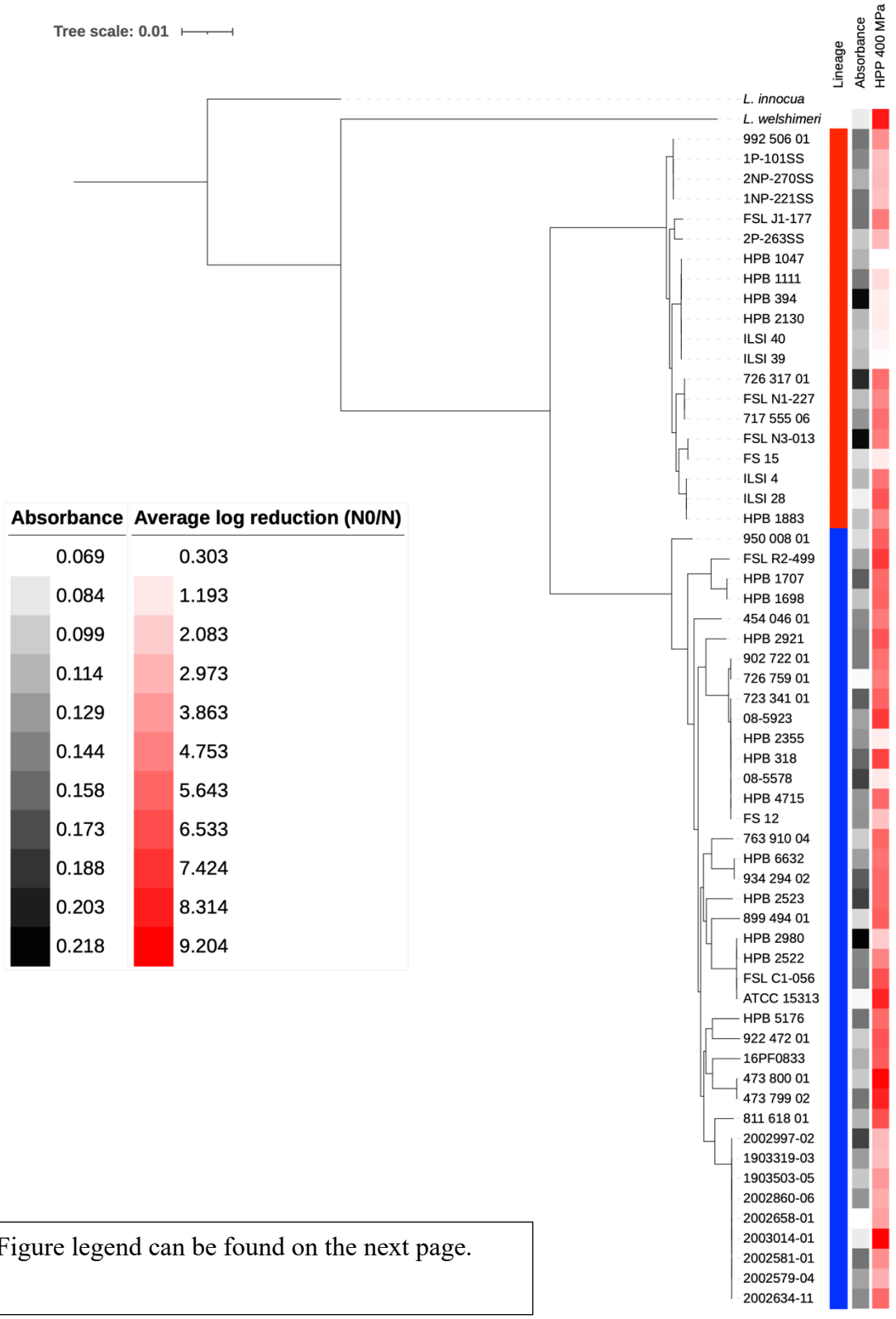

Figure legend can be found on the next page.

Figure 4.2 Core genome phylogenetic tree and heat map of 59 clinical, environmental, meat and dairy isolates of *Listeria monocytogenes* and 1 *Listeria welshimeri* using *Listeria innocua* as an outgroup. Each leaf indicates a single isolate. The tree was visualized on iTOL (https://itol.embl.de/). The solid color strip on the tree represents the phylogenetic lineages each isolate belongs to (red lineage I and blue lineage II). The black and white heat map shows the biofilm biomass absorbance reading at 595 nm. White represents the lowest biomass and black is the highest biomass. The red and white heat map indicates the average log reduction [log(N0/N)] of cells after HPP treatment at 400 MPa. Data in the heat map are shown as means of three independent experiments (n=3).

4.4. Discussion

4.4.1 Phylogenetic analysis of strains of *L. monocytogenes* differing with respect to their source of isolation

L. monocytogenes is one of few bacterial foodborne pathogens that can contaminate food in multiple ways, either through the raw material (produce, raw meat, and seafood or unprocessed and raw milk cheese), through environmental contamination, or through long-term persistence in food processing facilities (Kurpas et al., 2018; López et al., 2008; Muhterem-Uyar et al., 2015; Nesbakken et al., 1996). Past studies that aimed to discriminate whether specific phylogenetic clades in the species differ in their virulence or their ability to persist in food processing plants predominantly categorized strains on the basis of clonal complexes or sequence types, which comprise phylogenetically highly related strains with a relatively recent common ancestor (Chen et al., 2017; Cherifi et al., 2018; Cooper et al., 2021; Fagerlund et al., 2022; Pasquali et al., 2018; Yang et al., 2020). Strains of *L. monocytogenes* are more broadly categorized into four phylogenetic lineages based on molecular subtyping methods (Ward et al., 2008; Wiedmann et al., 1997). This study determined whether the ability to persist in food processing plants and the resistance to high pressure treatments are lineage specific traits. ANI analysis of the strains of *L. monocytogenes* used in this study showed that lineages III and IV share an ANI of less than 95%

