HETEROGENEITY IN PHENOTYPIC RESPONSE OF FOODBORNE PATHOGENS TO CONTROL MEASURES

A Dissertation
Submitted to the Graduate Faculty
of the
North Dakota State University
of Agriculture and Applied Science

By

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In Partial Fulfillment of the Requirements
for the Degree of
DOCTOR OF PHILOSOPHY

Major Program:
Molecular Pathogenesis

May 2019

Fargo, North Dakota

North Dakota State University
Graduate School

Title

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Foodborne pathogens are estimated to cause 48 million foodborne illnesses through consumption of contaminated food annually. Designing efficient control measures is vital to reducing foodborne illnesses. The modern trend toward preserving foods is using combinations of stresses (hurdle model). However, bacterial adaptation to one stress has the potential to increase resistance to subsequent stress, which is known as cross-protection. Due to recent outbreaks, contamination of ready-to-eat (RTE) foods by *L. monocytogenes* is a major food safety concern. *L. monocytogenes* nisin resistance increases when first exposed to NaCl and other stresses, such as low pH. In addition to environmental stressors, specific genomic elements can confer nisin resistance, such as the stress survival islet (SSI-1). We wanted to determine if SSI-1 was associated with salt-induced nisin resistance. Examining 48 *L. monocytogenes* strains when exposed to nisin and salt revealed that nisin resistance of *L. monocytogenes* strains increased when first exposed to NaCl. Deletion of SSI-1 demonstrated the role of SSI-1 in salt-induced nisin resistance. These data suggest that inducible nisin resistance in *L. monocytogenes* can be influenced by environmental conditions and the genetic composition of the strain, which should be considered when selecting control measures for RTE foods. Contamination of low moisture foods (LMFs) (a_w <0.85) by *Salmonella* is a major concern, as *Salmonella* can survive for a long time on LMFs. A common method to control *Salmonella* on LMFs is thermal treatment. LMFs can be stored for long periods of time before thermal treatment. There is a possibility of cross-protection when *Salmonella* is exposed to low a_w conditions followed by thermal treatment. 32 *Salmonella* strains were exposed to flaxseed for 24 weeks. Serovar Agona had a significantly lower death rate compared to Enteritidis and Montevideo (adj. $p<0.05$). At 24 weeks post inoculation, Agona had significantly higher thermal resistance than Enteritidis (adj.

p<0.05). Specific genomic elements can confer osmotic resistance, such as proU and mgtC. Δ*mgtC* had a higher death rate than wild type. However, deleting proU did not change survival rate. This study broadens our knowledge about heterogeneity of bacterial responses to stressors, which will help to design efficient control measures.

ACKNOWLEDGEMENTS

First, I would like to express my sincere gratitude to my Ph.D. advisor Dr. Teresa Bergholz, assistant professor of the Department of Microbiological Sciences at North Dakota State University (NDSU) for the continuous support of my Ph.D. study and related research, for her patience, motivation, and immense knowledge. Her guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and mentor for my Ph.D. study.

Besides my advisor, I would like to thank the rest of my thesis committee: Dr. John McEvoy, Dr. Sheela Ramamoorthy, and Dr. Clifford Hall, for their insightful comments and encouragement.

My special thanks go to Dr. John McEvoy the head of Department of Microbiological Sciences at NDSU for giving me the opportunity of starting my Ph.D. by allocating financial support for my first year of study. My sincere thanks also go to Dr. Peter Bergholz, assistant professor of the Department of Microbiological Sciences at NDSU who has supported me through my preliminary exams and also gave me chance to be the part of Graduate Program Committee as a student representative to participate in making decisions for graduate programs at Department of Microbiological Sciences at NDSU.

I thank college of agriculture, food systems, and natural resources for their financial support through Frank Bain Graduate Student Scholarship which decreased my financial burden.

I thank my fellow lab mates, our lab technician Megan Ramsett and all people in the Department of Microbiological Sciences and NDSU who helped me to achieve my goals.

Last but not the least, I would like to thank my family: my parents and my sister and brother for supporting me spiritually throughout writing this thesis and my life in general.

DEDICATION

This study is wholeheartedly dedicated to my beloved brother, Siamak Malek, who has been my source of inspiration and gave me strength when I thought of giving up, who continually provide his moral, spiritual, and emotional support.

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1. LITERATURE REVIEW

1.1. General introduction

Each year foods consumed in the United States of America (USA) contaminated with foodborne pathogens are estimated to cause 48 million episodes of foodborne illness, 128,000 hospitalizations, and 3000 deaths (Scallan *et al.*, 2011). Most illnesses are attributed to norovirus. In terms of hospitalization, nontyphoidal *Salmonella* spp., norovirus, *Campylobacter* spp., and *T. gondii* are the most reported pathogens; and most of the deaths are linked to nontyphoidal *Salmonella* spp., *T. gondii*, *L. monocytogenes*, and norovirus (Tauxe, 2002). Of the foodborne illnesses acquired in the USA, seven leading foodborne pathogens (namely; *Salmonella*, *Toxoplasma*, *Campylobacter*, norovirus, *L. monocytogenes*, *Clostridium perfringens*, and *Escherichia coli* O157) caused about 112,000 disability adjusted life year (DALY). DALY is a measure of total disease burden, expressed as the number of years lost due to ill-health, disability or early death (Hoffmann *et al.*, 2012). In this regard, non-typhoidal *Salmonella* and *L. monocytogenes* caused about 32,900 and 8,800 DALYs respectively (Scallan *et al.*, 2015).

Foodborne disease is defined as illness due to the consumption of contaminated food; food can be contaminated with microbial pathogens or a toxic substance. Some foodborne pathogens are zoonotic, which means that infectious pathogens or parasites originate in non-human hosts and show little or no signs of clinical disease. These pathogens live in the intestinal tract of healthy animals and can be transmitted to humans through fecal contamination of the environment and subsequent entry into the food supply. In addition to direct fecal contamination, during slaughter, poor sanitation practices (Podolak *et al.*, 2010), cross-contamination

(Kusumaningrum *et al.*, 2003), and poor facility and equipment design/inadequate maintenance (Ferreira *et al.*, 2014) are possible routes for pathogens entering in food supplies.

Different types of thermal control measures such as pasteurization, roasting, canning, x-ray, radiation, and blanching and non-thermal methods like high pressure, antimicrobial compounds, and dehydration have been used to control foodborne pathogens in the food supply. Each of these methods has different impacts on the bacterial cell which leads to bacteria inactivation.

The modern trend toward preserving foods is to use a combination of mild food preservation strategies (hurdle model) rather than a single extreme stress to make foodborne pathogens unable to grow or survive as they have to overcome many stressful conditions in succession or in parallel (Hill C, 2002). Even with new preservation techniques (Singh & Shalini, 2016), about a thousand foodborne outbreaks happen in the USA annually (CDC, 2016a). Cross-protection is a concept that may explain some insufficiencies of the hurdle model. A phenomenon that is referred to as stress cross-protection is when foodborne pathogens can sense their surroundings and respond to changed environmental conditions by expressing genes and producing proteins. The proteins activated/inactivated by the first stress (e.g., low pH) may also confer protection against a subsequent stress (e.g., heat).

To have an efficient combination of stressors using the hurdle model, knowing the bacterial phenotypic response to those stressors is important. Also, as different strains of varying genetic backgrounds have caused foodborne outbreaks, it is essential to examine the stress response of bacteria in a population of bacteria with genetic variation.

Contamination of RTE foods by *L. monocytogenes* is a major concern. The combination of salt and antimicrobial peptides such as nisin can be used to inactivate *L. monocytogenes* in

several RTE foods, including cheeses, deli meats, and cold smoked salmon (Gharsallaoui *et al.*, 2016). There is a potential for cross-protection between salt and nisin as salt-induced nisin resistance has been reported for a few *L. monocytogenes* strains (Bergholz *et al.*, 2013). Also, LMFs are a major concern for contamination by *Salmonella*. The most common method to control *Salmonella* on LMFs is thermal treatment. Since LMFs can be stored for a year or more before applying control measures, there is a potential of cross-protection between exposure to low moisture condition in LMFs and subsequent thermal resistance of *Salmonella*.

In this dissertation, response variability of *Salmonella* serovars when exposed to low moisture foods (LMFs) and thermal treatment and *L. monocytogenes* lineages and clonal complexes when exposed nisin and salt was investigated. The ultimate goal is to modify control measures for *Salmonella* and *L. monocytogenes* on LMFs and ready-to-eat (RTE) foods, respectively.

1.2. *Salmonella*

Salmonella is a Gram-negative facultative intracellular anaerobe which is a major cause of diarrheal disease globally, estimated to cause 93 million infections and 155,000 deaths each year (Ao *et al.*, 2015). Among foodborne diseases, diarrheal and invasive infections caused by non-typhoidal *S. enterica* resulted in the highest public health burden and has the highest DALYs (Kirk *et al.*, 2015). The most common initial symptoms of salmonellosis are nausea, vomiting, and non-bloody diarrhea; other symptoms may include fever, chills, abdominal pain, myalgias, arthralgias and headache. These symptoms are usually self-limited and not associated with intestinal damage (Sanchez-Vargas *et al.*, 2011). People of all age groups are susceptible to *Salmonella*; however immunocompromised, elderly and young children are at a higher risk (Gordon, 2008).

Contaminated food is by far the main source of human salmonellosis, with 86–95% of cases estimated to be foodborne (Majowicz *et al.*, 2010). Surveillance data of food-borne outbreaks in the USA from 1998 to 2016 indicate 2711 outbreaks due to *Salmonella*, resulting in 72,412 illnesses, 8,554 hospitalizations and 100 death (CDC, 2017). Those outbreaks were attributed to a variety of sources, including chickens, eggs, pork, turkey, beef, nuts as well as vegetables and fruits such as mango, melon, alfalfa sprouts, tomato, lettuce, and sweet basil (CDC, 2016b).

Salmonella is zoonotic in origin, and the most common reservoir for *Salmonella* is animals (Braden, 2006). A variety of animals from many environments have been found to harbor *Salmonella*, but food animals are the main route for spreading *Salmonella* to humans via the food supply (Callaway *et al.*, 2008). Chickens (Zhao *et al.*, 2001), turkeys (N. A. Cox, 2003), eggs (Braden, 2006), pigs (Oliveira *et al.*, 2005), and poultry (Gieraltowski *et al.*, 2016) can all be infected with *Salmonella*. The intestinal tracts of swine (Davies *et al.*, 1997), as well as that of beef and dairy cattle, can contain *Salmonella* (Callaway *et al.*, 2008). Additional outbreaks of salmonellosis have been linked to inappropriate pasteurization of dairy products (Hedberg *et al.*, 1992) or inadequately cooked ground beef (Mead *et al.*, 1999).

Due to healthy lifestyle recommendations, the consumption of fruit and vegetables continues to rise in the United States and the rate of foodborne illness caused by the consumption of these products remains high, representing a significant public health and financial issue. An increasing number of human salmonellosis outbreaks have been reported to be associated with the consumption of numerous plant products such as fresh cilantro (Campbell *et al.*, 2001), lettuce (Haley *et al.*, 2009), tomato (Greene *et al.*, 2008), sweet basil (Pezzoli *et al.*, 2008), sprouts, watermelon, cantaloupe (Hanning *et al.*, 2009) and serrano peppers (Caterina Levantesi,

2012). These outbreaks highlight that vegetables and fruits can be vehicles for the transmission of *Salmonella.*

Low water activity (a_w) is a barrier to growth for many pathogens, including *Salmonella*. Although LMFs do not support the growth of *Salmonella*, in recent years, there have been multiple outbreaks and recalls in LMFs products, such as cereal, chocolate, dog food, flour, nuts, nut butter, and spices (CDC, 1998; CDC, 2012; CDC, 2016a), which supports that *Salmonella* can survive at low a_w and LMFs are a vehicle for *Salmonella* transmission.

Salmonella can contaminate food through contaminated manure, irrigation water, fertilizers, wildlife, wash water, processing equipment, and packaging (Kaneko *et al.*, 1999). One probable source of contamination on LMFs may be contact with birds or rodents during storage or shipping (Mahon *et al.*, 1997; Waldner *et al.*, 2012). In addition, some seeds may be contaminated by animal feces (Brooks *et al.*, 2001).

Over the last 25 years, many outbreaks associated with LMFs such as dry cereal, peanut butter, spray-dried milk, infant formula, and nuts have been reported. Epidemiological and environmental surveys of LMF outbreaks have been used to propose cross-contamination through facilities, personnel, raw ingredients, and poor sanitation practices as likely ways of transferring *Salmonella* on LMFs (Podolak *et al.*, 2010). Current hypotheses propose cross contamination alone accounts for 57% of all contributing factors in the United Kingdom outbreaks (Powell & Attwell, 1998). The ability to survive under stresses contributes to the persistence of *Salmonella* in a food-processing environment (Kusumaningrum *et al.*, 2003); therefore increasing the risk of transmission of this pathogen to foods.

Over 2500 serovars of *S. enterica* have been recognized which belong to six subspecies of *S. enterica*. Among 2,500 serovars, less than 4% accounts for most human

infections (Abdullah *et al.*, 2018). Outbreaks of salmonellosis were attributed to several different *Salmonella* serovars including Enteritidis (Bartholomew *et al.*, 2014; CDC, 2004), Typhimurium (Anderson *et al.*, 2017), Agona (Jourdan-da Silva *et al.*, 2018), Anatum (Hassan *et al.*, 2017), Tennessee (CDC, 2007), Montevideo (Dominguez *et al.*, 2009), Poona (CDC, 2004), Wandsworth (Sotir *et al.*, 2009), Newport (Dallap Schaer *et al.*, 2010), Oranienburg (Kaneene *et al.*, 2010), Infantis (Imanishi *et al.*, 2014) and Stanley (Mahon *et al.*, 1997). Specifically, in terms of LMFs, *S.* Typhimurium, Enteritidis (Rangel-Vargas *et al.*, 2015), Agona (CDC, 1998), Tennessee (CDC, 2007), Bredeney (CDC, 2012), and Montevideo (CDC, 2016a) have been reported.

1.3. *Listeria*

The gram-positive bacterium *L. monocytogenes* is a non-spore forming, facultatively anaerobic rod that has been involved in outbreaks of foodborne disease (Buchanan, 2017). In the USA incidence of *L. monocytogenes* compared to other bacterial pathogens such as *Salmonella* is low. However, it is estimated that this foodborne pathogen causes approximately 1,460 hospitalizations annually in the USA, resulting in 260 deaths (Scallan *et al.*, 2011). In industrialized countries, the occurrence of listeriosis is 0.36 to 5 cases annually per million people (Eurosurveillance editorial, 2013; Ferreira *et al.*, 2014). However, the number of reported cases can be very low in countries with inadequate surveillance for this disease.

Surveillance data of foodborne outbreaks in the USA, from 1998 to 2017 indicate 80 outbreaks due to *Listeria*, resulting in 945 illnesses, 691 hospitalizations and 140 deaths (CDC, 2017). Listeriosis occurs mainly in persons at the extremes of age (neonatal period and old age), those with immunocompromising conditions (malignancies, diabetes mellitus, alcoholism, liver, renal and autoimmune diseases), and those undertaking immunosuppressive therapy. However,

the infection may also occur in people with no known risk factors (Swaminathan & Gerner-Smidt, 2007). The manifestations of listeriosis include septicemia, meningitis, and cervical infection of pregnant women. The early symptom of listeriosis is diarrhea which may precede bacteremia and meningitis. The presence of multiple abscesses in the brain stem (encephalitis) rarely occur (Ramaswamy *et al.*, 2007).

While outbreaks were attributed to a variety of foods including RTE foods (including meat and meat products, fish and fish products, milk and milk products), salads, bean sprouts, cantaloupe, and frozen vegetables (CDC, 2018). Since 2010, several listeriosis outbreaks have been linked to fresh produce (e.g., celery, lettuce, cantaloupe, sprouts, stone fruit, and caramel apples), and ice cream (Buchanan, 2017).

As *Listeria* is ubiquitously present in the environment, initial contamination of food may occur at any stage before consumption, including primary agricultural production, food processing, retail level, and consumer's homes (Walland *et al.*, 2015). So, there are two contamination routes: either directly from raw RTE products or indirectly via environmental or post-processing contamination (Walland *et al.*, 2015).

Raw meats including raw poultry are important sources of *Listeria* spp., especially *L. monocytogenes* (Baek *et al.*, 2000; Busani *et al.*, 2005; Dworkin *et al.*, 2001). Regardless of prevalence rates, RTE foods pose a higher risk for listeriosis than raw foods because raw foods generally are cooked before they are consumed. Nevertheless, raw or insufficiently cooked meats may serve as sources of cross-contamination of products that are intended to be consumed without heat treatment, and along with insufficient cleaning and sanitation, have been known as the main sources of postprocessing contamination of RTE meat products (Cordano & Rocourt, 2001; Salvat *et al.*, 1995). It is reported that in postprocessing contaminated RTE foods, *L.*

monocytogenes fast proliferation during cold storage is due to the absence of competitive microflora (Jorgensen & Huss, 1998).

Listeriosis outbreaks linked to milk and milk products, including pasteurized milk, chocolate milk, soft cheese, and butter have been reported (McLauchlin *et al.*, 2004). Since normal pasteurization procedures are enough to destroy *L. monocytogenes* in milk, the presence of *L. monocytogenes* in properly pasteurized products is most likely the result of post-pasteurization contamination (Dalton *et al.*, 1997).

L. monocytogenes has been isolated from produce, such as freshly cut fruit (Oliveira *et al.*, 2014) and fresh-cut vegetables (Vandamm *et al.*, 2013). Moreover, *L. monocytogenes* has been isolated from the vegetable growing environment (Esteban *et al.*, 2009). Prevalence of *L. monocytogenes* in fresh, including direct or indirect contamination from the environment, such as from soil, water, compost and feces has been reported (Strawn *et al.*, 2013a; Strawn *et al.*, 2013b).

Possible sources of *L. monocytogenes* contamination of foods in retail and food service operations include incoming products, food handling, and environmental sources such as utensils and equipment that under conditions of poor cleaning and sanitation may harbor pathogenic microorganisms or serve as vehicles of cross-contamination (Bryan, 2002). Regulations implemented by industries between 1998 and 2008 have reduced outbreaks from RTE red meats and poultry. However, listeriosis outbreaks from dairy products did not decrease (Cartwright *et al.*, 2013).

Currently, there are 17 identified *Listeria* species. *Listeria* species can be classified into *Listeria* sensu stricto (*L. monocytogenes, L. ivanovii, L. innocua, L. seeligeri, L. welshimeri,* and *L. marthii*) and *Listeria* sensu lato (*L. grayi, L. fleischmannii, L. floridensis, L. aquatica, L. new*

yorkensis, *L. cornellensis*, *L. rocourtiae*, *L. weihenstephanensis*, *L. grandensis*, *L. riparia*, and *L. booriae*) (Orsi & Wiedmann, 2016; Weller *et al.*, 2015). *L. monocytogenes* and *L. ivanovii* are pathogenic. *Listeria* strains were historically characterized by serotyping (Farber & Peterkin, 1991). There are 13 serotypes of *L. monocytogenes* that have been classified into 4 evolutionary lineages and 63 clonal complexes (CCs) by multilocus sequence typing (MLST). MLST is a method to group bacteria based on differences in base sequence of 7 housekeeping genes. 1/2a, 1/2b, 1/2c and 4b are the four *L. monocytogenes* serotypes that are commonly found in food, food-processing, or natural environments and are responsible for 98% of human listeriosis (Liu, 2006).

Among *Listeria* species, *L. monocytogenes* and *L.innocua* are generally the most frequently isolated from RTE foods in retail and food service locations, including meat and poultry products (Awaisheh, 2010; Farber & Daley, 1994; Sheridan *et al.*, 1994), fish and seafood products (Dominguez *et al.*, 2001; Handa *et al.*, 2005), dairy products (da Silva *et al.*, 1998; Rudol & Scherer, 2001; Torres-Vitela *et al.*, 2012), and produce (Uchima *et al.*, 2008). Previous reports have shown an increase in the prevalence of *L. monocytogenes* in RTE, vacuum packaged, sliced meat products where 95% of all *L. monocytogenes* belonged to Lineage 2, serotype 1/2a, with the remaining 5% varying between serotypes 1/2b, 3b, and 4b (Berzins *et al.*, 2009). Kramarenko et al. reported that 93% of all *L. monocytogenes* isolates found from meat products belonged to serotype 1/2a and 1/2c (Rothrock *et al.*, 2017).

1.4. Stresses encountered by bacteria in the food supply

Foodborne bacterial pathogens may encounter physical treatments such as heat, pressure, or osmotic shock, chemical treatments such as acids or detergents, and biological stresses such as bacteriocins during food production, processing, storage, and cooking. Each of these treatments

may result in bacterial cell injury and damage to cellular structures, including the cell wall, cell membrane, proteins, RNA, and DNA (Wesche *et al.*, 2009). The degree and type of damage depend on the nature and severity of the stress.

In general, foodborne pathogens sense their surroundings and altered environmental conditions by changes to protein structure, mRNA stability, and ribosome stability (Guisbert *et al.*, 2008), as well as accumulation of certain cellular metabolites such as guanosine phosphate, guanosine tetraphosphate (ppGpp), and guanosine pentaphosphate (pppGpp), each of which may trigger changes in gene expression that will help bacteria to survive by reprograming the cell (Liu *et al.*, 2015). Stresses may be sensed by two-component systems, which consist of a membrane-associated histidine kinase and a cytoplasmic response regulator (Stock *et al.*, 2000). The histidine kinase senses changes in an environmental parameter and the associated response regulator effects changes in cellular physiology, often via regulation of gene expression (Begley & Hill, 2010). These stress responses may lead to the production of proteins that can repair damage, maintain cell homeostasis or facilitate the removal of the stress agent (Begley & Hill, 2015).

Food preservation puts microorganisms in a hostile environment, in order to inhibit their growth or shorten their survival or cause their death (Leistner, 2000). Applying osmotic pressure is one method to preserve food products (Gutierrez *et al.*, 1995). The food industry may apply different methods such as adding salt in combination with other mild technologies as a general preservative and an antibacterial agent in RTE foods, seafood, fermented food, such as salami and cheese to preserve them (Desmond, 2006). Moreover, applying desiccation (drying) and adding sugar are the other osmotic dehydration techniques to preserve foods mainly in fruits and vegetables (Maftoonazad, 2010; Torreggiani & Bertolo, 2004). Bacteria may encounter osmotic

stress during a move to a hyperosmotic solution or due to dehydration. Moreover, bacteria on surfaces in the processing plant may encounter desiccation stress (Veluz *et al.*, 2012). Changes in osmolarity pose significant pressure on bacterial cells by causing swelling in a hypotonic environment or dehydration and shrinking under the hypertonic situation (Sleator & Hill, 2002). Shrinkage of cells layers, increase in intracellular salt concentrations and macromolecule are the main consequence of desiccation (Potts *et al.*, 2005). Changes in the biophysical properties of a cell reduced the fluidity of membrane lipids, and protein and DNA damages are the other effects of desiccation stress. Free-radical attack of phospholipids, DNA, and proteins are the main reason cells are sensitive to desiccation (Burgess *et al.*, 2016).

One of the conventional methods to preserve food is with heat. The key goals of the thermal processing of foods are to guarantee microbiological safety, improve the shelf life of food by the destruction of enzymes, toxins, etc. (Varghese *et al.*, 2014). The impact of heat on bacteria has been widely studied, and many different cellular changes have been reported. In general, the lethality of a thermal treatment will depend on the alteration of at least one critical component of bacterial cells. Nucleoids such as DNA and RNA are examples of a critical component. Even though, RNA and DNA are cellular elements with the highest thermostability (Earnshaw *et al.*, 1995), denaturation of DNA and damage of RNA with heat treatment have been reported (Iandolo & Ordal, 1966; Mackey *et al.*, 1991). It has been reported that the outer membrane and peptidoglycan wall are affected by heat treatment (Cebrian *et al.*, 2017). Also, protein denaturation and aggregation in bacterial cells as a consequence of heat treatment have been reported (Krasowska, 2014).

Chemical preservatives in foods has become a consumer concern and created a demand for natural and minimally processed food (Cleveland *et al.*, 2001). Many researchers pointed out

that antimicrobial peptides confirmed activity against several foodborne pathogens, and therefore, can help in food safety (Elayaraja *et al.*, 2014; Hintz *et al.*, 2015; Kraszewska *et al.*, 2016). The main benefit of using antimicrobial peptides is that it preserves the food without changing its quality and it is not harmful to human (Wang *et al.*, 2016). The mechanism of action of antimicrobial peptides mainly depends on the interaction of peptides with the cell membrane and its composition. Generally, antimicrobial peptides interact with the membrane by electrostatic interaction (Guilhelmelli *et al.*, 2013). Antimicrobial peptides have a membrane permeabilizing action. They can enter into the membrane causing its disruption (Bolintineanu & Kaznessis, 2011). Carpet model, toroidal pore model, barrel-stave model, and aggregate model are well-studied models for how antimicrobial peptides attach to the cell membrane of bacteria (Strempel *et al.*, 2015).

The carpet model also known as the detergent model is based on the accumulation of peptides around the membrane of microorganisms. When the peptide concentration reaches the maximum level, they penetrate into the lipid membrane of bacteria (Gazit *et al.*, 1996). The toroidal pore model is based on an interaction between the charged hydrophobic cell membrane of bacteria and the hydrophilic region of the peptide (bacteriocin). When the concentration peptides reach the maximum level, all the peptides change their orientation that they are perpendicular to the membrane (Barbosa Pelegrini *et al.*, 2011). The barrel-stave model is based on the assembly of antimicrobial peptides in the form of a bundle. The positive charge of peptide binds with the membrane similar monomer and aggregation of peptide leads to membrane disintegration and pore formation (Bahar & Ren, 2013). Aggregate model is based on electrostatic interaction between the hydrophilic region of the peptide and the phospholipids layer of membrane. Aggregation of peptides on the membrane form a sphere-like structure. The

aggregates contain a water molecule with the release of fluid, leading to membrane disruption (Rai *et al.*, 2016).

Antimicrobial peptides like bacteriocins can be used to control *L. monocytogenes* on RTE foods (Nilsson *et al.*, 1997). Bacteriocin (Pediocin) PA-1 produced by *Pediococcus acidilactici* PAC 1.0, has been used in RTE foods to control *L. monocytogenes*. Pediocin PA-1 binds to cytoplasmic membranes, then it enters in the membranes, and forms of the pore complex. This process finally leads to cell death that may occur with or without cell lysis (Rodriguez *et al.*, 2002). Nisin produced by *Lactococcus lactis* was evaluated to be safe for food by the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives in 1969 (Arauza *et al.*, 2009), and it has gained widespread application in the food industry. Nisin can be used to inactivate *L. monocytogenes* on several RTE refrigerated foods, including cheeses, deli meats, and cold smoked salmon. The inhibitory effect of nisin occurs at the cytoplasmic membrane. Nisin binds to lipid II, a peptidoglycan precursor, inhibits cell wall biosynthesis and creates pores which lead to disruption of the proton motive force, leakage of ions, hydrolysis of ATP and ultimately cell death (Wiedemann *et al.*, 2001). Leuconocin S is a small (molecular weight, 10,000) glycoprotein (Bruno & Montville, 1993). The mode of action is similar to that of nisin against *L. monocytogenes,* depletion of the proton motive force (Kaur *et al.*, 2011).

1.5. How bacteria adapt to stresses

Bacteria are exposed to many stressful environments during fecal-oral transmission to a new host and have an extensive repertoire of stress response mechanisms to allow survival under varied conditions. In non-host environments, bacteria may encounter acidic (Álvarez-Ordóñez, 2012), thermal (Murphy *et al.*, 2004), starvation (O'Neal *et al.*, 1994), oxidative (Wang *et al.*,

2010), and osmotic stress (Burgess *et al.*, 2016). The ability to adapt quickly to environmental changes is important for bacterial survival and virulence, and there are numerous stress response mechanisms that reduce the impact of stressors on the cell (Shen & Fang, 2012).

As mentioned previously, *Salmonella* may encounter thermal stress in the food processing environment, as heat treatment is a common control measure for microbes in foods. To overcome thermal stress, *Salmonella* may apply a wide range of strategies. *Salmonella* can sense temperature changes via thermosensors such as FourU (Waldminghaus *et al.*, 2007), TlpA (Gal-Mor *et al.*, 2006; Waldminghaus *et al.*, 2007), and HtrA (Clausen *et al.*, 2002). Thermosensors can regulate genes to express adaptive heat stress responses. In particular, sigma factors play a leading role in the thermal stress response. Two sigma factors are generally activated: heat shock sigma factor, σH (RpoH) which is a cytoplasmic thermal stress response regulator and extracytoplasmic function sigma factor, σE (RpoE) (Ades, 2008; Bashyam & Hasnain, 2004; Helmann, 2002). Expression of heat shock proteins including DnaK, DnaJ (Takaya *et al.*, 2004), HptJ (Milillo *et al.*, 2011), GrpE (Cimdins *et al.*, 2013), ClpP (Thomsen *et al.*, 2002), and HscAB (Dawoud *et al.*, 2017) regulated by RpoE, is important for maintaining protein function by folding and/or refolding and degrading misfolded proteins (Dawoud *et al.*, 2017).

In the case of desiccation, to increase osmotic pressure, the first strategy of *Salmonella* is taking potassium inside the cell. Two main transport systems are responsible for this function: Trk and Kdp (Spector & Kenyon, 2012). To maintain electroneutrality, the cell next induces the synthesis of glutamate by glutamate dehydrogenase and glutamate synthase (Csonka, 1989). At the high level of osmotic stress, RpoS regulates the induction of *ots*AB operons and as a consequence, trehalose synthesis is induced by the products of the *otsAB* operon (Spector &

Kenyon, 2012). Moreover, *Salmonella* can increase uptake or biosynthesis of other osmoprotectants such as betaine (N,N,N-trimethyl glycine), proline and trehalose to manage osmotic stress (Burgess *et al.*, 2016). To transport those compatible solutes, RpoS regulates the transcription of transporters such as ProP (Shiroda *et al.*, 2014; Spector & Kenyon, 2012), ProU (ProVWX), and OsmU (Finn *et al.*, 2013b; Shiroda *et al.*, 2014; Spector & Kenyon, 2012). *S.* Typhimurium uses OmpF and OmpC porins as channels to diffuse small hydrophilic molecules (Spector & Kenyon, 2012). The OmpF porin decreases the influx of solutes into the periplasm (Rychlik & Barrow, 2005).

L. monocytogenes also encounters numerous environmental stresses including low temperature (Miller *et al.*, 2000), acidic conditions (Ryan *et al.*, 2008), osmotic pressure (Sleator *et al.*, 2003), bile (Begley *et al.*, 2002) and bacteriocins (Begley *et al.*, 2010) during food preservation. *L. monocytogenes* can grow in suboptimal conditions by using numerous mechanisms. In terms of low temperature, *L. monocytogenes* encounters various forms of cold stress challenge at several stages in food-processing and storage environments. *L. monocytogenes* is a psychrotrophic microorganism that can grow at temperatures as low as 4°C. *L. monocytogenes* respond by changing the fatty acid composition of their cell-membrane lipids to similarly lower the liquid to solid phase transition temperatures. Therefore, the molecular adaptation measures adopted in cell-membrane lipids include a change in the fatty acid chain lengths, an alteration unsaturated fatty acid and a change in the type of branching at the methyl end of the fatty acids (Tasara & Stephan, 2006). Moreover, several studies showing uptaking of cryoprotective osmolytes such as glycine-betaine and carnitine in growth media enhances *L. monocytogenes* growth at low temperatures (Bayles & Wilkinson, 2000; Beumer *et al.*, 1994; Ko *et al.*, 1994). Also, in cold-adapted *L. monocytogenes* cells, the induction of Csp-

like proteins, as well as *cspA* gene transcripts, was reported (Chan *et al.*, 2007; Wemekamp-Kamphuis *et al.*, 2002).

L. monocytogenes is osmotically tolerant and can grow at > 10% NaCl. The organism can adapt to elevated osmolarity by accumulating compatible solutes or osmolytes (Al-Nabulsi *et al.*, 2015). This mechanism is facilitated by an amplified expression of genes (such as *opuCABCD* operon, *gbuABC* operon, *betL* (Chan *et al.*, 2007)) encoding for proteins involved in the transport of the particular compatible solutes (Cacace *et al.*, 2010). Similar to *Salmonella*, the first response mechanism to osmotic stress in *L. monocytogenes* is the upregulation of membrane transporters that uptake K^+ from the environment to help maintain cell turgor pressure (Brondsted *et al.*, 2003). The upregulation of glutamate metabolism genes, including Glu-tRNA ligase and the intracellular accumulation of glutamate, have been shown to enhance survival during osmotic stress in *L. monocytogenes* (Cotter *et al.*, 2001). By expressing *cspA* and *cspD, L. monocytogenes* can facilitate the repair of DNA lesions, as NaCl has been shown to induce DNA breaks (Dmitrieva *et al.*, 2004; Schmid *et al.*, 2009). It is reported that mechanosensitive channels can help *L. monocytogenes* to survival in hyper-osmotic and hypo-osmotic conditions (Sleator *et al.*, 2003).

Bacteriocin resistance of *L. monocytogenes* seems to depend on various factors. As an example, nisin resistance of *L. monocytogenes* has been associated with acid stress response. As the glutamate decarboxylase system is considered the most important system employed by this species to resist low pH stress (Begley *et al.*, 2010), they found the *gadD1* mutant of *L. monocytogenes* LO28 was susceptible to nisin, exhibiting a 10^2 -fold reduction survival in the presence of 300 mg nisin ml^{-1}. They also observed a 40% reduction in the intracellular ATP levels found in the DgadD1 mutant. Based on these data, Begley et al. proposed that, as nisin

activity ultimately leads to release of ATP and cell death, GadD1 may restore the intracellular ATP pools, leading to nisin resistance (Begley *et al.*, 2010).

Moreover, it has been shown that bacteriocin resistance is due to changing the bacterial cell envelope. Gram-positive bacteria have the *dlt* operon that codes for proteins required for the incorporation of D-alanine to teichoic acids (TAs) or lipoteichoic acids (LTAs). Mutations in the *dltA* gene result in increased sensitivity to bacteriocins due to a defective D-alanine incorporation to TAs or LTAs (Peschel *et al.*, 1999). A functional MprF protein is required for the biosynthesis of lysylphosphatidylglycerols, whose presence in the bacterial cytoplasmic membrane also reduces the net negative charge of the cell envelope, contributing to bacteriocin resistance (Peschel *et al.*, 2001). The response regulator VirR, which is part of the two-component signal transduction system (2CS) VirRS, positively controls the expression of both *dltA* and *mprF* in *L. monocytogenes* (Mandin *et al.*, 2005). Therefore, as expected, inactivation of VirR results in enhanced bacterial susceptibility to bacteriocins (Thedieck *et al.*, 2006). In addition, the SigB is an important mediator of the bacterial stress response and is also involved in the resistance of *L. monocytogenes* to bacteriocins. It is assumed that SigB may control membrane charge or lipid composition, as alteration of these characteristics affects bacteriocin binding or insertion. SigB may also regulate transporters involved in bacteriocin efflux (Begley *et al.*, 2006).

Alteration in composition of cytoplasmic membrane may also reduce a bacterium resistant to bacteriocins. A nisin-resistant variant of *L. monocytogenes* Scott A, isolated by exposure to increasing concentrations of nisin, was shown to produce less diphosphatidylglycerol and more phosphatidylglycerol than the parental strain (Verheul *et al.*, 1997). As nisin penetrates more deeply into lipid monolayers of diphosphatidylglycerol than those of other lipids, including phosphatidylglycerol, the resistance exhibited by the mutant was attributed to a reduction in the

diphosphatidylglycerol content of the cytoplasmic membrane (Verheul *et al.*, 1997). Crandall and Montville also reported that nisin resistance in *L. monocytogenes* ATCC 700302 involved alterations in cellular membrane composition, such as a lower ratio of C15: C17 fatty acids and the presence of more phosphatidylethanolamine and less phosphatidylglycerol and cardiolipin. All these changes may result in a less fluid cytoplasmic membrane and increased rigidity, which may prevent nisin from inserting into its target. Moreover, the decrease in phosphatidylglycerol content and, therefore, in the net negative charge of the lipid bilayer may also hamper nisin's ability to bind to and interact with the membrane (Crandall & Montville, 1998).

Regulators have also been shown to contribute to acquired resistance. For example, the LisRK 2CS is involved in *Listeria* susceptibility to nisin (Cotter *et al.*, 2002). The LisRK 2CS regulates the LiaFSRLm 3CS, which in turn regulates the *Listeria* response to environmental stresses (Fritsch *et al.*, 2011). Expression of a functional LiaFSRLm seems to result in extensive remodeling of the protein composition of the cytoplasmic membrane (Fritsch *et al.*, 2011) and in a decreased transcription of lmo2229/pbp2229, which codes for a protein similar to penicillin-binding protein (PBPs) (named PBP2229) (Collins *et al.*, 2012), rendering the cell more susceptible to nisin.

1.6. Variation in survival under different stresses

1.6.1. *Salmonella* on LMFs

Numerous studies have shown *Salmonella* ability to survive in dry processing environments (Binter *et al.*, 2011) and on dried surfaces (Kusumaningrum *et al.*, 2003), potentially leading to subsequent transfer and contamination of food (Hood & Zottola, 1997; Iibuchi *et al.*, 2010; Parkar *et al.*, 2001; Rossoni & Gaylarde, 2000). For example, the cause of the chocolate contamination in a production facility was due to an uncontrolled airborne spread

of dust in the factory environment (Craven *et al.*, 1975). Similarly, samples taken in an oil meal plant had *Salmonella* on the processing floor, in air dust and on equipment (Morita *et al.*, 2006). In another study, Hiramatsu et al., showed that *Salmonella* can survive on paper disks for 24 months (Hiramatsu *et al.*, 2005). Similarly, Margas et al., showed that *S.* Enteritidis remained viable on dry stainless-steel surfaces for at least one month (Margas *et al.*, 2014). More importantly, when present on LMFs, *Salmonella* can survive for long periods of time (Beuchat *et al.*, 2013), in some cases, up to 550 days (Uesugi *et al.*, 2006). Five outbreaks and 46 recalls in recent years (Centers for Disease & Prevention, 2013; McCallum *et al.*, 2013; Sheth *et al.*, 2011; Unicomb *et al.*, 2005) have been reported by CDC (CDC, 2015) which again confirms that *Salmonella* can survive in dry conditions.

LMFs have a long shelf life and if *Salmonella* can survive for a long period of time, it can present risks over that timeframe. Understanding variation in survival parameters among *Salmonella* serovars becomes necessary for risk assessment and challenge studies. Gruzdev et al., in 2011 studied the desiccation tolerance of *S.* Enteritidis, Hadar, Infantis, Newport and Typhimurium (SL1344) on polystyrene and on glass surfaces (Gruzdev *et al.*, 2011). In their study, *S.* Enteritidis had the highest (80% ± 9%) and *S.* Newport having the lowest (36% ± 3%) survival percentage (Gruzdev *et al.*, 2011). Moreover, Gruzdev et al., studied dehydration tolerance for five different *S.* Typhimurium strains and three other *S. enterica* serovars at 22 h at 25°C and 40% relative humidity (RH) and long-term persistence at 4°C, 40–45% RH for 12 weeks (Gruzdev *et al.*, 2012b). Results from the dehydration tolerance study showed a log reduction ranging from 0.31±0.25 to 2.12±0.44 $\log_{10}$ CFU. *S.* Typhimurium strain #311 (SL 1344) had the lowest reduction whereas *S.* Typhimurium strain #323 showed the most reduction. The other three *Salmonella* serovars showed similar survival parameters under 22 h dehydration.

However, significant differences were observed among strains under long-term persistence conditions. Serovars Enteritidis and Newport showed similar log reductions of 1.20 ± 0.44 and 1.84 ± 0.74 $\log_{10}$ CFU, respectively, at 12 weeks of storage. On the other hand, serovar Infantis was found to be more susceptible with 3.2 ± 0.50 $\log_{10}$ CFU reduction observed at week 12. *S.* Typhimurium SL 1344 strain showed high dehydration tolerance compared with the other Typhimurium strains and the three *Salmonella* serovars (Gruzdev *et al.*, 2012b).

Comparing the desiccation resistance of fifteen *Salmonella* isolates on stainless steel discs stored at 33% humidity and 25°C showed that at the initial time *S.* Typhimurium DS and *S.* Typhimurium strain FH/St/165 were the most sensitive strains with a log reduction of 2.08±0.92 and 1.84±0.57 $\log_{10}$ CFU surface^{-1}, respectively. One-month post storage, the highest $\log_{10}$ reduction of 4.3 ± 0.9 log CFU surface^{-1} was for *S.* Typhimurium DS and the lowest reduction was for *S.* Muenchen, *S.* Typhimurium DT104, and *S.* Typhimurium HR with 1.5±0.50, 1.3±0.60 and 1.6 ± 0.50 $\log_{10}$ CFU surface^{-1}, respectively (Margas *et al.*, 2014).

Several foods can be heat treated to kill foodborne pathogens, and such processes are abundantly used in the food industry to our benefits. Heat treatments in various forms, such as dry heat treatment, steam treatment or vacuum steam pasteurization, liquid heat treatment and oil roasting are often implemented. Such processes are usually used to treat foods that cannot be consumed raw or foods that are at high risk of contamination due to foodborne pathogens.

LMFs like almonds and peanut butter are known to be contaminated with *Salmonella*. Although these foods could be consumed raw, the presence of pathogens necessitates heat treatment to kill the pathogens. Variation in stress response among *Salmonella* serovars has been well documented, which poses a challenge to suitably select a temperature and time condition to inactive pathogens without significant deterioration of sensory properties or nutritional values.

However, many of the heat inactivation studies were conducted using laboratory broth medium for studies that evaluate variability in stress response phenotypes among different *Salmonella* serovars.

Bayne et al. found that all tested *Salmonella* strains were sensitive to heat but *S.* Senftenberg, 775W appeared as the most resistant (Bayne *et al.*, 1965). Anellis et al, studied the heat resistance of seven *Salmonella* serovars by pasteurization of liquid whole egg at 60°C and pH value of 5.5 and 8. The most heat resistant serovar in both pH was *S.* Senftenberg 775W with D-values of 9.5 and 1.5 min at pH 5.5 and 8, respectively. Interestingly, *S.* pullorum 13117 was the least resistant serovar with D-values of 0.4 and 0.14 min at pH 5.5 and 9 (Solowey, 1984). Ng et al. tested heat resistance phenotypes of 75 different serovars of *Salmonella* at 57°C for 10 min; *S.* Senftenberg 775W was again found to be the most heat resistant with a D-value of 30 min. However, other serovars had an observed D-value of less than 5 min. (Ng *et al.*, 1969).

Evaluating the heat resistance of eight *Salmonella* strains belonging to different serovars in pork meat showed *S.* Potsdam I33 was the most heat resistant strain in pork meat, with D-values of 4.80, 1.57 and 0.30 min at 58°C, 60°C and 63°C, respectively. The most sensitive strain was *S.* kingston I124, with a D-values of 2.79, 0.92 and 0.24 min, at 58°C, 60°C and 63°C, respectively (Quintavalla *et al.*, 2001). Moreover, examining the heat resistance of 94 *S. enterica* strains belonging to different serovars in culture broth at 58°C showed D-values ranging from 0.79 to 2.67 min. Interestingly, the most resistant strains in pork meat showed the least resistance in TSB (Tryptic Soy Broth) (Quintavalla *et al.*, 2001).

Similarly, studying the variation in heat resistance for 40 serovars in TSBYE (Tryptic Soy Broth Yeast Extract) medium aliquots in water bath at 57°C for 13 min showed that *S.* New-haw was the most resistant strain and *Salmonella* Typhimurium the most sensitive strain to the

heat stress. The difference in log reduction between the two serovars during the treatment process was approximately 2.5 Log_{10} CFU ml^{-1}. Other isolates were more consistently distributed throughout this range (Sherry *et al.*, 2004).

Challenging 60 strains of *Salmonella* classified in 9 serovars to heat stress at 57°C in TSB without dextrose for 20 min showed Montevideo had the highest inactivation rate of k_{heat} ~ $1.1h^{-1}$ and Enteritidis had the least inactivation rate of k_{heat} ~ 0.8 h^{-1} (Lianou & Koutsoumanis, 2013).

Studies on the variation in phenotypic response among *Salmonella* serovars to heat stress in artificially inoculated foods are almost non-existent. However, multiple strains that are known to contaminate foods into a cocktail mixture have been used for inactivation studies. Although such studies do not show variability among serovars, they show the need for stringent inactivation parameters for supposedly a representative cocktail of strains. For example, studying the heat resistance kinetics of 10 different serovars at 60°C in beef gravy showed significant differences in symptomatic D-values among serovars, with an estimation of approximately 20% coefficient variability (CV) among the serovars. Also, differences in thermal resistance among strains of the same serovars have been noted (Juneja *et al.*, 2003).