to each other and to lineages I and II. Strains from Lineage I and II share an ANI of just over 95%. An ANI of 95% is generally used as threshold for the delineation of novel species, i.e. as a threshold below which strains differ with respect to relevant physiological and ecological properties (Konstantinidis & Tiedje, 2005). This study shows that strains that differ with an ANI between 94-96% also differ with respect to relevant ecological properties. Human clinical and foodborne outbreak pathogenic strains of *L. monocytogenes* are commonly linked to lineage I and II while lineage III and IV are commonly isolated from animals with clinical symptoms. In a chapter review on the taxonomic designation of *Escherichia coli*, the author discusses the need to reconsider taxonomic designations of five "cryptic" clades isolated from environmental samples that are less virulent and are thought to be divergent isolates from *E. coli* (Walk, 2015). Current designation of *L. monocytogenes* into the four phylogenetic clades is based on ribotyping pattern methods with further classification by serotype and multilocus sequence typing (MLST). While all strains of *L. monocytogenes* species contain a virulence gene cluster or *Listeria* pathogenicity island-1 (LIPI-1), only lineage I and II are known to cause illness in humans (Kreft et al., 1999; Orsi et al., 2011). The virulence of *L. monocytogenes* lineages is an important factor to consider for any taxonomic implications of the low inter-lineage ANI values. If strains of only two lineages are commonly found in the food supply and pose a threat to human health, then taxonomic re-organization of the species would facilitate control and regulation of *L. monocytogenes* in the food supply. If lineages III and IV include pathogenic or opportunistic pathogenic strains, then a new description of species may lead to *nomina periculosi* (Parker et al., 2019).

An initial assessment for the differential distribution of lineage I strains and of lineage II strains of *L. monocytogenes* in clinical and food isolates was based on analysis of rarefied set of genomes from NCBI. The genome dataset was rarefied to include about 40 genomes per category, which

avoids that the uneven distribution of genomes per category in GenBank confounds the data analysis. The dataset is nevertheless confounded because the "dairy" category includes raw milk cheese and products from pasteurized milk. Likewise, "meat" includes fresh and RTE meat. In addition, NCBI metadata is missing or ambiguous in several cases, e.g. by lack of differentiation whether the strains originate from the animal (sheep, cow) or from meat (mutton, beef). Despite these limitations, logistic regression analysis from this study shows an overall trend nearing significance of a higher proportion of lineage II strains in all source categories except for produce. Overall, the categories of environment, meat and seafood had the highest proportion of lineage II isolates (65.9, 68.8 and 68.4%, respectively). This is not surprising as serotypes and clonal complexes within lineage II clade are more common to persist in these environments (O'Connor et al., 2010). The food processing environment is thus the most likely source for *L. monocytogenes* contamination in RTE food products. Multiple listeriosis outbreaks from produce have been linked to isolates in lineage I and the source of contamination was the raw unprocessed product (Angelo et al., 2017; Aolo Ureli et al., 2000; Laksanalamai et al., 2012; Schlech et al., 1983). From this study, within the produce category, most stains were lineage I and a likely source of contamination could be the raw fruit or vegetable.

While the use of the metadata has provided more information on the source of contamination and ecology of *L. monocytogenes* lineages, it is important to note that the data set is still relatively small. Other large-scale studies that have assessed genomic differences among isolates have used a larger data set which lowers margin of error and is better for prediction of overall trends. While the data set in this study is small and has limitations from the published GenBank categorization, it is evident that at the ecology of the lineage-level phylogeny *L. monocytogenes* plays a role in source of isolation, and in the route of transmission to food and then to humans. An overall trend

was observed in which Lineage I strains are more commonly associated with raw, unprocessed food products while lineage II strains are common in food processing facilities and in processed foods. Understanding route of contamination and ecology of *L. monocytogenes* are important factors to consider when designing control measures to reduce risk of foodborne outbreaks and increase food safety.

4.4.2 Strain level persistence of lineage I and lineage II isolates of *L. monocytogenes* in food processing plants.

Current cleaning and sanitizing practices are in place to control bacterial load and eliminate *L. monocytogenes* on food contact surfaces. Although these control practices are in place, *L. monocytogenes* continues to persist in food processing plants and can pose a threat to public health. Analysis of these persistent strains can further document the lineage-specific preference for different routes of contamination of food products. To date, three studies documented persistence of strains of *L. monocytogenes* with the criteria fewer than 20 SNPs and sampling of more than 3 month apart (Alvarez-Molina et al., 2021; Daeschel et al., 2022; Palaiodimou et al., 2021). This study analysed an additional set of strains from one processing facility, and re-analysed genomes from four additional processing facilities (Burnett et al., 2022; Lachmann et al., 2021; Palaiodimou et al., 2021; Pasquali et al., 2018). It is widely accepted in outbreak investigations that two isolates that differ in fewer than 20 SNPs represent the same strain and have originated from the same source and a very recent common ancestor (Pightling et al., 2018). The number of SNPs can also provide information on the persistence of an isolate in food processing facilities (Cherifi et al., 2018; Daeschel et al., 2022).

From our analysis of 5 facilities (1 local and 4 published literature), 7 persisting strains from 4 facilities were all lineage II, and 1 strain from 1 facility was lineage I. Of 18 isolates obtained from 13 different sampling sites in a newly built Spanish meat plant, 5 persistent strains were from lineage II (Alvarez-Molina et al., 2021). Jagadeesan et al. (2019) determined that 4 strains isolated from a cold-smoked salmon food processing facility persisted for more than 18 years and were all from lineage II. The analysis of isolates of *L. monocytogenes* that were collected during routine inspections of U.S. food processing facilities by the U.S. Food and Drug Administration (FDA) across four years identified 15 persisting strains in nine facilities. Of these 15 strains, 8 are assigned to lineage II, 5 to lineage I and 2 could not be assigned to one of the two lineages based on the information of the sequence type (Daeschel et al., 2022). Of note, the analysis by Daeschel et al., 2022 excluded meat processing facilities which are not under FDA's jurisdiction. Pulsed-field gel electrophoresis (PFGE), which is less accurate than SNP analysis, identified 5 persistent strains isolated from a fish processing facility were in lineage II and 1 from lineage I (Wiktorczyk-Kapischke et al., 2022). In total, our analyses and meta-analyses document 24 persisting strains of *L. monocytogenes* in lineage II but only 7 strains of lineage I, again strongly suggesting that persistence in processing plants resulting in post-processing contamination of RTE foods with a long refrigerated shelf life is much more likely for strains belonging to lineage II.