Examining the heat inactivation kinetics of stationary-phase cultures of *S.* Enteritidis PT4 showed survival profiles among different isolates. Based on their report, isolate A, human stool sample, had the highest D-value (26.8 min at 52°C) and isolate I, a chicken carcass sample, had the lowest D-value (16.8 min at 52°C) (Humphrey *et al.*, 1995).

As heat treatment is a common control measures for LMFs, the inactivation of *Salmonella* on almonds and walnuts using various types of thermal treatments have been widely investigated. Heat treatment by oil roasting at 121°C showed that a 4-log reduction was achieved

for *S.* Enteritidis PT30 in 0.73 and 0.98 min when stored at 4 and 23°C, respectively (Abd *et al.*, 2012). In another similar study, oil roasting of almonds at 127°C showed a 4 log reduction in 0.66 and 0.74 min for *S.* Senftenberg 775W and *S.* Enteritidis PT30, respectively (Du *et al.*, 2010). Harris et. al, evaluated the inactivation of two *Salmonella* serovars was conducted using hot water at 88°C on almonds. In their study, a D-values of 0.39 and 0.37 min were observed for *S.* Enteritidis PT30 and *S.* Seftenberg 775W, respectively (Harris *et al.*, 2012).

In low moisture inactivation studies, no significant differences may have been observed between serovars given that only two serovars were tested and that the heat treatment temperatures were significantly greater. It is important to note that phenotypic variability in *Salmonella* serovars may not be observed when exposed to high heat treatments rather more pronounced differences may be observed at lower heat treatment temperatures. More research on the topic would allow for an increased understanding of the phenotypic differences among *Salmonella* serovars under thermal heat treatment processes.

1.6.2. *L. monocytogenes* exposure to salt and nisin

As mentioned previously, bacteriocins have been used to control *L. monocytogenes* in RTE foods. However, it has been reported that *L. monocytogenes* can resist through nisin. Examining the nisin sensitivity of 27 of *L. monocytogenes,* 4 of the *L. innocua* and one of *L. ivanovii* showed that the tested strains of *L. innocua* were as resistant as the most resistant *L. monocytogenes* whereas the strain of *L. ivanovii* was very sensitive. To be more precise, strains NCTC 5105 and BL 88/7 were particularly sensitive and were inhibited by < 200 IU ml^{-1} and 200-400 IU ml^{-1} nisin respectively, at pH 6.8 and 37°C whereas 1000-2000 IU ml^{-1} was required to inhibit many strains at these conditions (Ferreira & Lund, 1996).

Examining nisin and pediocin A sensitivity of 381 *L. monocytogenes* strains showed that only two out of 381 strains were able to grow weakly on 500 IU ml^{-1}. However, only four strains (1%) were very sensitive towards nisin and could not grow at 10 IU ml^{-1}. On the other hand, 67.5% of strains could not grow at the lowest concentration of pediocin. 20 strains (5.2%) were able to grow at all three pediocin concentrations. 34 strains were able to grow very weak at 100 AU ml^{-1} but were able to grow at all the concentrations tested (Rasch & Knochel, 1998).

Examining the survival of 30 clinical and meat origin *L. monocytogenes* isolates in presence of carnitine and NaCl, as osmotic stress, revealed that 11 (73%) of the 15 strains of meat origin had a lower maximum cell density in defined media with NaCl and carnitine than in defined media (DM) alone. By contrast, only 5 (33%) of the 15 strains of clinical origin showed the same characteristic, with the remaining strains displaying a higher maximum cell density in DM with NaCl and carnitine than in DM alone (Dykes & Moorhead, 2000). Bergholz et al., reported that growth of *L. monocytogenes* under salt stress varies among lineages. Lineage 1 strains grew at a significantly faster rate under 6% NaCl at 37°C compared to lineage 2 strains (Bergholz *et al.*, 2010).

CC2 and CC11 exhibited greater salt tolerance compared to the other CCs (Hingston *et al.*, 2017). Kale et al. analyzed the salt tolerance of 104 of *L. monocytogenes* strains. 13 out of 104 strains (12.5%) were found to be tolerant up to 12.5% high salt concentration followed by 65 (62.5%) strains tolerant to up to10% salt concentration (Satyajit B. Kale, 2017).

1.7. Control measures

After the 2001 and 2004 Salmonellosis outbreaks related to raw almonds, achieving a minimum 4-log reduction of *Salmonella* population became mandatory in California, the world's largest almond producer (Pan Z *et al.*, 2012). Control- measures for *Salmonella* on LMFs can be

classified as chemical, thermal, and non-thermal methods. *S.* Enteritidis is sensitive to chemical treatments. Besides the nature of the chemical, its concentration and temperature, and the treatment time play a key role in the reduction of *Salmonella* (Foods, 2006). Propylene oxide (Danyluk *et al.*, 2005), organic acid sprays (Pan Z *et al.*, 2012), and chlorine dioxide (Benarde *et al.*, 1967) are common chemicals that have been used to control *Salmonella* on LMFs. Thermal treatment such as hot air (heating with dry hot air (129 to 154°C) (Jeong *et al.*, 2009), hot water (60-80°C) (Bari *et al.*, 2009), and steam (Bari *et al.*, 2010) treatments mostly transfer heat to the surface of the product by convection and then to the center of the product by a conduction mechanism are traditional heat treatment methods to reduce *Salmonella* on LMFs. On the other hand, some non-traditional heat treatments like infrared (IR) (Brandl *et al.*, 2008) and radio frequency (RF) (Gao M *et al.*, 2011) are an electromagnetic wave, which is more effective in heat transfer than the conventional convection and conduction systems. Although laboratory studies show that IR and RF pasteurization techniques are effective for LMFs, these control measures remain to be tested for their suitability at the industrial level. In order to control the LMFs quality, non-heat treatment techniques including electron beam irradiation (Prakash A *et al.*, 2010) and hydrostatic pressure (Goodridge L.D *et al.*, 2006) have been used.

In the case of almond, among all mentioned technologies, the FDA has, to date, approved only polyphenol oxidase, hot water, steam, and hot oil processing as suitable methods to achieve a minimum 4-log reduction of *Salmonella*. It is important to note that all techniques are not applicable to all types of LMFs. Most importantly, the proposed technologies must be demonstrated to cause no significant degradation to the sensory quality and nutritional characteristics of LMFs (Pan Z *et al.*, 2012).

Control measures for *L. monocytogenes* in RTE foods can be classified into four categories. Pasteurization (Juneja, 2003; McCormick *et al.*, 2003; Muriana *et al.*, 2002) and irradiation including Gamma irradiation (Thayer *et al.*, 1998) and Electron beam (Foong *et al.*, 2004) are treatment techniques that have been used to control *L. monocytogenes*. Chemical antimicrobials like the salt of lactate (sodium lactate and sodium diacetate), sodium diacetate (Qvist *et al.*, 1994), trisodium phosphate (Capita *et al.*, 2001), NaCl, and chemically synthesized short-chain peptide (Appendini & Hotchkiss, 2000) are food preservatives that have been used to control *L. monocytogenes* in RTE foods. On the other hand, bio-preservation techniques including using lactobacilli, probiotic bacteria, and bacteriocins are suitable alternatives to chemical preservatives (Jacobsen *et al.*, 2003). Nisin is the most commercially important bacteriocin that has been used to control *L. monocytogenes* in RTE meats (Franklin *et al.*, 2004). Other bacteriocins such as reuterin (El-Ziney *et al.*, 1999), and purified sakacin P (Katla *et al.*, 2002) have been used to decrease *L. monocytogenes*. As bio-preservation techniques, some plant extracts have shown inhibitory effects against *L. monocytogenes*. For example, eugenol (clove extract) and pimento extract significantly inhibited the growth of *L. monocytogenes* inoculated in cooked beef slices (Hao *et al.*, 1998). The other technique to control *L. monocytogenes* is high-pressure processing as a non-thermal method (Lucore *et al.*, 2000). Level of pressure, treatment temperature, exposure time, pH, water activity, and food composition impact the effectiveness of high-pressure techniques (Zhu M *et al.*, 2006).

1.8. Gaps in knowledge

Applying combination of stressors is a common method to preserve food, which is known as the hurdle model. For example, simultaneous combinations of ultraviolet radiation and ozone, induced synergistic inactivation of *S.* Typhimurium poultry on processing equipment (D-value

was > 0.8 log CFU/mL) (Diaz M.E *et al.*, 2001). The combination of nisin and cinnamon, enhanced inactivation of *S.* Typhimurium to a non-detectable level in apple juice (Yuste & Fung, 2004). Synergetic effect of ultrasound, mild heat, and slightly acidic electrolyzed water, a type of electrolyzed water with a pH value of 5.0-6.5, is regarded as an effective antimicrobial agent in the recent decades, on *Salmonella* and *L. monocytogenes* was examined. They found the combined treatment achieved about 3.0 log CFU/g reduction in the two pathogens on fresh-cut bell pepper (Luo & Oh, 2016). Synergistic effect of *Listeria*-active class IIa bacteriocins with high pressure on *L. monocytogenes* in ham has been reported (Marcos *et al.*, 2008).

Although many stress combination methods are efficient, numerous foodborne illnesses show that not all those combinations are effective. As an example, desiccated *Salmonella* cells showed resistance to elevated NaCl or bile salts compared to non-desiccated cells (Gruzdev *et al.*, 2011). Also, exposure to UV irradiation for 25 min resulted in complete eradication of non-desiccated *Salmonella* cells, whereas only a 3-log reduction in survival occurred for desiccated cells (Gruzdev *et al.*, 2011). After a 1-hour exposure of *Salmonella* to dry heat, desiccated cells demonstrated a 3.1-log reduction at 100°C, whereas non-desiccated cells showed an 8-log reduction at 100°C (Gruzdev *et al.*, 2011). Also, exposure of *L. monocytogenes* to acidic pH can lead to increased resistance to subsequent exposure to lethal concentrations of H_2O_2 and ethanol (Lou & Yousef, 1997). Exposure of *L. monocytogenes* to osmotic stress can lead to cross-protection against other stresses, such as low temperature (Schmid *et al.*, 2009) and bile salts (Begley *et al.*, 2002). Increased resistance to antimicrobials that can be applied to foods, such as bacteriocins, can occur when *L. monocytogenes* is first exposed to low pH (van Schaik *et al.*, 1999) or osmotic stress (Bergholz *et al.*, 2013).

RTE foods are a major concern for contamination by *L. monocytogenes*. Recently, an outbreak of *L. monocytogenes* linked to deli-sliced meats and cheeses has been reported (CDC, 2019). Combination of nisin and salt can be used to inactivate *L. monocytogenes* in several RTE refrigerated foods, including cheeses, deli meats, and cold smoked salmon. Cross-protection in presence salt and nisin has been observed among a few *L. monocytogenes* strains (Bergholz *et al.*, 2013). Because different *L. monocytogenes* strains in lineage 1 and 2 are associated with many RTE food outbreaks, we are interested to examine cross-protection concept of salt and nisin in a population of *L. monocytogenes* strains. Also, innate nisin resistance and salt-induced nisin resistance of *L. monocytogenes* strains have been reported, but the actual mechanism behind that is not clear. SSI-1 has been shown to be variably present or absent among *L. monocytogenes* strains (Ryan *et al.*, 2010). We wanted to assess the impact of this accessory genome component on both innate and salt-induced nisin resistance among strains representing lineages 1 and 2.

Contamination of LMFs by *Salmonella* is a major food safety concern, and since 2010, sixteen outbreaks due to Salmonella on LMFs have been reported (CDC, 2015; CDC, 2018). While LMFs can be stored for a year or more before applying control measures, most studies have not assessed the impact of storage on subsequent thermal resistance. The gap in the knowledge is that we do not know if exposure to low moisture condition on LMFs impacts thermal resistance of Salmonella. While several different Salmonella serovars have been associated with LMF outbreaks, and studies have shown survival of Salmonella on LMFs can vary among serovars and strains (Andino et al., 2014; Finn et al., 2013a; Hiramatsu et al., 2005), we do not know if different serovars response differently when exposed to heat treatment.

Moreover, although it is assumed that *Salmonella* use the same mechanism as osmotic stress in LMFs, survival mechanism of *Salmonella* on LMFs is unknown.

This study will help us to broaden our knowledge about heterogeneity of bacterial responses to combination of stressors. Moreover, results of this study can help to design effective control measures for foodborne pathogens particularly *Salmonella* and *L. monocytogenes* on LMFs and RTE foods, respectively.

2. GENETIC AND ENVIRONMENTAL FACTORS INFLUENCE *LISTERIA MONOCYTOGENES* NISIN RESISTANCE

2.1. Abstract

Aims: *L. monocytogenes* nisin resistance increases when first exposed to NaCl and other stresses, such as low pH. In addition to environmental stressors, specific genomic elements can confer nisin resistance, such as the stress survival islet (SSI-1). As SSI-1 is variably present among *L. monocytogenes* strains, we wanted to determine if SSI-1 was associated with salt-induced nisin resistance.

Methods and Results: The presence of SSI-1 was determined using PCR for 48 strains of *L. monocytogenes*. When combined with multi-locus sequence typing data, we found that the distribution of SSI-1 is clonal, where strains from clonal complexes (CC) 2, 6 and 11 do not have SSI-1, while strains from CCs 3, 5, 7 and 9 contain SSI-1. The impact of SSI-1 on salt-induced nisin resistance was dependent on CC. The average log decrease after 24 h exposure to nisin at 7°C under salt-inducing conditions was 2.6 ± 1.1 for CC 9 strains and 2.3 ± 0.7 for CC 11 strains, which was significantly lower survival compared to the other CCs, such as 1.3 ± 0.3 for CC 6. Deletion of SSI-1 from a CC 7 strain demonstrated the role SSI-1 plays in salt-induced nisin resistance, as the deletion mutant had lower resistance compared to the parent strain.

Conclusions: These data suggest that inducible nisin resistance in *L. monocytogenes* can be influenced by environmental conditions as well as the genetic composition of the strain, which should be considered when selecting control measures for ready-to-eat foods.

Significance and impact of the study: The foodborne pathogen *L. monocytogenes* can grow in suboptimal conditions, including low temperature and high osmolarity, which makes it a safety concern for ready-to-eat foods. When using antimicrobial peptide inhibitors such as nisin,

it is important to understand how food components can impact antimicrobial resistance across the genetic diversity of *L. monocytogenes*.

Keywords: *L. monocytogenes*, nisin, salt stress, resistance, variation

2.2. Introduction

Robust stress resistance capabilities combined with distribution in an array of environments that encompass the food supply make the foodborne pathogen *L. monocytogenes* a significant food safety concern. *L. monocytogenes* encounters numerous environmental stresses during transmission from food to humans, and there are many examples where exposure to one stress influences the ability of the pathogen to grow or survive upon exposure to a subsequent stress. For example, exposure to acidic pH can lead to increased resistance to subsequent exposure to lethal concentrations of H_2O_2 and ethanol (Lou & Yousef, 1997). Exposure of *L. monocytogenes* to osmotic stress can lead to cross-protection against other stresses, such as low temperature (Schmid et al., 2009) and bile salts (Begley et al., 2002). Increased resistance to antimicrobials that can be applied to foods, such as bacteriocins, can occur when *L. monocytogenes* is first exposed to low pH (van Schaik et al., 1999) or osmotic stress (Bergholz et al., 2013). As ready to eat (RTE) foods are a major concern for contamination by *L. monocytogenes*, it is important to consider these stress resistance properties and the potential for cross-protection when developing and applying hurdles, i.e., combinations of stresses that act synergistically to inhibit microbial growth (Leistner, 2000).

In addition to environmental effects, stress resistance properties of *L. monocytogenes* can be influenced by genetic factors, such as the presence or absence of genes in the accessory genome. For example, *L. monocytogenes* strains that possess the LG1 genomic island containing emrE have greater resistance to benzalkonium chloride (Kovacevic et al., 2015), a commonly

used sanitizer in food processing facilities (To et al., 2002). Genes encoding proteins involved in cadmium resistance are also variably present or absent among *L. monocytogenes* strains (Parsons et al., 2016), and can be carried on plasmids (Mullapudi et al., 2010). Another example is the 5-gene stress survival islet (SSI-1), which provides resistance to low pH, high osmolarity, and nisin (Begley et al., 2010; Ryan et al., 2010). SSI-1 contains 2 genes encoding hypothetical proteins of unknown function (lmo0444 and lmo0445), a gene encoding a protein involved in bile tolerance (pva, lmo0446), and two genes encoding proteins involved in the glutamate dependent acid resistance system (gadD1 and gadT1, lmo0447 and lmo0448) (Ryan et al., 2010). Deletion of the entire SSI-1 in *L. monocytogenes* strain LO28 led to reduced growth at high salt concentrations and at low pH, as well as reduced survival on hot dogs at 4°C (Ryan et al., 2010). Deletion of *gadD1* in strain LO28 led to markedly reduced survival compared to the parent strain under lethal nisin stress at 37°C (Begley et al., 2010).

Bacteriocins such as nisin can be used to control *L. monocytogenes* on RTE foods (Nilsson et al., 1997). Nisin was evaluated to be safe for food by the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives in 1969 (Arauza et al., 2009), and it has gained widespread application in the food industry. Nisin can be used to inactivate *L. monocytogenes* on a number of RTE refrigerated foods, including cheeses, deli meats, and cold smoked salmon. The inhibitory effect of nisin occurs at the cytoplasmic membrane. Nisin binds to lipid II, a peptidoglycan precursor, interferes with cell wall biosynthesis and create pores which leads to disruption of the proton motive force, leakage of ions, hydrolysis of ATP and ultimately cell death (Wiedemann et al., 2001). Variation in nisin resistance has been observed among *L. monocytogenes* strains, both in laboratory medium and on cold smoked salmon at 7°C (Tang et al., 2013). We also know that prior exposure to salt stress

can increase subsequent nisin resistance (Bergholz et al., 2013). As the SSI-1 has been shown to be variably present or absent among *L. monocytogenes* strains (Ryan et al., 2010), we wanted to assess the impact of this accessory genome component on both innate and salt-induced nisin resistance among strains representing lineages 1 and 2.

2.3. Materials and methods

2.3.1. Strains used in this study

A total of 48 *L. monocytogenes* strains were evaluated in this study (Table 1). Strains were obtained from the Food Safety Lab at Cornell University and from the Centers for Disease Control and Prevention. Stocks were stored at -80°C in Brain Heart Infusion (BHI) broth (Criterion, Hardy Diagnostics, California, USA) with 15% glycerol.

Table 1. *L. monocytogenes* strains used in this study.

Strain	Lineage	Serotype	Source	10G-ST*	CC†	SSI‡
FSL F2-0091	1	4b	human sporadic	2	2	-
FSL F2-0656	1	4b	human sporadic	2	2	-
FSL F2-0661	1	4b	human sporadic	2	2	-
FSL J1-0116	1	4b	pate outbreak	2	2	-
FSL J1-0220	1	4b	1979 vegetable outbreak, human isolate	2	2	-
FSL J1-0020	1	4b	1987 Philadelphia outbreak	9	2	-
ScottA	1	4b	1983 Boston milk outbreak	9	2	-
FSL S4-0848	1	4b	environment	39	2	-
G6054	1	1/2b	1994 chocolate milk outbreak	1	3	+
FSL F2-0369	1	1/2b	RTE pasta salad	1	3	+
FSL J1-0049	1	3c	human sporadic	1	3	+
FSL L3-0051	1	1/2b	RTE salmon	1	3	+
FSL R2-0154	1	1/2b	smoked seafood	1	3	+
FSL F6-0386	1	1/2b	smoked seafood	20	3	+
L2624	1	1/2b	2011 cantaloupe outbreak	7	5	+
FSL C1-0406	1	1/2b	RTE food	7	5	+
FSL J1-0169	1	3b	human sporadic	7	5	+
FSL J2-0064	1	1/2b	cattle	7	5	+

Table 1. *L. monocytogenes* strains used in this study (continued)

Strain	Lineage	Serotype	Source	10G-ST*	CC†	SSI‡
FSL L4-0060	1	1/2b	processing plant environment	7	5	+
FSL L4-0400	1	1/2b	processing plant environment	7	5	+
FSL R2-0182	1	1/2b	smoked seafood	7	5	+
H7858	1	4b	1998 hot dog outbreak	4	6	-
FSL E1-0201	1	4b	soil	4	6	-
FSL F3-0950	1	4b	human sporadic	4	6	-
FSL N3-0692	1	4b	soil	4	6	-
FSL N3-0780	1	4b	cattle feces	4	6	-
FSL R2-0763	1	4b	2002 turkey deli meat outbreak	4	6	-
10403S	2	1/2a	human skin lesion	69	7	+
L2626	2	1/2a	2011 cantaloupe outbreak	3	7	+
L2676	2	1/2a	2011 cantaloupe outbreak	3	7	+
L1846	2	1/2a	2009 hog head cheese outbreak	3	7	+
FSL F3-0631	2	1/2a	human sporadic	3	7	+
FSL F3-0744	2	1/2a	human sporadic	3	7	+
FSL F6-0084	2	1/2a	human sporadic	3	7	+
FSL R6-0896	2	1/2a	processing plant environment	3	7	+
EGDe	2	1/2a	asymptomatic pregnant	6	9	+
LO28	2	1/2c	lab strain	61	9	+
FSL J1-0125	2	1/2c	human sporadic	6	9	+
FSL R2-0561	2	1/2c	human sporadic	6	9	+
FSL J1-0022	2	1/2c	human sporadic	13	9	+
F6854	2	1/2a	human sporadic	5	11	-
J0161	2	1/2a	2000 turkey deli meat outbreak	76	11	-
FSL F2-0141	2	1/2a	human sporadic	5	11	-
FSL F2-0405	2	1/2a	human sporadic	71	11	-
J0221	2	1/2a	human sporadic	82	11	-
J0847	2	1/2a	human sporadic	83	11	-
F4235	2	1/2a	1987 Philadelphia outbreak	5	11	-
2009L-1023	2	1/2a	2009 Mexican-style cheese outbreak	83	11	-

* sequence types based on 10 gene MLST described by den Bakker et al., 2010† clonal complex based on the Pasteur Institute typing scheme ‡ PCR based detection of the presence (+) or absence (-) of the 5 gene SSI-1

2.3.2. Determining presence/absence of the stress survival islet SSI-1.

Strains were inoculated from -80°C freezer stocks into Brain Heart Infusion (BHI) broth and incubated at 37°C for 16-18 h. The strains were plated on BHI agar and incubated at 37°C to obtain isolated colonies. For each strain, a single colony was suspended in 40 µl of lysate buffer (50 µg ml^{-1} Proteinase K in TE pH 8.0), incubated at 55°C for 10 min followed by 80°C for 10 min. Cell debris was pelleted by centrifugation at 10,000 x g for 30 s, and lysates were stored at -20°C. Lysates were used as a template for a PCR assay designed to detect whether each strain carried SSI-1. Primers were designed with Geneious v6.1 from 16 available *L. monocytogenes* genomes representing genetic lineages 1 and 2 (Table 2). PCR reactions contained 5x Flexi buffer, 2U GoTaq (Promega, Madison Wisconsin) 0.2 m mol dNTPs, 2 m mol $MgCl_2$, and 0.3 m mol each of the three SSI-1 detection primers. PCR consisted of an initial hold at 95°C for 10 min, followed by 35 cycles of 95°C for 1 min, 54°C for 1 min, and 72°C for 1.5 min. The presence of SSI-1 was determined by amplification of a 1087 bp product from primers lmo0448F and lmo0449R (where only strains with SSI-1 will contain a homolog of lmo0448) and the absence of SSI-1 was determined by amplification of a 1385 bp product from primers lmo0443F and lmo0449R, the genes that are directly flanking the region where SSI-1 would be inserted.