Isolates that have adapted to processing facilities have acquired unique genes that could allow for resistance to controls and remain persistent. Lineage II isolates are genetically diverse compared to lineage I and it has been hypothesised that this indicates a competitive advantage (Borucki et al., 2005; Bruhn et al., 2005).

4.4.3 Lineage-specific resistance to pathogen intervention methods including sanitation resistance and high pressure treatment

One hypothesis for the persistence of isolates obtained from food processing facilities has been linked genes involved in increased resistance to sanitizers such as benzalkonium chloride (BC) (Maury et al., 2019; Pirone-Davies et al., 2018). Muchaamba et al. (2022) determined that clonal complex 121 strains found in lineage II carry BC resistance genes, which are hypothesized to contribute to survival in food processing facilities. A higher proportion of resistant genes to BC was identified in persistent strains isolated from a slaughterhouse compared to non-persistent strains (Cherifi et al., 2020). While lineage II isolates are less virulent than lineage I due to truncated *inlA* gene, with increased resistance to sanitizers, lineage II commonly persist in food processing facility and can still cause foodborne illness. In addition to stress resistance, biofilms are suggested to play a role in the persistence of *L. monocytogenes* in food processing faculties (Lappi et al., 2004). Biofilms are surfaced-attached microbial communities that are held together by extracellular polymeric substance (EPS). *L. monocytogenes* biofilm formation in food processing facilities begins with adhesion of planktonic cells to a surface. Once attached, a monolayer of microcolonies form and the biofilm matures (Moretro & Langsrud, 2004). The sessile cells in the mature biofilm can detach and contaminate food or other surfaces and spread throughout the facility. Biofilm formation impedes removal of bacteria by cleaning protocols and enhances bacterial resistance to sanitizers when compared to planktonic cells of the same strain (El-Azizi et al., 2016). This study found no difference in biofilm production between strains of lineage I and lineage II. The biofilms in this study were grown in diluted TSB for 48 h at 37 °C. While nutrient deficiency is likely typical for many sites that are colonized by *L. monocytogenes*, it may enhance the adhesion *of L. monocytogenes* to the substrate but it also impedes biofilm

maturation over a prolonged period (Lee et al., 2019). Previous studies are inconclusive as some reported that lineage I isolates have increased biofilm production compared to lineage II (Djordjevic et al., 2002) while others suggest that lineage II isolates are better biofilm formers (Borucki & Call, 2003; Maury et al., 2016; Norwood & Gilmour, 1999). In processing plants, *L. monocytogenes* lives in complex microbial communities that include strong biofilm forming organisms such as *Pseudomonas* and *Staphylococcus* (Fagerlund et al., 2021; Puga et al., 2018). The ability of *L monocytogenes* to form biofilms in single culture is likely inconsequential for its ability to persist as part of microbial communities in food processing plants.

High pressure processing is a non-thermal post processing technology used to inactivate pathogenic microorganisms to increase food safety. Commercial parameters for RTE meat products are 600 MPa for 3-7 min at cold or room temperature (Bolumar et al., 2021). Many regulatory agencies require a 5-log reduction of *L. monocytogenes* when treated with HPP. In meat products treated with 600 MPa, Hayman et al., 2004, reported that a 5-log reduction of *L. monocytogenes* was dependant on strain. However, in liquid food products treated at the same pressure, the treatment is sufficient to comply with regulations (Usaga et al., 2021). In the current study, a high pressure treatment of 500 and 600 MPa in broth was sufficient to comply with the regulations and reduce cell numbers of all strains of *L. monocytogenes* to below the detection limit. When the applied pressure was decreased to 400 MPa, less than 5-log reductions was observed for more than half (58%) of the strains of *L. monocytogenes*. The increased inactivation when applied pressure is increased has previously been reported for *L. monocytogenes* (Duru et al., 2020; Simpson & Gilmour, 1997). Bruschi et al., (2017), investigated strain variability by HPP when treated at various pressures. The authors found that of the 14 strains of *L. monocytogenes* treated with 400 MPa, more than 5-log reduction was observed for only 2 of the strains and the lethality

of the treatment ranged from 0.06 to ~3.6 logs for the surviving strains. Unfortunately, at 400 MPa, the treatment lethality was not significate enough to comply to regulatory requirements. While strain variability is well documented, this study compared the pressure resistance of 59 *L. monocytogenes* isolates and found that lineage I strains were significantly more resistant to 400 MPa compared to strains belonging to lineage II. The differences in pressure resistance of *L. monocytogenes* have been attributed to strain variability but the link to the lineage of the strains has not been reported. Den Bakker et al., (2008) reported higher recombination events in lineage II strains compared to lineage I strains based on molecular typing techniques. Significant decline in relative gene expression of SigB and PrfA by HPP at 400 and 600 MPa was observed in pressure resistant *L. monocytogenes* S2542 (Bowman et al., 2008). Interestingly, comparative genomics of lineage I and II reveal that the region encoding for the Bgl-family antiterminator that is required for PrfA-dependent regulation of virulence (or generally gene expression) genes is absent in all lineage I isolates (Call et al., 2003; Hain et al., 2012; Zhang et al., 2003). This would explain why there was a decline in gene expression of PrfA when the *L. monocytogenes* S2542 was treated as this is a lineage I isolate, and this region is not present. The differential pressure resistance observed in lineage I strains in this study is not clear; however, genetic diversity between the two lineages may explain strain variability. Genetic diversity has also resulted in differences in sanitizer resistance between lineages. Understanding these differences is important to design interventions to control the spread of *L. monocytogenes*.

4.5 Conclusion

The ecology of *L. monocytogenes* is important to consider as different controls could help reduce risk of outbreaks. Lineage I strains are likely to contaminate the raw product (raw meat, raw cheese

and milk or fruits and vegetables) and lineage II strains are more likely to persistent in processing facilities and contaminate food products post-processing. Although results from this demonstrate no differences in biofilm formation between lineages, lineage I strains are more pressure resistant than strains belonging to lineage II. While lineage I isolates are more virulent than lineage II, lineage II isolates are proven to be more resistant to sanitizers used in processing facilities. The different contamination routes and phenotypic response to intervention methods observed by *L. monocytogenes* from lineage I and II demonstrate the need for different control measures to minimize the spread and contamination of this pathogen in food processing facilities.