2.3.3. Growth conditions prior to nisin resistance assays

Strains were streaked from culture stocks stored at -80°C onto BHI agar and incubated at 37°C overnight. A single colony was transferred into 5 mL BHI and incubated at 37°C, 230 rpm, for 20 h. Cultures were transferred 1:100 to BHI at 7°C and incubated without shaking at 7°C for 50 h. At this point, cultures are in exponential phase, based on previous work (Bergholz et al., 2012). These exponential phase cultures were then used for the nisin resistance assays.

2.3.4. Nisin resistance assays

Nisin resistance was measured for strains exposed to two different growth conditions (BHI and BHI + 6% NaCl) followed by exposure to nisin in the presence or absence of 6% NaCl. Exponential-phase cultures (~3 x 10^7 CFU ml^{-1}) were transferred 1:10 into BHI broth and BHI + 6% NaCl (VWR, Amresco, Ohio, USA) and incubated at 7°C for 200 min. In previous work, this time point reflected 10% of the length of lag phase when *L. monocytogenes* is adapting to growth in the presence of NaCl (Bergholz et al., 2012), and also the maximum induction of nisin resistance by exposure to 6% NaCl (Bergholz et al., 2013). After 200 min of incubation, cultures in BHI and BHI + 6% NaCl each were transferred 1:5 into BHI + 2mg ml^{-1} Nisaplin (Danisco, Copenhagen, Denmark) (equivalent to 50 ppm nisin, as Nisaplin contains 2.5% nisin) and BHI + 6% NaCl + 2 mg ml^{-1} Nisaplin. This resulted in a total of 4 different test conditions for each strain: i) growth in BHI and exposure to nisin in BHI, ii) growth in BHI and exposure to nisin in BHI + 6% NaCl, iii) growth in BHI + 6% NaCl and exposure to nisin in BHI, and iv) growth in BHI + 6% NaCl and exposure to nisin in BHI + 6% NaCl. All cultures were sampled prior to nisin exposure and after 24 h of incubation at 7°C in each medium containing nisin. Cultures were plated on BHI agar, and plates were incubated overnight at 37°C. Colonies were enumerated using a Q-Count instrument (Spiral Biotech, Norwood, MA). Initial densities of *L. monocytogenes* strains in the nisin test media ranged from 4 x 10^6 to 7x 10^6 CFU ml^{-1}. The limit of detection for the nisin assays was 10 CFU ml^{-1}. Survival experiments in each of the 4 test conditions were conducted for two independent cultures of each strain, and data were used to calculate the log decrease in cell density after 24 h of exposure to nisin under each test condition.

2.3.5. Mutant construction

In-frame deletion of SSI-1 in *L. monocytogenes* strain 10403S was created by using the splicing-by-overlap-extension (SOE) method as previously described (Bergholz et al., 2012). Primer sequences used for SOE are listed in Table 2. In-frame deletion was verified by sequencing the region amplified from primers TB161 and TB162.

2.3.6. Construction of MLST based phylogeny

A phylogeny was constructed based on a previously described 10 genes multi-locus sequence typing (MLST) scheme (den Bakker et al., 2010). This scheme includes DNA sequences of partial open reading frames for 10 genes: *ldh*, lmo0490, *prs*, *sigB*, *polC*, *rarA*, lmo1555, *pbpA*, *addB*, and lmo2763, and nucleotide data for these genes were obtained from either the Foodmicrobetracker.com database (Vangay et al., 2013) or Genbank, when genome sequences for the strain were available. The phylogeny was constructed using the Jukes-Cantor and neighbor-joining methods implemented in Geneious 6.1 with 5,000 bootstrap replications. The dendrogram was rooted with sequence from a lineage 4 *L. monocytogenes* strain, FSL J1-0208. Clonal complexes are based on the Pasteur Institute typing scheme, and were assigned based on previous comparisons between the 10 gene MLST and the 7 gene MLST scheme (Tang et al., 2015), as well as data available through the Pasteur sequence typing database (Cantinelli et al., 2013; Ragon et al., 2008).

Table 2. Primers used for SSI detection and SSI deletion.

Primer	Purpose	Sequence (5'→3')
lmo044	SSI detection	TTAAAAGAAGCGCAAAATGAAAGT
lmo044	SSI detection	TTTTAATTCCGTTTTTCATCTATGGT
lmo044	SSI detection	CCAAGTACACACTGCATAAGC
TB158	SSI deletion	CATATTCATACTCATATTTCCTCCTTC
TB159 soeC	SSI deletion	GAAGGAGGAAATATGAGTGAATATGATGA AATAAGAGGTGGAAAAATG
TB160	SSI deletion	GTACTGCAGTAGAAAACTAAAAGATTAATT
TB161	SSI deletion verification	AAACAGAAACAATGGAAAAATTCGC
TB162	SSI deletion verification	TTCTTTTCTTTGTTTTGTCCTTCATGA

2.3.7. Statistical analysis

The log decreases in cell density after 24 h of exposure to 2 mg ml^{-1} Nisaplin was calculated as the difference in log10 CFU ml^{-1} at time zero and after 24 h in medium with nisin. Analysis of variance (ANOVA) was implemented using the mixed procedure in the software program SAS v.9.3 (SAS Institute, Cary, NC) with a linear model that included CC, presence or absence of SSI-1, strain, replicate, and treatment (each of the 4 growth and nisin exposure combinations), all as fixed effects. To assess the overall effects of lineage and presence or absence of SSI-1 on nisin resistance, a two-way ANOVA was utilized. The Tukey multiple-correction procedure was applied to all ANOVA results. Adjusted p values of <0.05 were considered significant.

2.4. Results

2.4.1. The presence or absence of SSI-1 is clonal

The presence or absence of SSI-1 was determined by PCR assay, and results were confirmed for selected strains (e.g. L2624, H7858, 10403S) with available genome sequences. Over 50% (26/48) of the strains evaluated carried SSI-1, and when this information was overlaid on a MLST-based phylogeny of these 48 *L. monocytogenes* strains, it is evident that the presence or absence of SSI-1 is clonal (Figure 1). Lineage 1 strains belonging to CC 3 and CC 5 have SSI-1, while strains from CC 2 and CC 6 do not. In lineage 2, all strains tested from CC 7 and CC 9 have SSI-1, while CC 11 strains do not. This clonal framework was used to group strains for assessment of nisin resistance among the different CCs.

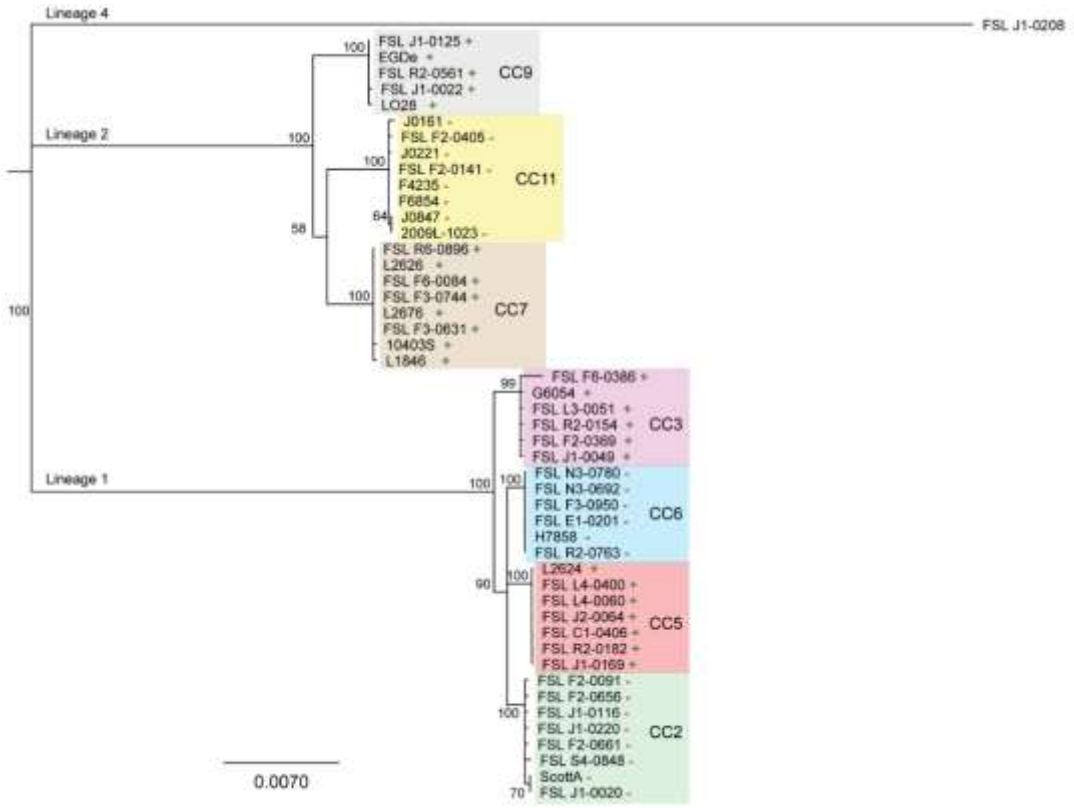

Figure 1. *L. monocytogenes* phylogeny based on multi-locus sequence data.
The scale bar at the bottom denotes number of nucleotide differences per 100 sites. Boxes denote clonal complexes (CCs) identified in the Pasteur MLST database. Branch tips are labeled with each strain name, followed by whether the SSI-1 was detected based on PCR or from genome sequences when available. + indicates presence of the SSI-1, - indicates absence of the SSI-1.

2.4.2. Nisin resistance increases significantly in the presence of salt

As NaCl is added to the growth medium, the test medium, or both, nisin resistance increases for the majority of strains (Figure 2). Overall, nisin resistance is significantly higher when strains are first exposed to BHI + 6% NaCl then exposed to nisin in the presence of NaCl (adj. $p < 0.05$) as compared to when strains are not exposed to NaCl during growth or the nisin assay (Table 3). Innate resistance to nisin, as measured by growth in BHI and survival in BHI + nisin, varied among the CCs, with strains in CC 6 and CC 7 having significantly higher resistance compared to the other CCs (adj. $p < 0.05$). Addition of NaCl to the growth medium led

to significantly improved nisin resistance for strains in CC 3, CC 5, and CC 11, with survival increasing by 0.7 to 0.8 log CFU ml^{-1}compared to their innate nisin resistance (adj. $p < 0.05$). Addition of NaCl to the nisin assay medium led to significantly greater nisin resistance for all CCs compared to their innate nisin resistance, and addition of NaCl to the growth and nisin assay media led to the greatest resistance to nisin for all CCs (Table 3). Addition of NaCl to the growth and nisin assay media led to an approximately 2 log increase in survival compared to the levels of innate nisin resistance across all CCs.

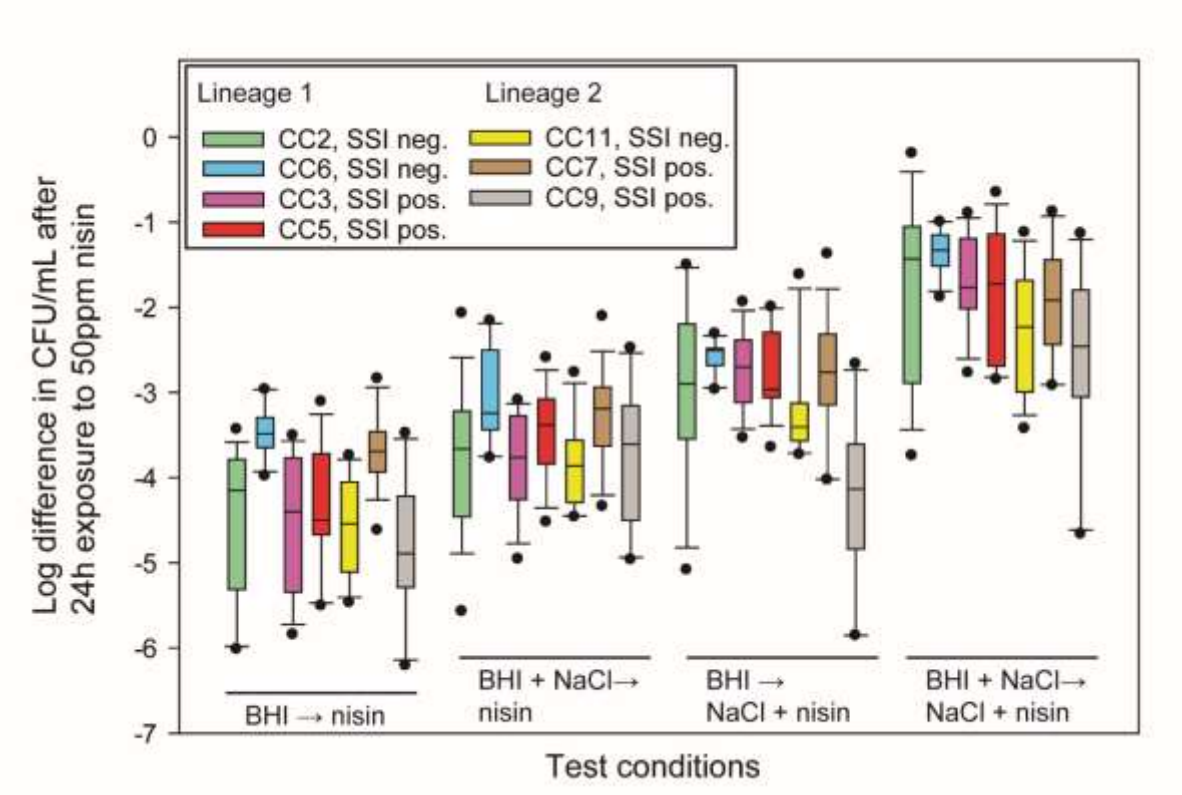

Figure 2. Distribution of survival data after 24 h exposure to Nisaplin by CC and growth and assay conditions.
Boxplots represent the distribution of log differences after 24 h exposure to 2mg ml^{-1} Nisaplin. Boxes represent the 25th to 75th percentile of the values; whiskers represent the 10th and 90th percentiles. The horizontal bar indicates the median for each condition. Filled circles represent values outside the 10th to 90th percentiles

Table 3. Average log decrease in *L. monocytogenes* populations following 24 h exposure to Nisaplin under each growth and assay condition.

	average log decrease after 24h exposure to 2mg ml^{-1} Nisaplin							
CC	BHI → BHI + Nisaplin*		BHI + NaCl → BHI + Nisaplin		BHI → BHI + NaCl + Nisaplin		BHI + NaCl → BHI + NaCl + Nisaplin	
2	4.5 ± 0.9	Aa	3.8 ± 0.9	Aa	3.0 ± 1.0	Ab	1.8 ± 1.1	Ac
3	4.5 ± 0.8	Aa	3.8 ± 0.6	Ab	2.7 ± 0.5	Bc	1.7 ± 0.5	Ad
5	4.3 ± 0.7	Aa	3.5 ± 0.5	Ab	2.8 ± 0.5	Bc	1.8 ± 0.7	Ad
6	3.5 ± 0.3	Ba	3.1 ± 0.6	Ba	2.6 ± 0.2	Bb	1.3 ± 0.3	Ac
7	3.7 ± 0.4	Ba	3.3 ± 0.6	Ba	2.8 ± 0.7	Bb	1.9 ± 0.7	Ac
9	4.8 ± 0.8	Aa	3.8 ± 0.8	Aab	4.2 ± 1.0	Cb	2.6 ± 1.1	Bc
11	4.5 ± 0.6	Aa	3.8 ± 0.5	Ab	3.2 ± 0.6	Ac	2.3 ± 0.7	Bd

* means and standard deviations are presented for each CC for each assay condition. Means followed by a different capital letter within a column are statistically different (adj. $p < 0.05$). Means followed by a different lowercase letter within a row are statistically different (adj. $p<0.05$).

2.4.3. Nisin resistance varies by CC

Innate nisin resistance varied among CCs, with CC 6 and CC 7 having greater innate resistance to nisin compared to the other CCs (Table 3). The average log decrease after 24 h exposure to nisin was 3.5 ± 0.3 for CC 6 strains, and 3.7 ± 0.4 for CC 7 strains (Table 3). Innate nisin resistance for all other CCs tested was significantly lower (adj. $p<0.05$) in comparison, with greater log reductions ranging from 4.3 to 4.8. CC 6 and CC 7 also had significantly greater nisin resistance (adj. $p< 0.05$) compared to the other CCs when NaCl was added to the growth medium (Table 3). With the addition of NaCl only to the nisin assay medium, CC 9 strains had significantly lower nisin resistance compared to the other CCs (adj. $p<0.05$). When NaCl was added to both the growth and the assay media, CC 9 and CC 11 had significantly lower nisin resistance compared to the other CCs (adj. $p<0.05$).

2.4.4. Influence of lineage and SSI-1 on nisin resistance

A two-way ANOVA was used to assess the overall effects of lineage and SSI-1 on nisin resistance. A significant interaction between lineage and presence or absence of SSI-1 was not identified, indicating that any effects of lineage and SSI-1 were independent of each other. Examining the effect of lineage on nisin resistance found that overall nisin resistance was higher for lineage 1 strains under the two test conditions where NaCl was present in the nisin assay medium ($p < 0.001$). When strains were grown in BHI and exposed to nisin in BHI with NaCl, average differences in cell density after 24 h exposure to nisin were 2.8 ± 0.7 log CFU $ml^{-1}$for lineage 1 and 3.3 ± 0.9 log CFU ml^{-1} for lineage 2. When strains were grown in BHI with NaCl and exposed to nisin in BHI with NaCl, average differences in cell density after 24 h exposure to nisin were 1.7 ± 0.8 log CFU ml^{-1} for lineage 1 and 2.2 ± 0.8 log CFU ml^{-1} for lineage 2. Including the presence or absence of SSI-1 as a factor in the comparison of nisin resistance between the lineages found no significant differences in nisin resistance due to the presence or absence of SSI-1 under any of the four test conditions.

2.4.5. Inactivation of SSI-1 in 10403S affects salt-induced nisin resistance

While overall effects of the presence or absence of SSI-1 on nisin resistance were not detected, likely due to confounding factors of the overall genetic differences among the CCs, there were notable differences in nisin resistance among the CCs that belong to lineage 2. CC 7 strains consistently had higher nisin resistance than strains from CC 9 or CC 11, under all 4 of the test conditions (Table 3). CC 7 do possess SSI-1, and we assessed the impact of inactivating SSI-1 by deletion mutation on one representative strain from CC 7, 10403S. Nisin resistance was measured for 10403S and 10403S ΔSSI-1 under the 4 growth and assay conditions. The absence of SSI-1 did not impact nisin resistance under 3 of the 4 conditions (Figure 3). Deletion of SSI-1

did significantly (adj. p <0.05) lower salt-induced nisin resistance compared to the parent strain, with an average log difference of 2.9 ± 0.1 log CFU ml^{-1} for 10403S and 4.1 ± 0.2 for 10403S ΔSSI-1.

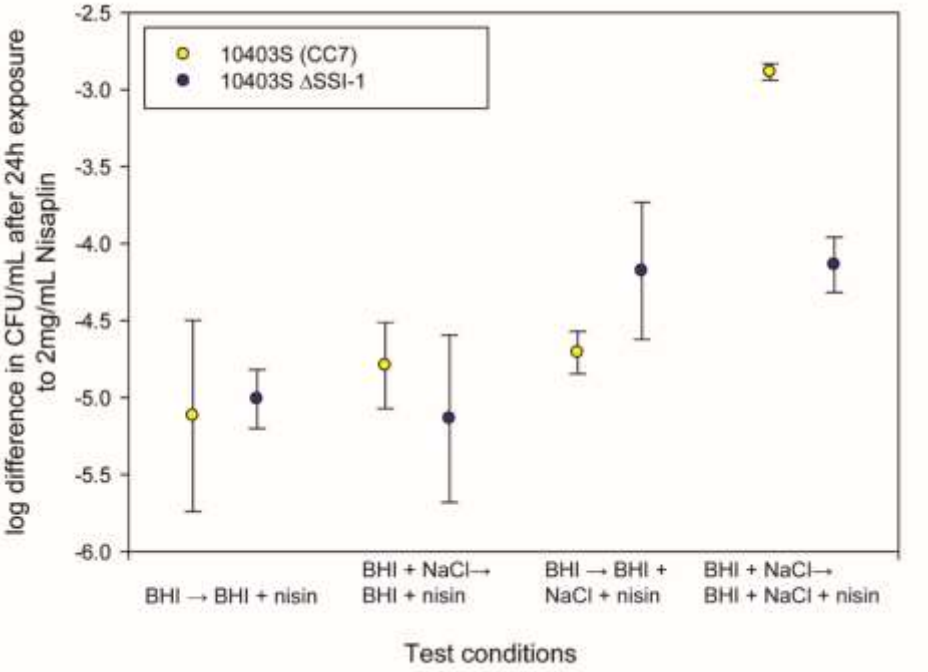

Figure 3. Average nisin resistance of strain 10403S (yellow circles) and 10403S ΔSS1-1 (black circles) under each of the 4 growth and test conditions.
Averages and standard deviations are calculated from 3 replicates of each strain under each test condition.