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5.0 General discussion and conclusion

Listeria monocytogenes and *Escherichia coli* O157:H7 have been the causative agents in multiple foodborne outbreaks. Large foodborne outbreaks such as the 2008 listeriosis outbreak and the 2012 *E. coli* beef recall have prompted the industry and government officials to re-evaluate the intervention protocols to control the growth and proliferation of foodborne pathogens. After the 2008 listeriosis outbreak, the Canadian Food Inspection Agency (CFIA) developed policies to control *L. monocytogenes* in ready-to-eat meats (Government of Canada, 2011; Weatherill, 2009). In the beef industry, thermal inactivation, such as carcass rinsing, peroxyacetic acid spray and steam pasteurization, were the most common interventions implemented to control enterohemorrhagic *E. coli* O157:H7 (Rajić et al., 2007). Despite the implementation of these protocols and procedures, the risk for foodborne outbreaks caused by these pathogens remains.

High pressure processing (HPP) is a non-thermal processing technology aimed to reduce the prevalence of foodborne pathogens and increase food safety. Although HHP has been proven to reduce numbers of pathogens, there is wide variability in the inactivation of *E. coli* and *L. monocytogenes*. The inactivation is dependent on several factors including the food matrix, physiological conditions (growth temperature and phase) and process parameters (Garcia-Hernandez et al., 2015; Li et al., 2016; Teixeira et al., 2016). The mechanism of bacterial inactivation is not fully understood; however, the cell membrane is the primary target for injury induced by pressure (Pagán & Mackey, 2000). Although the cell membrane is damaged, the bacterial cell can survive and in ideal growth conditions, cells can repair and divide during storage. The use of HPP as a post-processing technology on low pH foods is advantageous because if cells are present, the treatment will damage the membrane and the acid in the food acts as an addition

hurdle to kill microorganisms (Alpas et al., 2000). HPP is not only used on low pH foods and the lethality of HPP treatment on bacterial inactivation is dependent on several other factors within the food matrix (Hayman, Kouassi, et al., 2008; Li et al., 2016; Morales et al., 2006; Yuan et al., 2017). The effects of pressure on inactivation in complex matrices needs more focus for both *E. coli* and *L. monocytogenes*. Deconstructing the food matrix into its basic macronutrients would provide insight on how the constituents aid in pressure resistance. The aim of the research reported in Chapter 2 was to investigate impact of fat on pressure resistance of *Escherichia coli* in beef and yogurt. Four ground beef and five yogurt samples with different fat content were inoculated with resistant or sensitive strains of *E. coli* and treated at 600 MPa, 20 or 30°C for 3 min. To eliminate the effects of adiabatic heating, sample temperatures were adjusted prior to pressure treatment. The impact of HHP was dependent on strain and fat content in ground beef and yogurt. In beef with a fat content of >15%, resistance of all strains decreased ($p<0.05$). At 20°C, an increase in fat content from 15.5 to 24.4% increased treatment lethality from 3.2 to 9.3±0.6 log CFU/g for pressure sensitive *E. coli* MG 1655. In ground beef with 35% fat, pressure resistant *E. coli* DM 18.3 was reduced by only 4.0 log CFU/g when processed at 20°C but reduced by 9.0 log CFU/g when processed at 30°C. In yogurt, the impact of the fat content on treatment lethality was limited. The reduction of cell counts ranged from less than 2 to more than 8 CFU/g, indicating that lethality of HHP resistance to pressure was highly strain dependent. The pressure resistance of *E. coli* is dependent on strain, fat content, food matrix and adiabatic temperature changes. Adiabatic temperature changes are important to consider when investigating the pressure resistance of *E. coli* in a food matrix. It is still not clear why the increase in fat increases the lethality of the treatment as the opposite was hypothesized. Future direction for this study would be to investigate the impact of protein and oxidative stress caused by lipid oxidation on pressure resistance. Oxidative stress

could increase the sensitivity of *E. coli* as the reactive oxygen species are produced by lipid oxidation but to prove this, a thiobarbituric acid reactive substance (TBARS) assay would need to be completed. If lipid oxidation occurs during HPP, the assay measures the absorbance of malondialdehyde (MDA) and thiobarbituric acid (Ohkawa et al., 1978). Proteins in the meat matrix may also contribute to the lethality of the treatment and would be worth investigating.

Biofilms are a complex network of bacterial cells that are held together by exopolysaccharide (EPS) material and adhere to surfaces (Costeton et al., 1995). Biofilms are a concern in food processing environments because there is a potential for growth and contamination of food and potentially an outbreak depending on the organism. Sanitation protocols implemented by food processor aim to prevent contamination of pathogenic microorganism onto food contact surfaces and food products. Sessile cells in biofilms are more resistant to sanitizers compared to planktonic cells of the same organism (Aryal & Muriana, 2019; Chavant et al., 2004; Cruz & Fletcher, 2012). Conveyor belts and meat slicers have been identified as biofilm hotspots in meat processing facilities because they are hard to clean (Wagner et al., 2020). In the listeriosis outbreak in 2008, the ready-to-eat meat product was contaminated during slicing on a meat slicer that had not been adequately sanitized (Weatherill, 2009).

The aim of the research in Chapter 3 was to investigate if sessile cells of *L. monocytogenes* in single-, dual- and multi-species biofilms on RTE chicken meats and in broth solutions are more resistant to HPP compared to planktonic cells. Sessile cells were transferred onto ready-to-eat (RTE) chicken meat using methods that mimic the transfer from a meat slicer onto the product. In addition to determining the pressure resistance of sessile cells in a food system, sessile cells were also detached from stainless steel coupons and treated. Meat and detached cells were treated at 600