2.5. Discussion

Initial assessments of the presence or absence of SSI-1 looked for associations between serotype and/or lineage and the presence of the genomic islet. SSI-1 was found to be mostly absent from strains of serotype 4 (Ryan et al., 2008), mostly present in strains of serotype 1/2c (Hein et al., 2011), and variably present or absent in serotype 1/2b (Hein et al., 2011; Ryan et al., 2010). Here we show that the presence or absence of SSI-1 can be assessed by CC, where all strains in a CC either have the islet or do not. For lineage 1, CCs that are predominated by

serotype 4b, such as CC 2 and CC 6, do not have SSI-1, while CC 3 and CC 5, predominated by serotype 1/2b, do carry SSI-1. While not all of the major CCs are represented in our study, searches of available genome sequences in combination with known CC designation supports the trend that presence or absence of SSI-1 is clonal. SSI-1 is not found in genomes of strains belonging to lineage 1 CC 1 (e.g. F2365, SLCC2378, LL195) or CC 4 (e.g. L312, Clip80459, or 07PF0776). Other common CCs from lineage 2 include CC 8, which does have SSI-1 (e.g. Lm1823 and 08_5579) and CC 121, which does not have SSI-1 (e.g. La111, N53-1, Lm_1880).

Variation in a number of stress resistance phenotypes has been observed in *L. monocytogenes*, though in some cases when viewed by serotype or lineage, differences were not evident. Recent genomic data from ~1,700 *L. monocytogenes* isolates highlight the existence of important genomic differences in known virulence and stress response genes among the different sublineages and CCs (Moura et al., 2016). Classification by lineage or serotype may not adequately reflect the genetic basis for these phenotypic differences, while CC may be more appropriate. A comparison of growth phenotypes in defined minimal medium at 16°C identified significant differences among CCs, with strains from CC 11 and CC 7 exhibiting significantly faster rates of growth compared to those from CC 3 (Tang et al., 2015). Virulence phenotypes also seem to cluster by CC, with strains from CCs 1, 4, and 6 more commonly isolated from patients with few immunosuppressive comorbidities compared to strains from CCs 9 and 121, which are commonly isolated from significantly immunocompromised patients (Maury et al., 2016). Virulence assays in mice confirmed greater virulence capabilities of CCs 1, 4, and 6, which demonstrated significantly greater virulence in mice compared to CCs 9 and 121 (Maury et al., 2016). Here we show that nisin resistance phenotypes also cluster by CC, with strains from CCs 6 and 7 having significantly greater innate nisin resistance compared to the other CCs

evaluated, and CCs 9 and 11 having significantly lower salt-induced nisin resistance compared to the other CCs evaluated.

Previous studies have implicated the SSI-1 as a contributor to nisin resistance in *L. monocytogenes*, specifically *gadD1* (lmo0447) one of the 5 genes on SSI-1 (Begley et al., 2010). Targeted deletion of both SSI-1 or just *gadD1* in *L. monocytogenes* strain LO28 did lead to a nisin sensitive phenotype at 37°C under both sub-lethal and lethal nisin exposure (Begley et al., 2010). We constructed a non-polar deletion mutant of SSI-1 in strain 10403S, and while absence of SSI-1 did not significantly influence innate nisin resistance, it did significantly impact salt-induced nisin resistance. While both LO28 and 10403S do naturally possess SSI-1, they do belong to different CCs: LO28 to CC 9 and 10403S to CC 7. Begley et al. also compared nisin resistance among 5 strains that did and 5 that did not have SSI-1, and found that overall, those that possessed SSI-1 did have greater nisin resistance. It is interesting to note that their data also showed a high level of variability in the nisin resistance phenotype, with at least one of the 5 SSI-1 negative strains exhibiting a high level of nisin resistance (Begley et al., 2010). Based on the significant differences in nisin resistance phenotypes that we present here, it is possible that genetic background plays a larger role in overall nisin resistance phenotypes than simply the presence or absence of SSI-1. Cell envelope stress response systems, including those regulated by VirR (Kang et al., 2015) and LiaR (Bergholz et al., 2013), are known to contribute to nisin resistance at low temperatures and in the presence of NaCl. Variation in regulation or expression of these systems could also contribute to the varying nisin resistance phenotypes observed.

The data presented here demonstrate that both genetic and environmental factors can influence nisin resistance among *L. monocytogenes* strains. The presence of SSI-1, an accessory genome islet of five genes previously found to influence nisin resistance, is clonal. Differences in

nisin resistance at low temperature were observed among CCs, in both innate nisin resistance and salt-induced nisin resistance. Our data confirm that nisin resistance does increase with exposure to NaCl, which had previously been assessed for only six strains. Taken together, these results highlight that inducible nisin resistance can be affected by environmental conditions, such as osmotic stress induced by NaCl, as well as the genetic composition of the strain.

2.6. Funding information

This work was funded in part by the National Institute of Food and Agriculture, U.S. Department of Agriculture, under Project No. ND02426, as well as by a Graduate Fellowship to S.M. from the NDSU Graduate School, and a McNair Research Scholarship to K.K.

2.7. Acknowledgments

We thank Dr. Cheryl Tarr and Dr. Martin Wiedmann for providing the strains used in this study.

2.8. Conflict of interest

The authors declare no conflicts of interest.

3. VARIATION IN SURVIVAL AND THERMAL RESISTANCE AMONG *SALMONELLA* SEROVARS ON A LOW WATER ACTIVITY FOOD

3.1. Abstract

Salmonella contamination of low moisture foods (LMFs) is a major food safety concern. LMFs are identified by their low a_w (a_w <0.85) and can be stored for lengthy periods of time before use. Low a_w conditions can impact thermal resistance of *Salmonella*, and thermal treatment is a common method to control *Salmonella* on LMFs. To determine the impact of storage on a LMF on subsequent *Salmonella* thermal resistance, we evaluated long term survival and thermal resistance for 32 strains of *Salmonella* inoculated onto flaxseed. The 32 strains represented four *Salmonella* serovars and were stored on flaxseed for 24 weeks. After 24 weeks, average log reductions were 4.0 ± 0.8, 3.6 ± 0.7, 3.4 ± 0.7, and 3.3 ± 0.7 $\log_{10}$ CFU/g for serovars Enteritidis, Montevideo, Tennessee, and Agona, respectively. Strains from serovar Agona had a significantly lower average k_{max} compared to serovars Enteritidis and Montevideo during storage on flaxseed (adj. p<0.05). Thermal resistance was measured by determining $D_{71°C}$ for all strains inoculated onto flaxseed at 0, 8 , 16, and 24 weeks post-inoculation with vacuum steam pasteurization. Thermal resistance of *Salmonella* changed over time of storage in a serovar-dependent manner. Average initial $D_{71°C}$ for the serovars were similar, with 1.0 ± 0.4, 1.3 ± 0.4, 1.2 ± 0.4, and 1.5 ± 0.4 min, respectively, for serovars Enteritidis, Montevideo, Tennessee, and Agona. $D_{71°C}$ did not change significantly over storage time for serovars Enteritidis and Montevideo but did change for serovars Tennessee and Agona. Average $D_{71°C}$ for serovar Tennessee significantly increased from 0.7 ± 0.3 and 0.9 ± 0.4 at 8- and 16 weeks post-inoculation to 1.5 ± 0.6 minutes at 24 weeks post-inoculation (adj. p<0.05). Average $D_{71°C}$ for serovar Agona significantly increased from 0.9 ± 0.4 and 1.1 ± 0.4 at 8 and 16 weeks post-

inoculation to 1.8 ± 0.9 min at 24 weeks post-inoculation (adj. $p<0.05$). Significant differences in $D_{71°C}$ were observed among the serovars at 24 weeks post-inoculation, where average $D_{71°C}$ for serovar Agona was 1.8 ± 0.9 min, significantly higher than 1.0 ± 0.2 min for serovar Enteritidis (adj. $p<0.05$). These data highlight that *Salmonella* thermal resistance can change over storage time on a LMF, and these differences are dependent on serovar. Effects of storage time should be considered when evaluating control measures for *Salmonella* on LMFs.

3.2. Introduction

Outbreaks of foodborne illness attributed to low moisture foods (LMFs; $a_w< 0.85$) have become a recurring concern (Sanchez-Maldonado *et al.*, 2018). *Salmonella* spp., *Bacillus cereus*, *Cronobacter sakazakii*, *Clostridium* spp., *E. coli* O157:H7, and *Staphylococcus aureus* are foodborne pathogens reported to cause foodborne outbreaks associated with LMFs (Beuchat *et al.*, 2013). Among these pathogens, *Salmonella* spp. have a long history of outbreaks in LMFs, including peanut butter (Sheth *et al.*, 2011), chocolate (Werber *et al.*, 2005), infant formula (Jourdan *et al.*, 2008), almonds (Ledet Muller *et al.*, 2007), and dried spices (Rabsch *et al.*, 2005), among others. Since 2010, the Center for Disease Control (CDC) has reported 16 outbreaks due to *Salmonella* in LMFs, which caused 877 illnesses, 273 hospitalizations, and two deaths (CDC, 2015; CDC, 2018). *Salmonella* present on LMFs leads to product recalls, resulting in significant costs to the food industry. For example, 361 food recalls occurred due to *Salmonella* contamination of LMFs from 2003 and 2011 (Dey *et al.*, 2013) and 12 food recalls occurred in 2018 (FDA, 2018).

When present on LMFs, *Salmonella* can survive for long periods of time (Beuchat *et al.*, 2013); examples from studies on almonds include survival up to 550 days (Uesugi *et al.*, 2006) and even two years (Limcharoenchat *et al.*, 2019). Since *Salmonella* can survive for long periods

of time in LMFs, exposure to low a_w over time could impact the efficacy of applied control measures, such as thermal treatments. Thermal resistance of *Salmonella* is affected by a_w, where lower a_w can lead to significant increases in thermal resistance (Goepfert *et al.*, 1970; Mattick *et al.*, 2001). When utilizing heat as a control measure, *Salmonella* are known to have exceptional heat resistance when present in foods with low a_w. During application of dry heat, greater decimal reduction times are observed as a_w decreases. For example, in wheat flour at a_w=0.57, $D_{75°C}$ values for *Salmonella* were ~30 minutes, and increased to 150 minutes at a_w=0.26 during dry heat treatment (Archer *et al.*, 1998). Smith and Marks reported that the $D_{80°C}$ for *Salmonella* Enteritidis PT 30 in wheat flour at a_w = 0.6 was 1.3 min, but it increased to 7.3 min at a_w = 0.3 (Smith & Marks, 2015).

Compared to dry heat treatment, treatment with steam or superheated steam has shown greater log reduction of *Salmonella* on LMFs, with a shorter exposure time. For example, the *D*-value of *Salmonella* on almonds exposed to dry heat was 27 min at 80°C (Limcharoenchat *et al.*, 2019), while others found the use of steam or super-heated steam reduced the *D*-values of *Salmonella* on almonds to 4.87–6.68 s and 9.2 s at 100°C, respectively (Ban, 2018; Ban & Kang, 2016). Researchers have observed varying effects of storage on a LMF over time on subsequent *Salmonella* thermal resistance. Survival of *Salmonella* at 70°C significantly increased after a six-day incubation in peanut oil (a_w 0.52) (Fong & Wang, 2016a). However, no change in thermal resistance of *S.* Enteritidis PT 30 on almonds after storage for 68 weeks was observed (Limcharoenchat *et al.*, 2019).

A number of different *Salmonella* serovars have been associated with LMF outbreaks, and studies have shown survival of *Salmonella* on LMFs can vary among serovars and strains (Andino *et al.*, 2014; Finn *et al.*, 2013a; Hiramatsu *et al.*, 2005). For example, during nine

months storage of inoculated chocolate at room temperature, *S.* Eastbourne was still detected, however, *S.* Typhimurium was unrecoverable after six months (Tamminga *et al.*, 1976). *S.* Montevideo could not be recovered after four days from inoculated poultry feed while almost 3 logs CFU/g of *S.* Enteriditis and *S.* Typhimurium were recovered after seven days (Andino *et al.*, 2014). *S.* Hartford and *S.* Tennessee demonstrated significantly higher persistence on chia seeds compared to *S.* Enteriditis, *S.* Tompson, and *S.* Typhimurium (Fong & Wang, 2016b). In addition, thermal resistance of *Salmonella* can vary among serovars and strains under low a_w conditions. Examination of the impact of low a_w created by sucrose on thermal resistance of eight *Salmonella* strains at 57.2 ± 0.1°C showed that *S.* Infantis and *S.* Tennessee were grouped as the most sensitive strains and *S.* Alachua, *S.* Anatum, *S.* Montevideo, and *S.* Senftenberg were classified as the most resistant strains (Goepfert *et al.*, 1970). Another study showed that *S.* Thompson and *S.* Tennessee were the most resistant to heat inactivation (49°C) compared to six other serovars inoculated on dry corn flour (10-15% moisture content) (VanCauwenberge *et al.*, 1981). Comparing thermal tolerance of six *Salmonella* serovars at $a_w = 0.65$ showed serovars Senftenberg 775W, Java, and Agona were the least heat-tolerant isolates (Mattick *et al.*, 2001). The majority of studies that evaluated variation in *Salmonella* survival among strains and serovars have used abiotic surfaces or lab medium maintained at a low a_w, which does not necessarily account for the physiochemical features or native microbiota that may be inherent to LMFs. When survival on different LMFs have been evaluated, strains representing the diversity of *Salmonella* involved in LMF outbreaks have typically not been assessed.

Previous work from our group demonstrated that vacuum steam pasteurization can be used to effectively reduce *S.* Enteriditis PT 30 on different LMFs, including flaxseed, sunflower kernels, and quinoa (Shah *et al.*, 2017). The present study was conducted to evaluate the

response variability of 32 strains representing four serovars of *Salmonella* during long term storage at low a_w and subsequent heat treatment aiming at (i) characterizing the strain variability of these behaviors, and (ii) assessing the impact of long term association with LMFs and thermal resistance of these strains.

3.3. Materials and methods

3.3.1. Strains used in this study

A total of 32 strains of *Salmonella* were evaluated in this study (Table 4). *Salmonella* culture stocks were stored at -80°C in Brain Heart Infusion (BHI) broth (Criterion, Hardy Diagnostics, California, USA) with 15% glycerol.

Table 4. *Salmonella* strains used in this study.

Strain	Serovar	Source
FSL R8-0288	Enteritidis	Human clinical
FSL S10-1621	Enteritidis	Environmental soil
FSL S10-1623	Enteritidis	Environmental soil
FSL S10-1644	Enteritidis	Environmental farm
FSL S10-1646	Enteritidis	Environmental farm
FSL F6-0963	Enteritidis	Human clinical
ATCC BAA-1045	Enteritidis	Food
FSL R9-4060	Enteritidis	Human clinical
FSL R8-2812	Montevideo	Environmental farm
FSL R8-3417	Montevideo	Environmental farm
FSL R8-3658	Montevideo	Environmental farm
FSL R8-3659	Montevideo	Environmental farm
FSL R8-4923	Montevideo	Human clinical
FSL R9-1588	Montevideo	Environmental farm
FSL_R8-3881	Montevideo	Human clinical
FSL R8-3706	Montevideo	Human clinical
FSL R8-2240	Tennessee	Environmental farm
FSL R9-2434	Tennessee	unspecified
FSL R9-2435	Tennessee	unspecified
FSL R9-2436	Tennessee	unspecified
FSL S10-1757	Tennessee	Environmental farm
FSL R6-0198	Tennessee	Human clinical
FSL R6-0494	Tennessee	Human clinical
FSL R8-5221	Tennessee	Human clinical
FSL R8-8615	Agona	Environmental soil
FSL R8-8619	Agona	Environmental soil
FSL S10-1750	Agona	Environmental soil
FSL S10-1759	Agona	Environmental farm
FSL S10-1760	Agona	Environment farm
FSL S10-1761	Agona	Environmental farm
FSL M8-0485	Agona	Food
FSL S9-0322	Agona	Food

3.3.2. Inoculation of flaxseed

The inoculation protocol previously described by Shah et al. (Shah *et al.*, 2017) was used with minor modifications. Briefly, bacterial freezer stocks were streaked on BHI agar plates for isolation and grown overnight at 37°C. For each strain, a colony was transferred to 5 ml BHI broth and incubated at 37°C for 20 h. The overnight broth culture (250 µl) was plated uniformly onto BHI agar plates (100 mm × 15 mm) using a sterile spreader (Fisher Scientific Inc, Waltham, MA), and plates were incubated at 37°C for 24 h.

To inoculate 2500 g flaxseed, about 833 g seeds were divided into three Whirl-Pak bags (Nasco Inc, Fort Atkinson, WI). To achieve 8 log CFU/g, the bacterial lawns from 16 plates were collected, while for 6 log CFU/g, the bacterial lawn from one half of a plate was collected with a sterile spreader and mixed into a sterile beaker containing 2.5 ml sterile water. The bacterial suspension was poured into the flaxseed in a Whirl-Pak bag and mixed by hand for 3-5 min to obtain a homogenous distribution of bacteria. Following the same inoculation protocol, samples of flaxseeds were inoculated separately with all 32 strains. To test the impact of bacterial density on thermal resistance, two strains per serovar were randomly selected and inoculated at 8 and 6 $\log_{10}$ CFU/g. Three biological replicates were conducted for this test. All inoculation procedures were conducted in a bio-safety cabinet.

3.3.3. Assessing the homogeneity of *Salmonella* inoculated on flaxseed

To assess the homogeneity of *Salmonella* inoculated on flaxseeds, eight 25 g samples were randomly taken and plated in duplicates at the time of inoculation (0 h), 24, 48, and 72 h post-inoculation for two strains of each serovar. To plate samples, inoculated seeds were weighed in a whirl pack bag and Butterfield dilution buffer was added in appropriate amounts. Bags were homogenized by masticator (IUL instruments, Spain) for 90 s and appropriate serial

dilutions were plated in duplicate onto modified Tryptic Soy Yeast Extract (TSAYE) agar with ferric ammonium citrate (J.T. Baker Inc., Phillipsburg, NJ) and sodium tetrathionate (VWR Inc, Radnor, PA). The plates were incubated at 37°C for 24-48 h and black colonies indicative of *Salmonella* were enumerated with a Q-Count reader (Advanced Instruments Inc., Norwood, MA). In addition to these initial tests for homogeneity of inoculation, four 25 g randomly selected samples were enumerated to confirm homogeneity for each experiment at the time of inoculation. A standard deviation of < 0.5 log CFU/g was deemed an acceptable range to indicate homogeneity of the inoculum for each inoculated batch of flaxseeds. In a study by Limcharoenchat et al., the accepted standard deviation of pre- and post-fabrication inoculation methods of paste date was < 0.5 log CFU/g (Limcharoenchat *et al.*, 2018).

3.3.4. Water activity (a_w) equilibration and storage of inoculated flaxseed

The a_w of flaxseed prior to inoculation was measured using an Aqualab 4TE a_w meter (Aqualab Inc, Pullman, WA). After inoculation, a_w was measured to estimate the necessary amount of lithium chloride anhydrous 99% -20 Mesh (Alfa Aesar Inc, Ward Hill, MA) to equilibrate a_w to the original level. The inoculated flaxseeds were transferred to a sterile stainless-steel tray (12″ × 9″). The stainless-steel tray was placed in a closed chamber (Coleman cooler 24″ × 16″, Coleman Company, Inc., Kingfisher, OK). 15–30 g of lithium chloride anhydrous 99% -20 Mesh was weighed in plastic trays (Fisher Scientific Inc, Hampton, NH) and saturated with water. Trays of saturated LiCl were placed adjacent to the stainless steel trays in the closed chamber to reduce the a_w. The a_w of inoculated flaxseed was equilibrated to the initial a_w of flaxseed within 48 h. After a_w equilibration, inoculated seeds were divided in 16 Whirl-Pak bags and to maintain constant a_w during storage, bags were vacuum sealed in individual Mylar bags. All Mylar bags were stored at 22 ± 1°C. One bag was opened per sampling time point.

Samples were taken from bags and *Salmonella* were enumerated at 2, 4, 6, 8, 12, 16, 20, and 24 weeks. For the thermal resistance experiment, vacuum steam pasteurization was conducted on samples at 0, 8, 16, and 24 weeks post-inoculation.

3.3.5. Survival on flaxseed over time

Over 24 weeks storage, two whirl pack bags, each containing 25 g inoculated flaxseed were picked from stored Mylar bag and the number of bacteria was enumerated after 2, 4, 6, 8, 12, 16, 20, and 24 weeks. The samples were weighed in a sterile plastic bag and Butterfield dilution buffer was added in appropriate amounts. These bags were homogenized by masticator for 90 s and appropriate serial dilutions were spread plated in duplicate on modified TSAYE supplemented with ferric ammonium citrate and sodium thiosulfate. The respective agar plates were incubated at 37±2°C for 24 h. Following incubation, the colonies were counted using a Q-Count. In this experiment, 32 strains were evaluated for survival and two biological replicates have done for each strain.

3.3.6. Thermal treatment using vacuum steam pasteurization system

The lab scale vacuum steam pasteurization (VSP) system used in this study has been described previously (McEvoy *et al.*, 2001). This system consists of a process chamber to which a vacuum was applied to provide conditions under which steam condensed at temperatures below 100°C. Steam was introduced from an external boiler. During treatment, the pressure inside the chamber can be kept between preselected values, enabling temperature control at ±2°C from the set point. Seeds inoculated at 8 logs CFU/g were divided into 25 g portions and were placed into sterilized cotton bags (Uline Inc, Pleasant Prairie, WI). Three bags of each bacterial strain were treated at 71±2°C for 0.5, 0.75, 1,1.25, 1.5, 2, and 3 minutes representing one technical replicate for that bacteria. A thermocouple (Thermoworkers Inc, American Fork, UT) was put in one bag

contain 25 g flaxseeds to measure the temperature inside the bag during thermal treatment and temperature was recorded with data logger (Onset Inc, Bourne, MA) during the processing cycles. Three inoculated samples that were not heat treated were used to determine the initial level of *Salmonella*. Thirty-two strains were tested in this experiment and it was repeated two times for each strain. To test the impact of bacterial density on thermal resistance, seeds inoculated at 8 and 6 logs CFU/g was heat treated post a_w equilibration at week 0. After thermal treatment, *Salmonella* survival were enumerated using TSAYE agar plates as described previously.

3.3.7. Survival/Inactivation modelling and statistical analysis

Survival parameters were estimated using GInaFiT Version 1.7 in Microsoft excel (Geeraerd *et al.*, 2005). The most appropriate survival model was determined to be a Geeraerd-tail model for the survival study. Survival parameters of the Geeraerd-tail model were estimated using the following equation (Geeraerd *et al.*, 2000):

$$N = (N_0 - N_{res}) * e^{(-kmax*t)} + N_{res}$$

Where N is the bacterial population (CFU/g) considering time t (min), N_0 is the bacterial population (CFU/g) prior to treatment, N_{res} is the observed residual population considered to be resistant to the treatment, and k_{max} ($\log_{10}$ CFU/g/min) is the maximum rate of inactivation.

For each bacterial strain, two samples for survival study and three samples for inactivation study were used to conduct the experiments in two replicates providing 4 and 6 counts, respectively, at each time point for each strain. The duplicate counts obtained for each sample in CFU/g were averaged and log transformed. Time points where the limit of detection was reached were excluded from the D value determination. D values were calculated from the slope determined by regression using Proc Reg in SAS V9.4 (SAS Institute Inc., Cary, NC,

USA). Two-way Analysis of variance (two-way ANOVA) was conducted using Proc GLM in SAS V.9.4., where D value was the response variable with serovar and time as the main effects, and serovar * time as the interaction effect. The Tukey test was used to adjust for multiple comparisons, and an adjusted p value < 0.05 was considered significantly different.

3.4. Results

3.4.1. Survival on flaxseed over time varies among serovars

The average a_w of flaxseeds before inoculation was 0.53 ± 0.03, which was then equilibrated to 0.50 ± 0.02 within 48 h after inoculation with *Salmonella* (Table. 5). A_w over 24 weeks of storage remained similar. The homogeneity of the inoculum on flaxseed was examined by enumerating eight randomly selected 25 g samples at the time of inoculation (0 h), 24, 48, and 72 h post-inoculation for eight randomly selected strains. The standard deviations were determined from the 8 samples for each strain and ranged from ± 0.06 to ± 0.2 $\log_{10}$CFU/g. Based on these results, we used a standard deviation of < 0.5 $\log_{10}$CFU/g as an indicator of inoculum homogeneity, which was similar to previous results for *Salmonella* on date paste (Limcharoenchat *et al.*, 2018). For inoculation of each *Salmonella* strain onto flaxseed, homogeneity tests were performed by enumeration of four 25 g randomly selected samples to confirm the standard deviation was < 0.5 log CFU/g at the time of inoculation. The average initial number of Enteritidis, Montevideo, Tennessee, and Agona was 8.8 ± 0.3, 8.8 ± 0.3, 8.7 ± 0.4, and 8.9 ± 0.2 $\log_{10}$CFU/g, respectively. Non-linear reduction of *Salmonella* cells was observed over the 24 weeks storage period (Figure 4). After 24 weeks, the average counts of serovars Enteritidis, Montevideo, Tennessee, and Agona on flaxseed were 4.8 ± 0.9, 5.2 ± 0.7, 5.2 ± 0.6, and 5.5 ± 0.6 $\log_{10}$CFU/g, respectively, showing a reduction of 4.0 ± 0.8, 3.6 ± 0.7, 3.4 ± 0.7, and 3.3 ± 0.7 $\log_{10}$ CFU/g, respectively from week 0. The maximum rate of reduction for

serovars Enteritidis, Montevideo, Tennessee, and Agona was 0.60 ± 0.14, 0.50 ± 0.14, 0.48 ± 0.10, and 0.37 ± 0.11 $\log_{10}$ CFU/g/week, respectively. The maximum rate of reduction (k_{max}) varied among serovars, where strains of serovar Agona had a significantly lower average k_{max} compared to serovars Enteritidis and Montevideo (adj. $p<0.05$) (Figure 5). Differences in k_{max} were also examined among strains and *S.* Enteriditis FSL S10-1644 had a significantly higher rate of reduction (0.76 ± 0.11 $\log_{10}$ CFU/g/week) compared to *S.* Agona FSL S10-1759 (0.29 ± 0.03 $\log_{10}$ CFU/g/week (adj.$p<0.05$).

Table 5. Average a_w of flaxseeds inoculated with *Salmonella* serovars over 24 weeks of storage at 22°C

Serovar	Initial	Week 8	Week 16	Week 24
Enteritidis	0.53 ± 0.03	0.48 ± 0.02	0.49 ± 0.02	0.47 ± 0.03
Montevideo	0.50 ± 0.02	0.50 ± 0.02	0.50 ± 0.02	0.50 ± 0.02
Tennessee	0.49 ± 0.02	0.49 ± 0.01	0.49 ± 0.02	0.48 ± 0.02
Agona	0.50 ± 0.03	0.49 ± 0.02	0.49 ± 0.02	0.48 ± 0.02

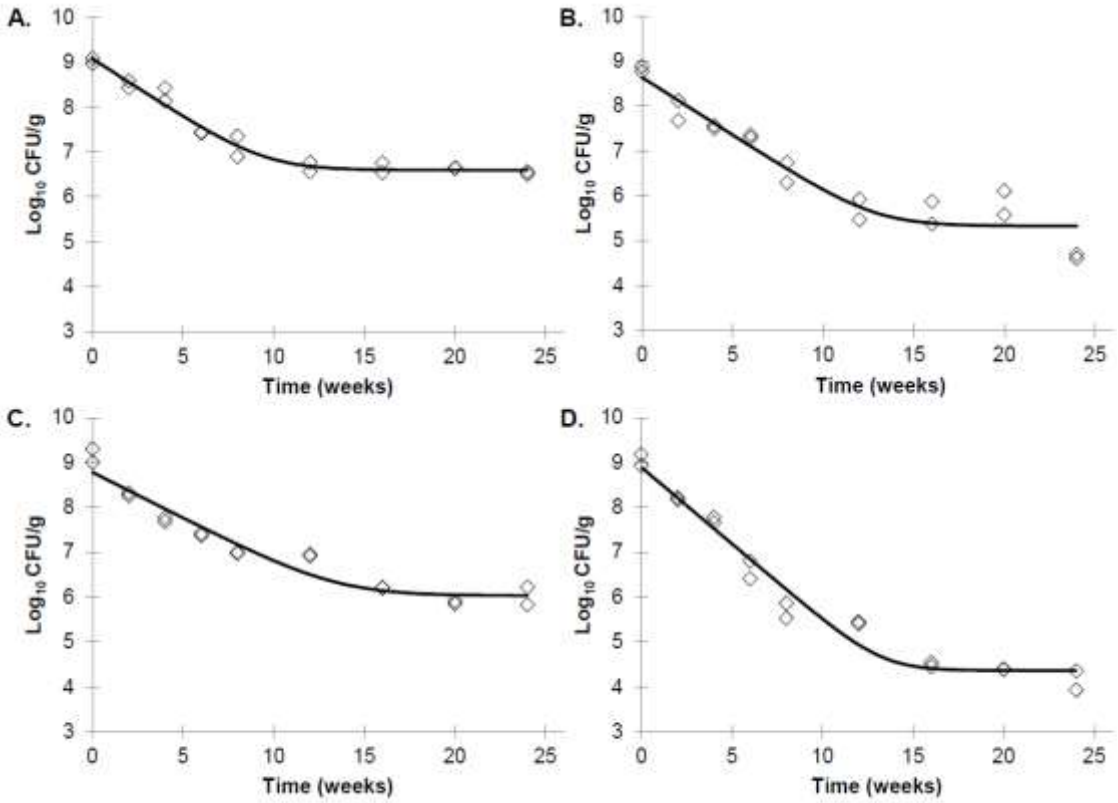

Figure 4. Non-linear reduction of *Salmonella* over 24 weeks on flaxseed at 22 ± 1°C. Open diamonds are the average plate counts of two technical replicates for a biological replicate at each time point. Lines are the curves generated from the Geeraerd model. A. *S.* Agona FSL M8-0485. B. *S.* Tennessee FSL R8-5221. C. *S.* Montevideo FSL R8-3881. D. *S.* Enteritidis FSL S10-1646

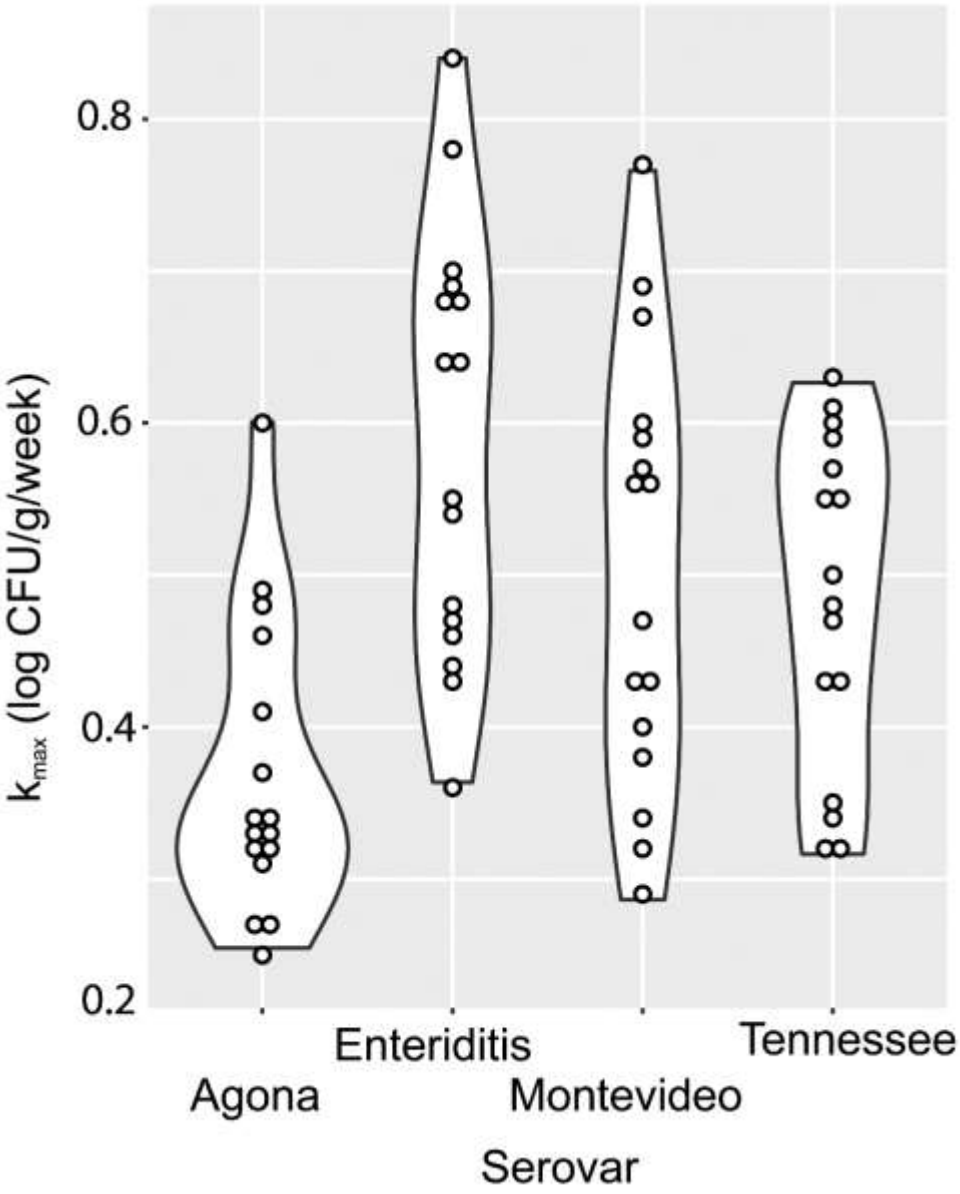

Figure 5. Average *kmax* values for 4 *Salmonella* serovars on flaxseed over 24-week of storage. Boxes represent the distribution of maximum rate of reduction for two replicates of eight strains in each serovar when stored for 24 weeks at 22 ± 1°C.

3.4.2. Inoculum density does not impact thermal resistance

As the number of *Salmonella* cells on flaxseed decreased over time of storage, we wanted to assess the potential for density dependent effects on thermal resistance. A subset of eight strains, two from each serovar, were inoculated onto flaxseed at 8 $\log_{10}$ CFU/g and 6 $\log_{10}$

CFU/g, and heat treated with VSP following the two day a_w equilibration period. The average *D*-values for the eight strains at both inoculum levels are listed in table A1. When comparing average *D*-values between the two inoculum densities for each strain, only *S.* Enteritidis ATCC BAA-1045 exhibited significantly different *D*-values dependent on inoculum density ($p<0.05$; Figure 6). For this strain, the lower inoculum level of 6 $\log_{10}$ CFU/g led to a lower *D*-value, indicating an increased sensitivity to the thermal treatment. While there was variation among *D*-values for the other strains, none of the other *D*-values were determined to be significantly different depending on inoculum level (Table A1).

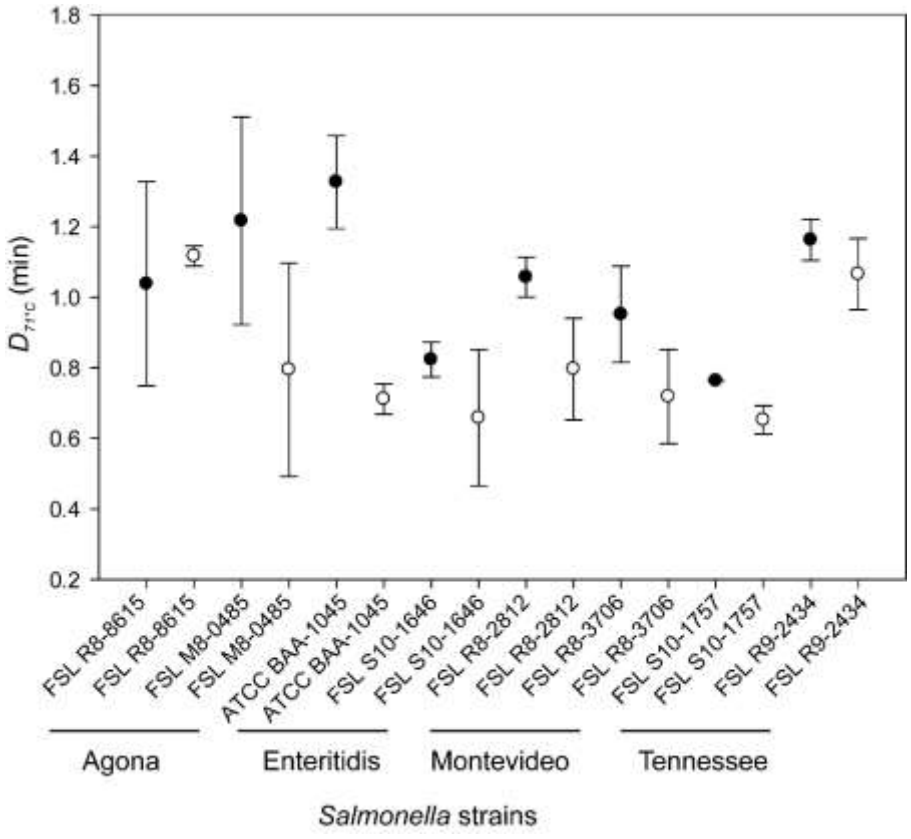

Figure 6. Average D71°C of 8 Salmonella strains at 8 log10CFU/g (black circle) and 6 log10CFU/g (white circle) inoculum levels.
Averages and standard deviations are calculated from three technical replicates for two biological replicates of each strain.

3.4.3. Thermal resistance differences among serovars at each time point of storage

The average $D_{71°C}$ of Enteritidis, Montevideo, Tennessee, and Agona at the initial time point were 1.0 ± 0.4, 1.3 ± 0.4, 1.2 ± 0.4, and 1.5 ± 0.4 min, respectively (adj. $p>0.05$) (Figure 7). 8 weeks post-inoculation, the average *D*-values of Enteritidis, Montevideo, Tennessee, and Agona were 1.1 ± 0.5, 1.1 ± 0.4, 0.7 ± 0.3, and 0.9 ± 0.4 min, respectively, which were not significantly different among serovars (adj. $p>0.05$). The average $D_{71°C}$ of Enteritidis, Montevideo, Tennessee, and Agona at 16 weeks post-inoculation were 1.2 ± 0.6, 1.3 ± 0.4, 0.9 ± 0.4, and 1.1 ± 0.4 min, respectively, which were not significantly different among serovars (adj. $p>0.05$). The average $D_{71°C}$ of Enteritidis, Montevideo, Tennessee, and Agona at 24 weeks were 1.0 ± 0.2, 1.4 ± 0.6, 1.5 ± 0.6, and 1.8 ± 0.9 min respectively. The $D_{71°C}$ for serovar Agona were significantly lower than those for serovar Enteritidis at 24 weeks post-inoculation (adj. $p<0.01$), indicating a greater thermal resistance.

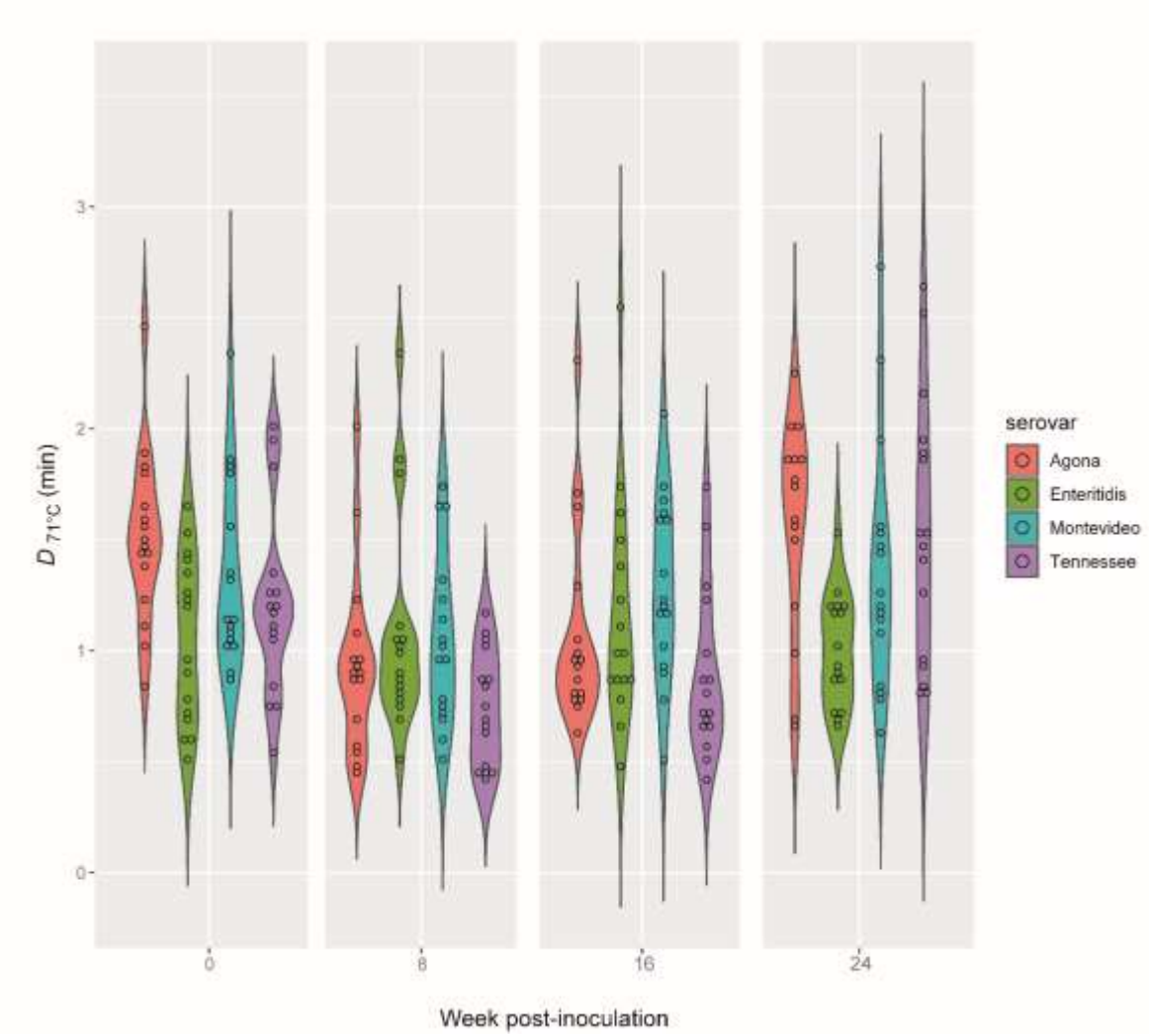

Figure 7. Average $D_{71°C}$ of 4 *Salmonella* serovars on flaxseed over 24 weeks storage. Boxes represent the distribution of $D_{71°C}$ of eight strains in serovars from two replicates at 0, 8, 16, and 24 weeks post-inoculation.

3.4.4. Thermal resistance increased over time of storage

To determine the effect of length of storage on subsequent thermal treatment, we compared the maximum inactivation rates of four serovars at four different time points by two-way ANOVA. The duration of storage under low moisture conditions significantly impacted thermal resistance in a serovar-dependent manner (serovar*storage time $p<0.05$). Although the overall thermal resistance decreased 8 weeks post-inoculation, it increased at 24 weeks for most

serovars. The average $D_{71°C}$ for Enteritidis were 1 ± 0.4, 1.1 ± 0.5, 1.2 ± 0.6, and 1.0 ± 0.2 min at 0, 8, 16, and 24 weeks post-inoculation, respectively (Figure 7). The average thermal resistance of serovar Enteritidis was constant over 24 weeks storage, with no significant differences over time (Figure 4). Average $D_{71°C}$ for serovar Montevideo were 1.3 ± 0.4, 1.1 ± 0.4, 1.3 ± 0.4, and 1.4 ± 0.6 min at 0, 8, 16, and 24 weeks post-inoculation, respectively, and did not change significantly over time (Figure 4). While the average $D_{71°C}$ were lower at 8 weeks post-inoculation, this difference was not significant. Average $D_{71°C}$ for serovar Tennessee were 1.2 ± 0.4, 0.7 ± 0.3, 0.9 ± 0.4, and 1.5 ± 0.6 min at 0, 8, 16, and 24 weeks post-inoculation, respectively. Thermal resistance of serovar Tennessee significantly increased 24 weeks post-inoculation compared to 8 weeks and 16 weeks post-inoculation (adj. $p<0.05$). The average $D_{71°C}$ for serovar Agona were 1.5 ± 0.4, 0.9 ± 0.4, 1.1 ± 0.4, and 1.8 ± 0.9 min at 0, 8, 16, and 24 weeks post-inoculation, respectively. Thermal resistance of serovar Agona significantly decreased from 1.5±0.4 to 0.9±0.4 min 8 weeks post-inoculation (adj. $p<0.05$). Similar to serovar Tennessee, thermal resistance of serovar Agona increased over time and average $D_{71°C}$ at 24 weeks post-inoculation was significantly higher than $D_{71°C}$ at 8 and 16 weeks post-inoculation (adj. $p<0.05$).