MPa for 3 mins at 20 °C. Results from this study showed that planktonic *L. monocytogenes* cells in broth are more sensitive to high pressure compared to planktonic cells on meat. This is not surprising as it is well known that the lethality of HPP in a liquid system is greater compared to a solid system (Li & Gänzle, 2016; Pérez-Baltar et al., 2020; H. Xu et al., 2009). It was hypothesized that sessile cells would be more resistant to pressure compared to planktonic cells because of the increased resistance observed to sanitizers. From this study, there is either no difference or in some cases planktonic cells on RTE chicken meat were more resistant to the pressure treatment. Finally, two methods were used to transfer sessile cells from coupon to RTE chicken sausage. Both methods sandwiched the meat between two coupons; however, in method 1 only one coupon was contained cells on the surface while method 2, the surface of both coupons were contaminated. There was very little difference observed between the two methods, but a greater concentration of *L. monocytogenes* was recovered from meat compared to detaching cells from stainless steel coupons using a cotton swab. Although, the hypothesis for this study was rejected and sessile cells are not more resistant to pressure, this study is the first to evaluate the pressure resistance of sessile cells. Furthermore, this study also provided information on the interaction of *L. monocytogenes* with species that were isolated from processing faculties. It is known that single species biofilms are not commonly found in nature and there is a diverse microbiome in processing facilities (Fagerlund et al., 2021; Giaouris & Simões, 2018; Møretrø & Langsrud, 2017). Understanding how *L. monocytogenes* interacts with other species can provide information on the biofilm network and if these interactions aid in resistance to controls aimed to reduce contamination. Future direction for this study is to use microscopy to examine the structure of *L. monocytogenes* biofilms with species found in meat processing faculties.

L. monocytogenes biofilms are believed to contribute to its long-term persistence in food processing facilities (Ferreira et al., 2014). When isolates are repeatedly isolated over 3 months or more, they are considered persistent (Cherifi et al., 2018). *L. monocytogenes* serotypes within lineage I and II are responsible for the majority of human and foodborne listeriosis cases (Orsi et al., 2011). *L. monocytogenes* is one of the few pathogenic microorganisms is believed to contaminate food multiple ways. Contamination by raw unprocessed foods is the first route of contamination and the second is contamination directly from the persistent strains in the facility (Fox et al., 2009; Lundén et al., 2002; Wiktorczyk-Kapischke et al., 2022). The ecology of these lineages differs with the majority of lineage II commonly isolated from food and food processing facilities (Gray et al., 2018). Stains isolated from meat faculties (lineage II) are less virulent than strains isolated from dairy processing facilities (lineage I) (Maury et al., 2019)

The aim of the research in Chapter 4 was to investigate if the source of isolation differed for strains in lineage I and II. Using a metadata found on GenBank, isolates were divided into seven ecological niches and selection was based on rarefaction. The selection criteria were date, country of origin and isolation source with a threshold of ~40 isolates within each category. A total of 398 *Listeria* isolates were investigated and genomes were annotated. Lineage II represented the highest proportion of isolate (59%) and 40% in lineage I. A logistic regression model was used to determine the probability of lineage I and II in each source of isolation. A non-significant likelihood ratio test indicated that the proportion of lineage I vs lineage II did not vary significantly within any of the different types of isolate source categories. In addition to using a likelihood ratio, an odds ratio coefficient was determined to indicate the odds lineage I vs II in each category. This low odds ratio was not significant ($P > 0.05$) but was identified as trend ($0.1 > P > 0.05$) for a higher proportion of lineage II strains in the categories of environment and meat. Furthermore,

strain level persistence in food processing plants was based on SNP analysis of isolates obtained with a distance of 3 or more months. A total of 34 isolates were analyzed to determine the SNPs. Of these 34 isolates, 26 were assigned to lineage II, 7 were assigned to lineage I and one to *L. welshimeri*. The results from this study confirm that lineage II was more likely to persist in food processing facilities compared to lineage I. Finally, the high pressure resistance and biofilm capabilities of isolates from dairy and meat were analyzed. A total of 60 strains were treated at 400, 500 and 600 MPa at 20 °C for 3 min. While no difference in biofilm formation was observed among isolates, the inactivation of *L. monocytogenes* by HPP is dependent ecology and phylogeny. Lineage I isolates were more resistant to pressure compared to lineage II. Future direction for this work is to use comparative genomics to investigate difference in virulence, sanitizer resistance, pressure resistance, and biofilm genes between lineage I and II. The phenotypic observations to pressure have been determined, comparing these components genetically can explain why lineage I are more resistant to pressure.

Overall, results from the work in this thesis have provided more information on the impact of high pressure processing on bacterial inactivation. HPP inactivation is dependent on food matrix, growth condition and finally the ecology and phylogeny of the targeted pathogen. HPP is not a suitable stand-alone pathogen intervention technology, however, can be used in context of a hurdle concept that considers the food matrix, the ecology, and the phylogeny of the target pathogen.

5.1 References

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Appendices

Appendix A

Supplementary tables S4

Table S4.1 *Listeria monocytogenes* isolates obtained from GenBank used for comparative genomics analysis

Country of origin	Source	Accession #
USA	Channel fish	GCF_000021185.1
N/A	Poultry	GCF_000209755.1
China	Milk	GCF_000218305.1
USA	Goat	GCF_000250715.1
Netherlands	cheese	GCF_000307065.1
Canada	coleslaw	GCF_000513615.1
Canada	meat	GCF_000513655.1
USA	pet food	GCF_000787895.1
China	blood	GCF_000801395.1
India	placenta	GCF_000806405.1
Sweden: Karlstad	blood from 86 year old patient	GCF_000808135.1
Austria	food processing environment	GCF_000960155.1
Austria	meat production plant	GCF_000970525.1
Austria	food processing environment	GCF_000972995.1
USA	Human clinical case	GCF_001027065.1
USA	cheese	GCF_001188025.2
Australia	Ovis aries	GCF_001188655.1
USA	Raw nectarines	GCF_001273535.1
Italy	baked ham	GCF_001280245.1
Italy	fermented meat product	GCF_001302395.1
Brazil	cheese	GCF_001317555.2
United Kingdom	Chicken	GCF_001454845.1
United Kingdom	Chicken	GCF_001454865.1
USA	Animal tissue	GCF_001454885.1
Ireland	cheese	GCF_001463975.1
Australia	dairy food production facility	GCF_001463985.1
Australia	meat food production facility	GCF_001463995.1
Ireland	cheese	GCF_001465135.2
missing	cheese	GCF_001466195.1
Switzerland	blood	GCF_001483405.1
Italy	blood	GCF_001513695.1

Table S4.1 cont.