3.5. Discussion

Studies have shown initial population decline of *Salmonella* during the first few weeks of storage on dry foods, followed by long-term persistence with slow, or no, decline over time (Abd *et al.*, 2012; Beuchat & Heaton, 1975; Beuchat & Mann, 2010; Blessington *et al.*, 2012; Blessington *et al.*, 2013; Burnett *et al.*, 2000; Farakos *et al.*, 2017; Limcharoenchat *et al.*, 2019). Here, after 24 weeks on flaxseeds at 22 ± 1°C, we observed average reductions of 3.7 ± 0.7, 3.7 ± 0.7, 3.5 ± 0.6, and 3.7 ± 0.7 $\log_{10}$ CFU/g for serovars Enteritidis, Montevideo, Tennessee, and

Agona, respectively. In many studies, cocktails of multiple *Salmonella* strains have been used to evaluate survival over time on LMFs. For example, a cocktail of five *Salmonella* strains from five different serovars on pecan halves decreased by 2.1 $\log_{10}$ CFU/g after 52 weeks at 21°C (Beuchat & Mann, 2010) and cocktail of five *Salmonella* strains on walnut kernels decreased by 2.3 $\log_{10}$ CFU/g after 52 weeks at room temperature (Blessington *et al.*, 2013). Additionally, a cocktail of five *Salmonella* strains on flour declined about 4 $\log_{10}$ CFU/g over one year storage at 23°C (Forghani *et al.*, 2019). Other studies have focused on evaluating survival characteristics of *Salmonella* on LMFs using *S.* Enteriditis PT 30 from the 2001 outbreak linked to almonds (Isaacs *et al.*, 2005). For example, *S.* Enteriditis PT30 reductions of 2.1 $\log_{10}$ CFU/g have been reported on almonds after 48 weeks of storage at 23°C (Uesugi *et al.*, 2006). *S.* Enteriditis PT30 at 23°C on almond decreased by 1.8 $\log_{10}$ CFU/g after 24 weeks (Abd *et al.*, 2012). Another study showed 2.3 $\log_{10}$ CFU/g and 1.2 $\log_{10}$ CFU/g reductions of *S.* Enteriditis PT30 after 68 and 70 weeks respectively on almonds stored at room temperature (Limcharoenchat *et al.*, 2019). We used multiple strains from four different serovars in this study, and included *S.* Enteritidis PT 30 (ATCC BAA-1045) in the set of strains for serovar Enteritidis to allow for direct comparisons to other published data. We found that *S.* Enteritidis PT30 (ATCC BAA-1045) had 3.2 ± 0.2 $\log_{10}$ CFU/g reduction after 24 weeks storage at 22±1°C on flaxseed. We observed greater reduction of *S.* Enteritidis PT30 (ATCC BAA-1045) on flaxseed over time compared to the data from almonds in the studies described above.

Salmonella survival kinetics can be affected by factors such as a_w, temperature, and food composition (Beuchat *et al.*, 2013; Podolak *et al.*, 2010). A_w is one of the most significant factors affecting survival of *Salmonella* (Gradl *et al.*, 2015; Keller *et al.*, 2013; Zhang *et al.*, 2017). As an example, by increasing a_w from ~0.5 to ~ 0.8, *Salmonella* cells decreased by 6 to 7 $\log_{10}$

CFU/g after 24 to 88 days of storage at 25°C. However, by decreasing a_w from ~0.5 to ~ 0.2, *Salmonella* cells decreased 2-3 $\log_{10}$ CFU/g after 88-280 days (Gradl *et al.*, 2015; Keller *et al.*, 2013; Zhang *et al.*, 2017). *S.* Montevideo and *S.* Typhimurium had greater survival at a_w = 0.18 than at a_w = 0.54 on whey protein stored at 35°C for 24 weeks (Santillana Farakos *et al.*, 2014). In our study, after a_w equilibration, inoculated flaxseeds were stored in individual vacuum sealed Mylar bags to minimize changes in a_w. Other survival studies have not reported a_w over the time of storage (Abd *et al.*, 2012; Uesugi *et al.*, 2006), which could impact survival rates if a_w changes over time. The natural chemical composition of the dry product could also impact the degree of *Salmonella* survival at low a_w. For example, S*almonella* survived for long periods of time in peanut oil (96 ± 8 days) and on chia seeds (94 ± 46 days) compared to significantly shorter survival times on the surface of peanut shells (42 ± 49 h) (Fong & Wang, 2016b). As the majority of studies have examined *Salmonella* long-term survival on almonds, further studies on other LMFs are required to fully understand the extent of LMF physicochemical properties on *Salmonella* survival.

As the number of bacteria decrease over time of storage, it may be the decreasing cell density that could have an impact on thermal resistance of *Salmonella*, rather than storage at low a_w for lengthy periods. To answer this question, we inoculated our flaxseed at two inoculation density levels (8 and 6 $\log_{10}$ CFU/g) and applied VSP. For the majority of strains, the inoculum density did not impact thermal resistance. Similar results were reported by Hilderbrandt *et al.* when they inoculated flour wheat at 7.75, 6.51, 4.59, and 2.78 $\log_{10}$ CFU/g levels and reported the thermal inactivation rate was not significantly impacted by the initial inoculation level (Hildebrandt *et al.*, 2016). In another study, thermal resistance of *Salmonella* in inoculated

peanut butter at 8 $\log_{10}$ CFU/g and 4 $\log_{10}$ CFU/g were not significantly different (He *et al.*, 2011).

Long-term persistence with slow, or no, decline over time means that *Salmonella* is able to survive in low a_w environments, which requires an essential adaptation at the cellular level (Deng *et al.*, 2012; Gruzdev *et al.*, 2012b; Li *et al.*, 2012). Encountering an environmental stress can lead to initial sub-lethal injury, which can enhance the resistance to a subsequently experienced stress, i.e. cross-protection (Gruzdev *et al.*, 2011). Studies on non-food materials have shown that reduction of a_w leads to increased thermal resistance of *Salmonella* strains (Goepfert *et al.*, 1970; Gruzdev *et al.*, 2011; Pena-Melendez *et al.*, 2014). As thermal treatments are common control measures for *Salmonella* on LMFs, there is a potential for cross-protection to thermal treatment due to low a_w exposure on LMFs. Studies demonstrating increased *Salmonella* thermal resistance on LMFs as a_w decreases have not considered the effect of long term association of *Salmonella* with the LMF (Archer *et al.*, 1998; VanCauwenberge *et al.*, 1981). Data on the impact of storage on actual LMFs on thermal resistance of *Salmonella* is limited. Six-day exposure to peanut oil (a_w ~0.52) led to increased thermal resistance of *Salmonella* at 70°C (Fong & Wang, 2016a). However, 48 weeks exposure of *Salmonella* on almonds did not increase thermal resistance of *Salmonella* when inoculated almonds were oil roasted at 121°C (Abd *et al.*, 2012). In these cases, a_w changes during storage time, an important factor for thermal resistance, were not evaluated. Limcharoenchat *et al*, stored inoculated almonds for 68 weeks, keeping a_w constant during storage, and did not find significant changes in *Salmonella* PT30 thermal resistance at 80°C (Limcharoenchat *et al.*, 2019).

Here we observed that thermal resistance of *Salmonella* serovars changed over time of storage, in a serovar dependent manner. Compared to thermal resistance at T_0, thermal resistance

of most serovars decreased 8 weeks post-inoculation, this reduction was significant for strains of serovar Agona (adj. $p<0.05$). After 8 weeks storage, *D*-values increased during the next 16 weeks. For Tennessee and Agona, this increase was significant (adj. $p<0.05$). Similar to the findings of Limcharoenchat *et al.*, 2019, we found that the thermal resistance of strains of serovar Enteritidis and Montevideo was constant over 24 weeks of storage. In contrast, the thermal resistance of serovars Agona and Tennessee changed over time of storage.

Changes in thermal resistance after exposure to low a_w vary among *Salmonella* serovars and strains. Goepfert et al. reported *S.* Infantis and *S.* Tennessee were more sensitive than *S.* Alachua, *S.* Anatum, *S.* Montevideo, and *S.* Senftenberg when encountering 57.2±0.1°C after exposure to low a_w in laboratory medium with sucrose (Goepfert *et al.*, 1970). Similarly, Mattick et al. found serovar Senftenberg 775W, Java, and Agona were the least heat-tolerant isolates when tested over a range of a_w from 0.65-0.9 (Mattick *et al.*, 2001). Studies that have investigated variation in phenotypic response among *Salmonella* serovars to heat stress in artificially inoculated foods are rare. VanCauwenberge *et al*. in 1981 reported that among eight *Salmonella* serovars, *S.* Thompson and *S.* Tennessee were more resistant to heat inactivation (49 °C) than the other serovars on dry corn flour (10-15% moisture content). Not all studies have shown increased thermal resistance variation among *Salmonella* serovars when they encounter low a_w condition. For example, Agona, Enteritidis, and Typhimurium were not significantly different in terms of thermal resistance when heat treated at 90°C in peanut butter. (Shachar & Yaron, 2006). Here we also found that at the initial time point there were no significant differences in thermal resistance among serovars, but the average thermal resistance of serovar Agona was significantly higher than that of serovar Enteritidis after 24 weeks of storage on flaxseed.

3.6. Conclusion

Data presented here demonstrate that there is heterogeneity in the phenotypic response of *Salmonella* serovars to survival on a LMF at a_w ~0.5 and subsequent thermal treatment. Changes in thermal resistance over storage time for some of the serovars suggests that these strains may be able to adapt to low a_w conditions over time, leading to increased *D*-values during thermal treatment. Mechanistic studies are needed to further explore the potential for adaptation over time under low a_w.

3.7. Acknowledgements

This work was supported by USDA-NIFA-2016-69003-24851 and by a NDSU EPSCoR award funded through the State of North Dakota and the North Dakota University System. We thank the Food Safety Lab at Cornell University for providing the strains used in this study.

4. MOLECULAR FACTORS IMPACTING SURVIVAL OF *SALMONELLA* ON A LOW MOISTURE FOOD

4.1. Abstract

Salmonella can survive for long periods of time on LMFs. Although the long-term survival of *Salmonella* in LMFs is an important issue, the underlying survival mechanisms that allow *Salmonella* to do so remain poorly understood. It is assumed that desiccation is the most probable stress on LMFs. Desiccation resistance mechanisms are based on osmotic pressure in liquid media with high levels of solutes. ProU is a transport system which plays a key role to transfer proline and glycine-betaine which are known as "osmoprotectant" to restore turgor pressure across the cell membrane. Although MgtC is required for survival of *Salmonella* inside macrophages and for growth in low Mg^{2+} media, upregulation of *mgtC* has been reported under desiccation and osmotic stress. To test the impact of these two genes on *Salmonella* survival on LMFs, deletion mutants of *proU* and *mgtC* were created by modified one-step gene inactivation method in *S.* Enteriditis FSL S10-1646. Measuring the maximum rate of reduction (*kmax*) of Δ*proU*, Δ*mgtC* and wild type on flaxseed over 7 weeks showed that deleting *proU* did not change the *kmax* of *S.* Enteriditis FSL S10-1646. However, deleting *mgtC* significantly decreased the *kmax* of *S.* Enteriditis FSL S10-1646 (adj. $p<0.05$). Here, just two possible genes were examined. As *Salmonella* can use complex mechanisms to combat low a_w condition, more studies should be done to find a clear picture of *Salmonella* long-term survival on LMFs.

4.2. Introduction

In the industry, LMFs can be stored for a year or more before applying control measures. For example, almonds have been stored for up to 6 weeks before sale and consumers may store LMFs for a year before consumption (Danyluk et al., 2006). Controlling *Salmonella* on LMFs is

challenging because *Salmonella* can survive for long periods of time in these type of foods (Beuchat et al., 2013). As an illustration, *Salmonella* can survive up to 550 days (Uesugi et al., 2006) and even 2 years (Limcharoenchat et al., 2019) in low a_w foods.

Although the long-term survival of *Salmonella* on LMFs is an important issue, the underlying survival mechanisms that allow *Salmonella* to do so remain poorly understood. Desiccation is a form of osmotic stress that is the most probable stress in low a_w foods that *Salmonella* encounters during storage. Most of what is known about osmotic stress is based on studies with high levels of solutes in liquid medium, rather than under desiccation.

Salmonella can generate turgor pressure by keeping the concentration of solutes in the cytoplasm higher than that found externally (Csonka & Hanson, 1991). A sudden increase in the osmolarity of the environment must be compensated for by an increase in the intracellular osmolarity. This adaptation is essentially a two-step process. The first strategy is up taking potassium by the Trk and Kdp transport systems (Spector & Kenyon, 2012). Subsequently, the concentration of osmoprotectants such as glycine-betaine, trehalose (Spector & Kenyon, 2012), proline, or proline-betaine (Burgess et al., 2016) increases either by synthesis or uptake from the environment, followed by an efflux of K^+-glutamate (Csonka, 1989).

Accumulation of osmoprotectants such as proline and betaine can help to restore turgor pressure across the cell membrane and protect enzymes from inactivation at high ionic strength (Le Rudulier et al., 1984). There are three known proline transporters; namely PutP, ProP, and ProU in *Salmonella*. In 1982, ProU as a third proline permease after PutP and ProP was recognized by creating Tn10-induced mutation in a gene (*proU*) (Csonka, 1982). Also, Jovanovich et al., studied the expression of ProU by using chromosomal operon fusions and found that within 10 min after the addition of 0.3 M NaCl to the culture medium, expression of

proU-lac, *S.* Typhimurium increased by 180-fold, which was 27-fold higher than that of the control (Jovanovich et al., 1988).

Many transcriptomic studies suggest that the ProU transport system contributes to the management of osmotic stress. For example, exposure of *S.* Typhimurium to 0.3 M NaCl caused *proU* expression to be induced after 4 min (Balaji et al., 2005). Similarly, upregulation of *proU* was observed in *S.* Typhimurium when exposed to NaCl and KCl (Finn et al., 2015). Also, transcriptomic analysis of desiccated. *S.* Typhimurium cells on aged broiler litter showed the upregulation of *proV* (Chen & Jiang, 2017). Li et al., reported that *proV* in *S.* enterica serovar Tennessee and *S.* Typhimurium LT2 were significantly induced by 12.5- and 14.9-fold under desiccation stress (Li *et al.*, 2012). *proU* was among the most highly upregulated genes following a 4 h desiccation on a stainless-steel coupon, where the contribution of ProU was confirmed by comparing the survival of wild type and Δ*proU* (Finn *et al.*, 2013b).

Expression of virulence-related genes under adverse conditions has been reported (Fang *et al.*, 2016; Johnson *et al.*, 1991). The MgtC required for survival of *Salmonella* inside macrophages and for growth in low Mg^{2+} media (Blanc-Potard & Groisman, 1997). In *Salmonella*, *mgtC* heads the *mgtCBR* operon, which specifies the inner membrane protein MgtC, This operon is codified in all *Salmonella* serovars in a very conserved SPI-3 region *(Amavisit et al.*, 2003). Transcriptiom from the *mgtCBR* operon is controlled by regulatory system PhoP/PhoQ (Groisman, 2001; Soncini *et al.*, 1996). Upregulation of *mgtC* (2.5 fold) reported under low a_w conditions (a_w 0.1) (Maserati et al., 2017). However, there are no additional studies available that examine the survival of Δ*mgtC* under desiccation stress.

It is assumed that mechanism of survival under low moisture conditions including LMFs is similar to osmotic stress (Finn et al., 2013a). Transcriptomic study of *Salmonella* on LMFs can

provide data on which genes are activated under these conditions. The only *Salmonella* transcriptomic study in LMF is in peanut butter oil ($a_w \sim 0.3$). Transcriptomic analysis of *Salmonella* cells in inoculated peanut oil which was stored for 72, 216, and 528 h suggested that non-coding RNAs play roles in *Salmonella* adaptation to desiccation stress (Deng et al., 2012). It is important to note that transcriptomic studies only provide an assessment of what genes are differentially expressed. Further studies are needed to confirm the role of non-coding RNAs to combat desiccation stress.

We were interested to know if *Salmonella* utilizes similar mechanisms to combat osmotic stress when present on LMFs. There is only one study that confirmed its result of transcriptomic study of desiccated cells by creating mutants of those detected genes and examining the survival of mutants under desiccation stress (Finn et al., 2013b). No studies here examine the survival of mutant *Salmonella* strains in actual LMFs. As ProU transport system is reported as an important system to combat osmotic/desiccation stress, and upregulation of *mgtC* reported recently under desiccation stress, we were interested to examine the importance of ProU system and *mgtC* on survival of *Salmonella* on flaxseed over time of storage.

4.3. Material and methods

4.3.1. Bacterial strains and growth condition

Bacterial strains and plasmids used in this study are summarized in Table 6. All strains were stored at -80°C in LB broth containing 15% glycerol.

Table 6. Strains and plasmids used in this study

Strain or plasmid	Reference
pKM208	(Datsenko & Wanner, 2000)
pKD3	(Datsenko & Wanner, 2000)
FSL S10-1646	
Δ*proVXW*	This study
Δ*mgtC*	This study

4.3.2. Mutant construction

S. Enteritidis FSL S10-1646 Δ*proVXW* and Δ*mgtC* strains were constructed by the modified one-step gene inactivation method for Enterohemorrhagic *E.coli* (EHEC) developed by Datsenko and Wanner and by Murphy et al (Datsenko & Wanner, 2000; Murphy & Campellone, 2003). Briefly, pKM208 plasmid contain ampicillin resistant gene extracted from DH5α was electroporated into *S.* Enteritidis FSL S10-1646.

As we found that the death rate of Enteritidis was initially significantly higher than that of Agona on flaxseed over 24 weeks storage, we decided to create a mutant in each serovar. *S.* Agona FSL M8-0485 and *S.* Enteritidis FSL S10-1623 were selected. We confirmed they were not ampicillin resistant. pKM208 was electroporated into both strains. Next pKD4 which has a kanamycin resistance marker, but we found that both strains became kanamycin resistance after transferring pKM208. We then screened all 32 strains for ampicillin and kanamycin resistance (Table 7). Because the majority of strains were kanamycin resistant, we selected to use pkD3 with chloramphenicol resistance marker. We selected FSL S10-1646, which was not chloramphenicol and ampicillin resistant (Table 7).

Recombinant PCR products containing a chloramphenicol resistance marker flanked by 38-bp sequences homologous to the upstream and downstream regions of the target genes were

generated from plasmid pKD3 (Datsenko & Wanner, 2000) by use of the primers listed in Table 8. The touchdown PCR condition included the following: (i) one cycle at 98°C for 30 s (ii) 20 cycles of 98°C for 10 s, annealing (for 30 s) at 55°C, decreasing to 50°C (at 0.5°C decrease per cycle), and 72°C for 2 min; (iii) another 20 cycles of 94°C for 10 s, 50°C for 30 s, and 72°C for 2 min; and (iv) a final cycle of 72°C for 2 min. As the melting temperature of *mgtC* H1P1 and *mgtC* H2P2 primers were lower than that for *proVXW*, annealing temperature of second 20 cycles for mgtC was set on 45°C for 30 s. PCR products were purified using a QIAquick DNA clean up kit (Qiagen, Valencia, CA). Purified PCR products were electroporated into Red recombinase-producing *S.* Enteritidis FSL S10-1646 containing pKM208 as described previously (Murphy & Campellone, 2003), and the transformants were identified on LB agar plates with 25 µg/ml Cl at 37°C. In-frame deletion was verified by sequencing the region amplified from primers in Table 8 and C2 primer from Datsenko and Wanner study (Datsenko & Wanner, 2000).

Table 7. List of strains that were screened for antibiotic resistance

Strain	Ampicillin (100mg/ml)	Chloramphenicol (20mg/ml)	Kanamycin (20mg/ml)
FSL R8-0288	S	S	R
FSL S10-1621	S	S	R
FSL S10-1623	S	S	R
FSL S10-1644	S	S	R
FSL S10-1646	S	S	S
FSL F6-0963	R	R	R
ATCC BAA-1045	S	S	R
FSL R9-4060	S	S	R
FSL R8-2812	S	S	R
FSL R8-3417	S	S	R
FSL R8-3658	S	S	R
FSL R8-3659	S	S	R
FSL R8-4923	S	S	R

Table 7. List of strains that were screened for antibiotic resistance (continued)

Strain	Ampicillin (100mg/ml)	Chloramphenicol (20mg/ml)	Kanamycin (20mg/ml)
FSL R9-1588	S	S	R
FSL_R8-3881	S	S	R
FSL R8-3706	S	S	R
FSL R8-2240	S	R	R
FSL R9-2434	S	S	R
FSL R9-2435	S	S	R
FSL R9-2436	S	S	R
FSL S10-1757	S	R	R
FSL R6-0198	S	S	R
FSL R6-0494	S	R	R
FSL R8-5221	S	S	R
FSL R8-8615	R	R	R
FSL R8-8619	R	R	R
FSL S10-1750	S	S	R
FSL S10-1759	S	S	R
FSL S10-1760	S	S	R
FSL S10-1761	S	S	R
FSL M8-0485	S	S	R
FSL S9-0322	R	R	R

R: Resistant. S: Sensitive

Table 8. Designed primers for *mgtC* and *proVXW* deletion

Primer	Purpose	Sequence (5'→3')
mgtC XF	*mgtC* deletion verification	GTGAATGCCCCGGGAGAA
proXVW XR	*proXVW* deletion	GATGGATCAAGCAGCGGC
mgtC H1P1	*mgtC* deletion	TCGTCCATGAATAAGAAGGTCTTTTTTACCTCCTTGCAT ACAATTACAAAGTGTAGGCTGGAGCTGCTTC
mgtC H2P2	*mgtC* deletion	TATCAGTTATTATCAAAAAATAAGTTAACGTTGTAATGC GGTCCGCATATATGGGAATTAGCCATGGTCC
proXVW H1P1	*proXVW* deletion	ATATCGACATAAGTAAATAACAGGAATCATTCTATTGC ATGGCAATTAAAGTGTAGGCTGGAGCTGCTT
proXVW H2P2	*proXVW* deletion	GCGCATGAACTGCGCTTTATCCGGCATAAAAAACGATC TTATTTCTGCGCATGGGAATTAGCCATGGTCC

4.3.3. Inoculation of flaxseed

The inoculation protocol previously described by Shah et al. (Shah et al., 2017) was used with minor modifications. Briefly, bacterial freezer stocks were streaked on BHI agar plates for isolation and grown overnight at 37°C. For each strain, a colony was transferred to 5 ml BHI broth and incubated at 37°C for 20 h. Overnight broth culture (250 µl) was plated uniformly onto BHI agar plates (100 mm × 15 mm) using a sterile spreader (Fisher Scientific Inc, Waltham, MA), and plates were incubated at 37°C for 24 h. Inoculation of flaxseed was conducted in a bio-safety cabinet. About 600 g seeds were added to whirl pack bags (Nasco Inc, Fort Atkinson, WI). To achieve 8 log CFU/g, the bacterial lawns from 16 plates were collected with a sterile spreader and mixed into a sterile beaker containing 2.5 ml sterile water. The bacterial suspension was poured into the flaxseed in a whirl pack bag and mixed by hand for 3-5 min to obtain homogenous distribution of bacteria. Following the same inoculation protocol, flaxseeds were inoculated separately with the 3 strains (*S.* Enteritidis FSL S10-1646, Δ*proVXW* FSL S10-1646, Δ*mgtC* FSL S10-1646). Three biological replicates were conducted for this test.