USA	granny smith apple	GCF_001557685.1
USA	Intact peach fruit, variety Snow King	GCF_001577875.1
Norway	salmon processing facility	GCF_001586305.1
Norway	poultry processing facility	GCF_001586315.1
USA	broccoli sprout	GCF_001594305.1
Australia	dairy cheese	GCF_001658145.1
Australia	meat environment	GCF_001658165.1
Australia	meat ham	GCF_001658175.1
Australia	meat	GCF_001658405.1
United Kingdom Ireland	meat environment	GCF_001658415.1
Switzerland	meat	GCF_001709385.1
Canada	food processing environment	GCF_001709525.1
Switzerland	food processing environment	GCF_001709715.1
Switzerland	Tuna sandwich	GCF_001710845.1
Canada	floor drain	GCF_001712265.1
Switzerland	carcasses	GCF_001712375.1
USA	chocolate ice cream	GCF_001720925.1
USA	clinical	GCF_001746875.1
missing	salmon	GCF_001752325.1
USA	chef salad	GCF_001827615.1
NA	Celery	GCF_001866355.1
NA	Environmental dairy, floor drain	GCF_001866385.1
USA	blood	GCF_001866435.1
USA	environmental	GCF_001911415.1
Canada: Calgary, AB	Food Processing Facility	GCF_001952735.1
Canada	blood	GCF_001952795.1
Malaysia	Salad	GCF_001984485.1
Malaysia	Fresh fish	GCF_001984505.1
Not applicable	Sprouts	GCF_001998945.1
Canada	Baby spinach	GCF_001998985.1
Not applicable	Baby spinach	GCF_001999085.1
Not applicable	Ground beef	GCF_001999105.1
USA	swab from facility	GCF_002016665.1
Ireland	cerebrospinal fluid	GCF_002028185.1
Ireland	blood	GCF_002028205.1
Uruguay	blood and lung culture	GCF_002101275.1
Romania	Food production environment	GCF_002103265.1

Table S4.1 cont.

Austria	raw fish sample from Turkey	GCF_002103335.1
Austria	Sausage from Russia	GCF_002103345.1
Austria	Bacon from Turkey	GCF_002103395.1
Austria	Falafel powder from Egypt	GCF_002103415.1
Austria	Eggs from China	GCF_002103425.1
Romania	Poultry sample from Republic of Moldavia	GCF_002103475.1
Romania	Butter sample from the Republic of Moldavia	GCF_002103485.1
Australia	Dairy	GCF_002114665.1
Australia	Dairy	GCF_002114675.1
Australia	Dairy	GCF_002114725.1
Australia	Dairy environmental	GCF_002115035.1
Australia	meat	GCF_002115065.1
Australia	Dairy	GCF_002115205.1
USA	white nectarine	GCF_002132305.1
USA	cheese	GCF_002132895.1
USA	Bagged Lettuce	GCF_002133255.1
USA	Honey smoked turkey breast lunch meat	GCF_002133345.1
Canada	blood	GCF_002213685.1
Canada	bacon flakes	GCF_002213745.1
Canada	CSF	GCF_002213805.1
Canada	cheese	GCF_002213825.1
Canada	cheese	GCF_002213865.1
Canada	stool	GCF_002213925.1
Canada	cheese	GCF_002213945.1
Canada	stool	GCF_002214065.1
Canada	imitation crab	GCF_002214105.1
Canada	Whipping cream	GCF_002214125.1
Canada	blood	GCF_002214145.1
South Korea	Lettuce	GCF_002220325.1
USA	cheese	GCF_002222785.1
Italy	blood	GCF_002444435.1
Italy	milk	GCF_002445255.1
Italy	smoked salmon	GCF_002445865.1
Italy	smoked salmon	GCF_002445925.1
Italy	smoked salmon	GCF_002445975.1
Italy	minced meat	GCF_002446175.1
Italy	ready to eat salad	GCF_002446535.1

Table S4.1 cont.

Italy	smoked salmon	GCF_002446575.1
USA	frozen vegetable	GCF_002487425.1
USA	mushroom	GCF_002488305.1
USA	raw salmon	GCF_002488755.1
USA	smoked salmon	GCF_002488895.1
USA	bean curd	GCF_002490105.1
USA	onion	GCF_002490225.1
Italy	bulk tank milk	GCF_002523275.1
Italy	swab (dairy plant - blue cheese)	GCF_002523555.1
Italy	bovine cheese blue	GCF_002523675.1
Italy	meat sheep	GCF_002523915.1
Italy	salmon	GCF_002524445.1
Italy	swab (dairy plant)	GCF_002524885.1
Italy	bovine cheese	GCF_002524965.1
Italy	smoked salmon	GCF_002525105.1
Italy	swine salami	GCF_002525615.1
Italy	raw milk	GCF_002525925.1
Italy	cheese	GCF_002525955.1
Italy	smoked salmon	GCF_002526095.1
Italy	smoked salmon	GCF_002526135.1
Italy	ice cream	GCF_002526435.1
Italy	ricotta	GCF_002526495.1
Italy	Meat (kebob)	GCF_002526835.1
Italy	cheese	GCF_002527025.1
Italy	cheese	GCF_002527055.1
Italy	bacon	GCF_002527075.1
Italy	salmon	GCF_002527415.1
Italy	butter	GCF_002527505.1
Italy	smoked salmon	GCF_002527635.1
Italy	sushi	GCF_002527795.1
Italy	chicken	GCF_002527885.1
Italy	salmon	GCF_002527915.1
Italy	cheese	GCF_002528095.1
Italy	sushi	GCF_002528155.1
Italy	raw milk	GCF_002528275.1
Italy	sushi	GCF_002528455.1
Italy	swab (meat plant)	GCF_002529605.1
Italy	raw milk cheese	GCF_002529715.1

Table S4.1 cont.

Italy	salami paste (production)	GCF_002529915.1
Finland	Equipment	GCF_002557815.1
Italy	rabbit meat processing plant environment	GCF_002776205.1
Italy	rabbit carcass	GCF_002776365.1
USA	human blood sporadic	GCF_002836935.1
Norway	meat processing facility	GCF 002843525.1
Norway	meat processing facility	GCF_002848505.1
USA	curly leaf spinach	GCF_002915005.2
USA	baby spinach	GCF_002915025.2
USA	Romaine Lettuce	GCF_002915045.2
USA	environmental swab	GCF_002915655.1
Chile	amniotic fluid	GCF_003002155.1
Chile	food processing environment	GCF_003002345.1
Chile	ham	GCF_003002535.1
Chile	smoked turkey ham	GCF_003002555.1
Chile	bologna sausage	GCF_003002575.1
Chile	blood	GCF_003002635.1
Chile	peritoneal fluid	GCF_003002675.1
Chile	beef	GCF_003002735.1
Chile	ice cream	GCF_003002775.1
Canada	Slaughterhouse-environment	GCF_003011575.1
Denmark	smoked fish	GCF_003030405.1
Switzerland	Salad	GCF_003097415.1
Switzerland	Salad	GCF_003097435.1
Switzerland	Salad	GCF_003097455.1
Argentina	cerebrospinal fluid	GCF_003121665.1
Singapore	blood	GCF_003121685.1
South Africa	production facility	GCF_003129705.1
South Africa	Blood culture	GCF_003129775.1
USA	Floor Drain (86-448-196 2a)	GCF_003173095.1
USA	Hot Dogs	GCF_003173145.1
USA	blood	GCF_003173215.1
Canada	cheese	GCF_003187115.1
Canada	Food - Raw Cow's Milk	GCF_003187225.1
Canada	Chopped Ham	GCF_003187405.1
Canada	Processed Cheese	GCF_003187805.1
Canada	Sundae	GCF_003188265.1
Canada	ice cream	GCF_003188385.1

Table S4.1 cont.

Canada	Parmesan Cheese	GCF_003189465.1
Canada	Prosciutto Cotto	GCF_003189625.1
Canada	chicken	GCF_003190005.1
Canada	Smoked Turkey	GCF_003190045.1
Canada	Feta Cheese	GCF_003190395.1
Canada	Raw Milk	GCF_003190405.1
Canada	Coleslaw	GCF_003190665.1
Canada	cheese	GCF_003190725.1
Canada	Salami	GCF_003191085.1
Canada	Raw Pork	GCF_003191165.1
Canada	beef	GCF_003191285.1
Switzerland	Milk, milk product	GCF_003265125.1
Italy	Sponge bags 2	GCF_003390115.1
Italy	meat products 2	GCF_003390255.1
India	Vaginal swab of Female	GCF_003409075.1
India	blood	GCF_003433415.1
Canada	blood	GCF_003433535.1
Switzerland	dryed meat	GCF_003586625.1
Switzerland	butter	GCF_003586665.1
Switzerland	environment	GCF_003587485.1
Switzerland	cooked vegetables	GCF_003587545.1
Switzerland	meat environment	GCF_003587565.1
Switzerland	cheese	GCF_003587625.1
Switzerland	salad	GCF_003587845.1
Liechtenstein	blood	GCF_003588085.1
Switzerland	raw sausage	GCF_003588155.1
Switzerland	cerebrospinal fluid (CSF)	GCF_003588985.1
Switzerland	feces	GCF_003589065.1
Switzerland	cheese	GCF_003589105.1
Switzerland	corn	GCF_003589185.1
Switzerland	mixed salad	GCF_003589225.1
Switzerland	milk	GCF_003589465.1
Switzerland	poultry	GCF_003589545.1
Switzerland	meat product	GCF_003589565.1
USA	raw cut celery	GCF_003607965.1
USA	semi soft cheese	GCF_003608105.1
USA	boiled ham	GCF_003608415.1
Chile	avocado	GCF_003608535.1

Table S4.1 cont.

USA	smoked turkey breast	GCF_003608735.1
USA	pork blood sausage	GCF_003608795.1
USA	beef jerky	GCF_003608935.1
USA	chinese sausage	GCF_003608945.1
missing	mint	GCF_003608975.1
USA	cantaloupe	GCF_003609035.1
USA	soft ripened cheese	GCF_003609055.1
USA	ready to eat product polish sausage link	GCF_003609105.1
Chile	fresh hass avocado	GCF_003609175.1
USA	Italian parsley	GCF_003609275.1
USA	basil	GCF_003609335.1
USA	blue veined and mold ripened cheese	GCF_003609355.1
USA	environmental swab	GCF_003668095.1
USA	roasted beef	GCF_003668115.1
Switzerland	milk	GCF_003703635.1
Spain	Abattoir	GCF_003966045.1
South Korea	Tuna Kimchi Gimbap (a type of Korean food)	GCF_003999665.1
USA	chocolate milk	GCF_004142705.1
Brazil	cerebrospinal fluid	GCF_004347315.1
USA	environmental swab	GCF_004763665.1
South Korea	blood	GCF_004771175.1
South Korea	blood	GCF_004771195.1
Algeria	Lentil salad	GCF_006348975.1
China	fruit	GCF_008014755.1
China	sushi	GCF_008014835.1
China	sushi	GCF_008014895.1
China	deli	GCF_008015035.1
China	vagina	GCF_008642175.1
China	aquatic product	GCF_008754425.1
China	environment	GCF_008754485.1
China	aquatic product	GCF_008754525.1
China	poultry	GCF_008754585.1
China	meat	GCF_008754615.1
China	aquatic product	GCF_008754665.1
China	aquatic product	GCF_008754715.1
China	vegetable	GCF_008754725.1
Poland	Green beans	GCF_008807195.1

Table S4.1 cont.

Poland	salami	GCF_008807495.1
Poland	broccoli	GCF_008807575.1
USA	peach	GCF_009648635.1
China	environmental swabs	GCF_009788555.1
missing	Herring	GCF_009807455.1
missing	Semi-finished fish	GCF_009807465.1
USA	Massachusetts listeriosis outbreak	GCF_009866905.1
New Zealand	Environment - Horticulture	GCF_009916185.1
Russia	Food products (fish)	GCF_011881955.1
Russia	Food products (beef)	GCF_011882015.1
Russia	Throat swab (meningitis)	GCF_011882085.1
Russia	Cerebrospinal fluid (meningitis)	GCF_011882105.1
Russia	Food products (fish)	GCF_011882125.1
USA	environment	GCF_012063765.1
USA	environment	GCF_012063825.1
Italy	radicchio	GCF_013122235.1
Estonia	Vacuum packaged sliced salted salmon product	GCF_013302955.1
South Africa	food	GCF_013389395.1
Poland	Processing environment	GCF_013433045.1
Germany	ready to eat mixed salad leaves	GCF_014059715.1
Germany	human listeriosis	GCF_014983945.1
Germany	clinical isolate	GCF_014987905.1
Canada	clinical	GCF_016126225.1
Austria	clinical	GCF_016126435.1
Austria	clinical	GCF_016126585.1
China	blood	GCF_016404955.1
Ireland	seafood factory FBO1	GCF_016919125.1
Senegal	breast milk	GCF_017115205.1
Senegal	vagina	GCF_017115385.1
France	blood	GCF_017115395.1
Mali	breast milk	GCF_017115445.1
China	Plant	GCF_017308805.1
China	patient	GCF_017308895.1
missing	mozzarella cheese	GCF_017314245.1
missing	dairy product	GCF_017314485.1
Netherlands	CSF	GCF_900037335.1
New Zealand	Original isolate from Factory - External Area	GCF_900162145.1

Table S4.1 cont.

New Zealand	mussel shell (raw)	GCF_900162315.1
New Zealand	Food - mussel (final product)	GCF_900162355.1
New Zealand	Food - mussel (final product)	GCF_900162465.1
New Zealand	Factory	GCF_900162535.1
New Zealand	Food - raw mussel	GCF_900162545.1
New Zealand	Food - smoked mussels	GCF_900162565.1
New Zealand	Food - coleslaw	GCF_900162585.1
United Kingdom	blood	GCF_900452905.1
Ireland*	Meat processing facility	GCF_018440765.1

*****_Listeria innocua_

Table S4.2 *Listeria* isolates and NCBI accession numbers used for SNP analysis

Strain	NCBI accession #	Reference
ISALM 31	GCF_002775915.1	(Pasquali et al., 2018)
ISALM 29	GCF_002775955.1	(Pasquali et al., 2018)
ISALM 27	GCF_002776205.1	(Pasquali et al., 2018)
ISALM 8	GCF_002776335.1	(Pasquali et al., 2018)
ISALM 9	GCF_002776305.1	(Pasquali et al., 2018)
ISALM 10	GCF_002776295.1	(Pasquali et al., 2018)
JLm 36	GCA_004457495.1	(Burnett et al., 2022)
JLm 20	GCA_004411205.1	(Burnett et al., 2022)
JLm 19	GCA_003920675.1	(Burnett et al., 2022)
JLm 17	GCA_004456755.1	(Burnett et al., 2022)
JLm 16	GCA_004386505.1	(Burnett et al., 2022)
JLm 15	GCA_004142425.1	(Burnett et al., 2022)
JLm 14	GCA_004457655.1	(Burnett et al., 2022)
19-LI00913-1	GCA_015444215.1	(Lachmann et al., 2021)
10-LI00913-0	GCA_015444145.1	(Lachmann et al., 2021)
19-LI00785-4	GCA_015444185.1	(Lachmann et al., 2021)
19-LI00773-0	GCA_015443095.1	(Lachmann et al., 2021)
19-LI00545-0	GCA_015443945.1	(Lachmann et al., 2021)
19-LI00373-0	CA_015443805.1	(Lachmann et al., 2021)
UCDL187	GCA_018440725.1	(Palaiodimou et al., 2021)
UCDL019	GCA_018440905.1	(Palaiodimou et al., 2021)
UCDL016	GCA_018446355.1	(Palaiodimou et al., 2021)
UCDL011	GCA_018440945.1	(Palaiodimou et al., 2021)
1903319-03		This study
2002581-01		This study
2003014-01		This study
2002997-02		This study
2002860-01		This study
2002578-04		This study
1903503-05		This study
2002634-11		This study
2002656-01		This study
2002997-04*		This study

**Listeria welshimeri*

Table S4.3 *Listeria* spp. strain and accession number used for ANI analysis

Species	Strain	Accession #	Lineage	Source
L. monocytogenes	Strain: FSL J1-208	GCF_000250715.1	IV	N/A
L. monocytogenes	Strain: LM850658	GCF_001188655.1	III	Ovis aries
L. monocytogenes	Strain: Finland 1998	GCF_000168595.2	II	N/A
L. monocytogenes	Strain: 02-6680	GCF_002213965.1	I	Environmental
Listeria cossartiae	FSL L7-1434	GCF_014224195.1	-	Soil
Listeria farberi	FSL L7-0072	GCF_014229345.1	-	Soil
Listeria innocua	FSL W9-0511	GCF_015276835.1	-	Water
Listeria marthii	FSL W9-0646	GCF_015275985.1	-	Water
Listeria welshimeri	Strain: NCTC11857	GCF_900187315.1	-	Decaying vegetation

Table S4.4 SNP analysis of *Listeria monocytogenes* strains isolated from food processing plants. Numbers indicate the number of SNPs

ISALM

	10	**27**	**29**	**31**	**8**	**9**
10		22567	22568	22561	0	6
27	22567		25	18	22567	22565
29	22568	25		23	22568	22568
31	22561	18	23		22561	22561
8	0	22567	22568	22561		6
9	6	22565	22568	22561	6	

JLm

	14	**15**	**16**	**17**	**19**	**20**	**36**
14		34	36	34	36	35	33
15	34		10	36	38	37	7
16	10	36		38	40	39	5
17	34	36	38		12	11	35
19	36	38	40	12		3	37
20	35	37	39	11	3		36
36	33	7	5	35	37	36	

LI00

	373-0	**545-0**	**773-0**	**774-0**	**785-0**	**913-0**	**913-1**
373-0		3	2	1	2	3	5
545-0	3		3	2	3	4	6
773-0	2	3		1	0	1	3
774-0	1	2	1		1	2	4
785-0	2	3	0	1		1	3
913-0	3	4	1	2	1		2
913-1	5	6	3	4	3	2	

Table S4.4. Cont.

	Alberta processing facility								
	4-11	**3-05**	**9-04**	**0-06**	**702**	**4-01**	**1-01**	**601**	**903**
4-11		1	1	1	8	4	0	2	3
3-05	1		0	0	7	3	1	1	2
9-04	1	0		0	7	3	1	1	2
0-06	1	0	0		7	3	1	1	2
7-02	8	7	7	7		10	8	8	5
4-01	4	3	3	3	10		4	4	5
1-01	0	1	1	1	8	4		12	3
8-01	2	1	1	1	8	4	12		3
9-03	3	2	2	2	5	5	3	3	