4.3.4. Water activity (a_w) equilibration and storage of inoculated flaxseed

The The a_w of flaxseed prior to inoculation was measured using an Aqualab 4TE a_w meter (Aqualab Inc, Pullman, WA). After inoculation, a_w was measured to estimate the necessary amount of lithium chloride anhydrous 99% -20 Mesh (Alfa Aesar Inc, Ward Hill, MA) to equilibrate a_w to the original level. The inoculated flaxseeds were transferred to a sterile stainless-steel tray (12″ × 9″). The stainless-steel tray was placed in a closed chamber (Coleman cooler 24″ × 16″, Coleman Company, Inc., Kingfisher, OK). 15–30 g of lithium chloride anhydrous 99% -20 Mesh was weighed in plastic trays (Fisher Scientific Inc, Hampton, NH) and saturated with water. Trays of saturated LiCl were placed adjacent to the stainless steel trays in

the closed chamber to reduce the a_w. The a_w of inoculated flaxseed was equilibrated to the initial a_w of flaxseed within 48 h. After a_w equilibration, inoculated seeds were divided in 16 Whirl-Pak bags and to maintain constant a_w during storage, bags were vacuum sealed in individual Mylar bags. All Mylar bags were stored at 22 ± 1°C. One bag was opened per sampling time point. Samples were taken from bags and *Salmonella* were enumerated at 0, 1, 2, 3, 4, 5, 6, and 7 weeks. For the thermal resistance experiment, vacuum steam pasteurization was conducted on samples at 0, 1, 2, 3, 4, 5, 6, and 7 weeks post-inoculation.

4.3.5. Survival on flaxseed over time

Over 7 weeks storage, two whirl pack bags, each containing 25 g inoculated flaxseed were picked from stored Mylar bags and the number of bacteria was enumerated after 1, 2, 3, 4, 5, 6, and 7 weeks. The samples were weighed in a sterile plastic bag and Butterfield dilution buffer was added in appropriate amounts. These bags were homogenized by masticator for 90 s and appropriate serial dilutions were spread plated in duplicate on modified TSAYE supplemented with ferric ammonium citrate and sodium thiosulfate. The respective agar plates were incubated at 37±2°C for 24 h. Following incubation, the colonies were counted using a Q-Count.

4.3.6. Survival modelling and statistical analysis

Survival parameters were estimated using GInaFiT Version 1.7 in Microsoft excel (Geeraerd et al., 2005). The most appropriate survival model was determined to be a Geeraerd-tail model. Survival parameters of the Geeraerd-tail model were estimated using the following equation (Geeraerd et al., 2000):

$$N = (N_0 - N_{res}) * e^{(-kmax*t)} + N_{res}$$

Where N is the bacterial population (CFU/g) considering time t (min), N_0 is the bacterial population (CFU/g) prior to treatment, N_{res} is the observed residual population considered to be resistant to the treatment, and k_{max} ($\log_{10}$ CFU/g/min) is the maximum rate of inactivation.

For each bacterial strain, two samples for survival study were used to conduct the experiments in three replicates providing 8 counts at each time point for each strain. The duplicate counts obtained for each sample in CFU/g were averaged and log transformed. One-way Analysis of Variance (one-way ANOVA) was conducted using Minitab 17 (Pennsylvania State University, State College, PA). For one-way ANOVA, K_{max} was considered as the response variable whereas the strains were used as the independent variables. Based on least squares means adjusted for significant interactions, the Tukey adjusted at p value < 0.05 were significantly different.

4.4. Results

4.4.1. Inactivation of *mgtC* in FSL S10-1646 strain affects survival on flaxseed

The average initial number of *S.* Enteritidis FSL S10-1646, Δ*proVXW* FSL S10-1646, and Δ*mgtC* FSL S10-1646 was 8.3±0.1, 8.4±0.2, and 8.2±0.1 $\log_{10}$ CFU/g, respectively. Non-linear reduction of *Salmonella* cells was observed over the 7-week storage period. After 7 weeks, the average counts of wild type, Δ*proVXW*, and Δ*mgtC* strains was 7±0.1, 6.9±0.1, and 6.9±0.1 $\log_{10}$ CFU/g, respectively, showing a reduction of 1.3 ± 0.2, 1.5±0.2, and 1.3± 0.2 $\log_{10}$ CFU/g, respectively from week 0. The maximum rate of reduction of wild type, Δ*proVXW*, and Δ*mgtC* strains was 0.61 ± 0.2, 0.5 ± 0.1, and 1.1 ± 0.2 $\log_{10}$ CFU/g/week respectively (Figure 8). The maximum rate of reduction varied among strains, Δ*mgtC* had a significantly higher average k_{max} compared to wild type and Δ*proVXW* (adj. p<0.05) (Figure 9).

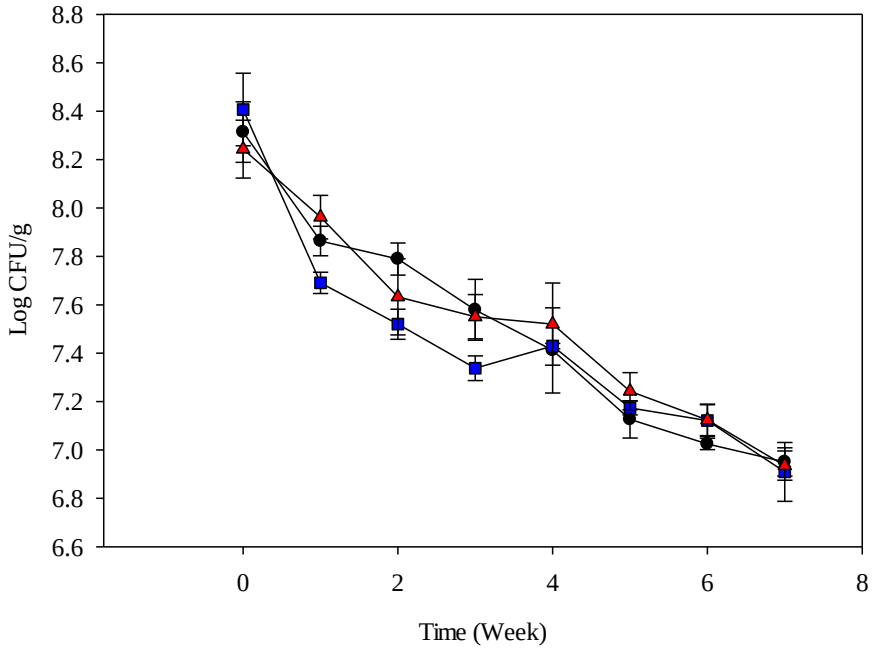

Figure 8. Reduction curve of *S.* Enteritidis FSL S10-1646 (black circle), *S.* Enteritidis FSL S10 1646 ΔproVXW (red triangle), *S.* Enteritidis FSL S10-1646ΔmgtC (blue square) over 7 weeks of storage on flaxseed at 22°C.

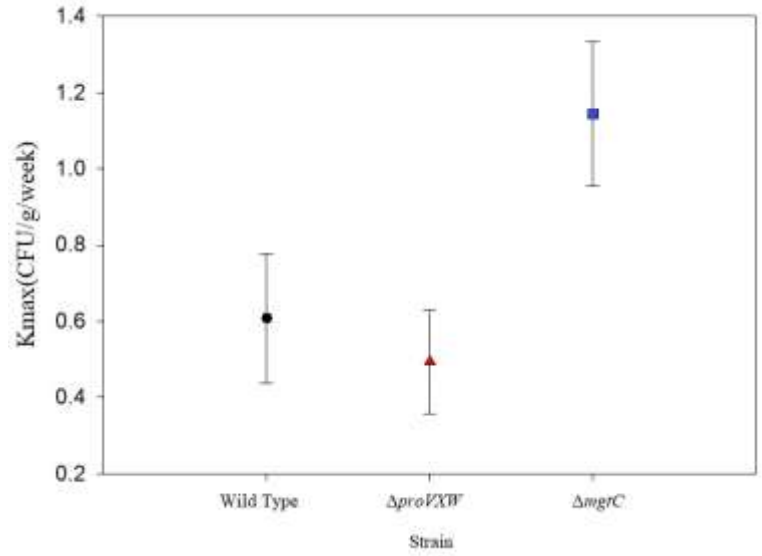

Figure 9. Average kmax of strain FSL S10-1646 (black circle), FSL S10-1646 ΔproVXW (red triangle), FSL S10-1646 ΔmgtC (blue square) after 7 weeks of storage on flaxseed at 22°C.

4.5. Discussion

ProU is a transport system that *Salmonella* can use to transport osmoprotectants. This system belongs to the ATP-binding cassette (ABC) superfamily of transporters. All ATP-type uptake systems are made up of three functional units: ATPases that supply the energy for the transport, integral membrane proteins that form pores, and extra cytoplasmic substrate-binding components that the substrate to the pore proteins (Biemans-Oldehinkel et al., 2006). For ProU in *Salmonella*, the ATPases, pore, and substrate binding protein are ProV, ProX, and ProW, respectively. ProU has a high affinity for glycine betaine that is essential for cell survival in media of high osmolarity (Cairney et al., 1985) but it can uptake proline in media of elevated osmolarity (Csonka, 1982; Dunlap & Csonka, 1985).

Transcriptomic analysis of desiccated cells suggested the upregulation of *proV* which is part of ProU system. For example, Li et al. observed the transcriptomic responses of *Salmonella* Tennessee K4643 and *S.* Typhimurium LT2 after 2 h of air-drying on a sterile filter paper disc at 11% RH. They found that the *proU* in *S. enterica* serovar Tennessee and *S.* Typhimurium LT2 were significantly induced by 12.5- and 14.9-fold, respectively (Li et al., 2012). They did not confirm the role of *proU* under desiccation stress.

However, our study shows that deleting the whole ProU system may decrease survival of *Salmonella*, but this reduction is not significantly different from the wild type strain. It is important to note that desiccation leads to activation of several stress response mechanisms in *Salmonella*. For example, after a 22 h dehydration on a plastic surface, 90 genes were upregulated is *Salmonella* cells. Among those, genes namely; *kdpFABC, aceA, rpoE, ddg, nifU, kdpA, hisABCDGH, glpK, ydaA,* and *fnr* were highly induced (>5 fold) (Gruzdev *et al.*, 2012a). Air-dried *S.* Typhimurium ST4/74 on a stainless-steel surface at 45% RH for 4 h showed an

upregulation of 79 genes. The upregulated genes include: genes involved in up-taking osmoprotectants [*proP*, *proU* , and *otsAB*], genes encoding OsmU ABC transporter system, global regulator *rpoE,* and genes encoding sigma E-regulatory proteins (*rseA* and *rseB*), which regulate *rpoE,* were highly upregulated (>5 fold) (Finn et al., 2013b). Maserati et al., reported 290 genes which are involved in metabolic pathways, transporter regulation, DNA replication/repair, transcription and translation, and, more importantly, virulence genes were up-regulated at a_w 0.11 (Maserati et al., 2017). So, deleting one operon of a compatible solute transporter may not be enough to see a significant difference in *Salmonella* survival.

Finn et al. analyzed transcriptome of air-dried *S.* Typhimurium ST4/74 on a stainless-steel surface at 45% RH for 4 h. They reported that the *proU* was among the most highly upregulated genes following a 4 h desiccation on a stainless-steel coupon. Examining the survival of wild type and Δ*proU* strains on stainless-steel coupons over 6 weeks showed the significant decrease in viability of Δ*proU* strain (Finn et al., 2013b). Also, they reported a fast average reduction of 4 and 5 $\log_{10}$ CFU/ml in wild type and Δ*proU* respectively one-week post inoculation, however, after one-week, wild type and Δ*proU* had a lower reduction of average 0.5 ± 0.1 and 0.3±0.1 $\log_{10}$ CFU/g reduction on flaxseed. Finn et al. reported about 6.5 and 7 $\log_{10}$ CFU/ml reduction wild type and Δ*proU* cells respectively in 6-week post inoculation. However, after 6 weeks 1.2 ±0.1 $\log_{10}$ CFU/ml reduction observed for both strains on flaxseed. Desiccated *Salmonella* cells on stainless-steel coupons may not expose to the similar conditions as those on LMFs. LMFs contain nutrients which may impact on their mechanisms of survival. Also, impact of the shape of LMFs on protecting bacteria has not been studied. We need more studies on LMFs to find the actual mechanisms that *Salmonella* use to combat low moisture condition on LMFs.

On the other hand, there is a hypothesis of time-line differences among induced operons and genes involved in short term and long-term adaptation to osmotic stress. *rpoS*, *proV*, and *proP* were induced earliest among the other genes (Balaji et al., 2005). However, several studies reported induction of the *kdp* operon is an early response to an osmotic shift and *proU* operon, are secondary consequences of accumulation of K^+ (Burg et al., 1996; Lucht & Bremer, 1994). Examining more than 7 weeks may give us better information about the long term impact of deletion of the proU system.

A global transcriptomic analysis comparing *S*. Typhimurium cells equilibrated to low a_w (a_w 0.11) and cells equilibrated to high water activity (a_w 1.0) showed the upregulation of *mgtC* (2.5 fold) (Maserati et al., 2017). Moreover, when *Salmonella* encountered 0.3 M NaCl, the mRNA corresponding to the *mgtC* coding regions increased five-fold. It is hypothesized that hyperosmotic stress promoting transcription of the *mgtCBR* coding region by decreasing the amount of cytosolic proline (Lee & Groisman, 2012). Although our result confirms the importance of *mgtC* for *Salmonella* survival under desiccation stress, *Salmonella* can survive even without *mgtC*, which again support this fact that complex mechanisms have been used by *Salmonella* to combat desiccation stress.

4.6. Conclusion

Data presented here demonstrate that deleting *proVWX* did not change the survival of *Salmonella* on flaxseed over 7 weeks of storage. I hypothesized that *Salmonella* might use other transporters such as ProP and PutP to transfer compatible solutes to combat low moisture condition on LMFs. Moreover, as we do not know the actual mechanisms, there is a possibility of using the other systems such as synthesizing osmoprotectants inside the cell instead of transferring them from outside. Also, we confirmed that *mgtC* probably plays a role to combat

low a_w conditions as death rate of Δ*mgtC* was higher than wild type. *Salmonella* can use complex mechanisms to combat low a_w condition. More study should be done to find a clear picture of *Salmonella* long-term survival on LMFs.

5. OVERALL CONCLUSIONS AND FUTURE STUDIES

5.1. Overall conclusions

In recent years, significant efforts have been focused on implementing a farm-to-fork approach to improve food safety. Despite major efforts from scientists, governments, and industry, foodborne outbreaks continue to be a major problem in modern society. For example, in 2016, 286 bacterial outbreaks happened which cause 5,481 illnesses, 641 hospitalizations.

The modern trend toward preserving foods is to use a combination of mild food preservation strategies (hurdle model) rather than a single extreme stress to make foodborne pathogens exhausted as they have to overcome many stressful conditions in succession or in parallel. Even though exposure to stress may result in bacterial cell injury and damage to several cellular structures, researchers have shown that foodborne pathogens can sense their surroundings and respond to changed environmental conditions by expressing genes that reprogram the cell and assist in survival. These stress responses may result in the creation of proteins that repair damage, maintain cell homeostasis, or helping to eliminate of the stress agent. Moreover, preexposure to one stress (e.g., low pH) may also confer protection against a different type of stress (e.g., heat), a phenomenon that is referred to as stress cross-protection. In the current study, we have found preexposure to salt induces nisin resistance of *L. monocytogenes* and preexposure to low a_w condition changed subsequent heat resistance of *Salmonella* strains. Thus, the emphasis of this study was on how combination of stressors may impact bacterial survival. Those strains exhibiting high cross-tolerance will more likely be involved in contamination of foods undergoing industrial processing.

To control foodborne illnesses knowing the ability of foodborne pathogen to survive in food and the environment is essential. The magnitude of stressors impact does not depend on

how closely related strains are or where they were isolated. In current study we found *Listeria* strains belonging to CC6 and CC7 have significantly higher innate resistance to nisin compared to strains belonging to CC2, CC3, CC5, CC9, and CC11. Strains in serovar Agona are significantly resistant to low a_w conditions compared to Enteritidis and Montevideo. However, heat resistance for *S.* Enteritidis was quite constant during storage time. It means strains which are resistant to one stressor do not behave similarly to other stresses, suggesting that more than one mechanism might be responsible for resistance and the mechanisms used may vary from one strain to another.

The discovery of strain-specific responses to food-related stresses in these and other studies may explain the observed differences in strain prevalence and persistence in certain environments. Furthermore, individual strain variation must be considered in future studies examining stress tolerance; such studies should ideally include a collection of strains from a given species rather than extrapolating findings from a single isolate. This thesis highlights the fact that when choosing isolates for designing control measures or inactivation models, it is important to be aware that each bacterial isolate is unique in its inherent ability to withstand stressors. Strain selection is a vital decision when designing and conducting studies aiming at the assessment of the behavior of bacterial pathogens in food products or in systems simulating food-related environments. In these projects we tried to include relevant outbreak- and food processing environment associated strains to have better idea about response variability among them.

In these two studies we deleted genes which were known for nisin (SSI-1) and desiccation resistance (*proVWX*) and *mgtC* in *L. monocytogenes* and *Salmonella* respectively. As our results showed, SSI-1 did not significantly influence innate nisin resistance of *L.*

monocytogenes. However, deleting SSI-1 decreases salt-induced nisin resistance. In case of *Salmonella*, although Δ*mgtC* showed lower inactivation rate than wild type, Δ*proVWX* was not significantly different from wild type. But both mutants were able to resist on flaxseed. We can generally conclude that bacteria used complex mechanisms to cope with stresses and by deleting one operon or specific genes we may not see significant differences. Moreover, in the both cases selection of those genes was based on laboratory experiments, such as transcriptomic studies on abiotic surfaces, which may not be a good representative of actual mechanism that pathogen use in the food matrix.

5.2. Future studies

5.2.1. Identify genes involve in *Salmonella* and *L. monocytogenes* survival under stressors

Based on our results and previous findings, it is possible that genetic background plays a larger role in overall nisin resistance phenotypes than simply the presence or absence of SSI-1. For example, cell envelope stress response systems, including those regulated by VirR (Kang et al., 2015) and LiaR (Bergholz et al., 2013), are known to contribute to nisin resistance at low temperatures and in the presence of NaCl. Although applying nisin and salt is a common method to control *L. monocytogenes* in RTE food,s we do not fully undrestood all the mechanisms that *L. monocytogenes* may apply to combat these stressors.

It is assumed that desiccation stress is a form of osmotic stress which we do not know if *Salmonella* encounters desiccation stress on LMFs. Most of the upregulated genes under desiccation in transcriptomic studies have not been confirmed by further studies such as creating deletion mutants. So, we do not know if the same mechanism used by *Salmonella* on LMFs and osmotic stress. Questions that we should answer are which genes are expressed when exposed to cell wall targeted antimicrobial peptides? Which genes are expressed under salt and nisin

condition? Which genes are expressed on actual LMFs over time of storage? Applying transposon sequencing (TnSeq) technique will allow us to answer those questions.

5.2.1.1. Expected outcomes

Finding the important genes under tested condition and broaden our knowledge about the mechanisms used by bacteria. Also, knowing the mechanisms can help scientists to design efficient control measures to control them.

For example, generating transposon mutant libraries and growing mutant pool under a test condition will help to find especial genes under tested condition (stress) which will help to make the idea of cross-protection clearer and then based on that we can design efficient control measures by following hurdle model concept. So, we can carefully select stressors and the order of application of them. As an illustration, maybe combination of salt and nisin is not a good idea and we should combine nisin or salt with the other control measures to prevent cross-protection and efficiently control *L. monocytogenes* in RTE foods.

Also, detecting genes by Tnseq method will give scientists a chance to design or discover new components to target those proteins which are necessary for bacteria to survive under stressor. For example, in the case of *Salmonella* if we find genes which help bacteria survival for a long period of time under low moisture condition, scientist can discover or create components to use during time of storage which eventually can target those expressed proteins which are important for *Salmonella* survival on LMFs and inactivate them.

5.2.2. Identify associations between specific genetic elements and survival phenotypes in *Salmonella* and *L. monocytogenes*

Data presented in this study demonstrate variability in survival among *Salmonella* serovars and *L. monocytogenes* CCs. Although there are some studies which confirm response

variability of strains and serovars when encountering stressors (Bergholz *et al.*, 2010; Gruzdev *et al.*, 2011; Gruzdev *et al.*, 2012b) examining the reason of this variation is rare. For example, Molecular investigation has shown point or larger mutations in specific genes such as *rpoS* are a potential reason for different acid resistance among *Salmonella* serovars and strains (Humphrey, 2004; Jorgensen et al., 2000).

Our questions here are: What is the reason of response variations among serovars? Why serovars reacted differently to the same stress condition? This information will help us to increase our knowledge about bacterial mechanism to combat stresses, meanwhile it can help us to design efficient control measures to control pathogens in food supplies.

With development of genome sequencing and the accumulation of whole genome sequences, comparative genomics analysis can help to identify unique features that distinguish serovars. Furthermore, the discrete absence or presence of specific genes can be an unstable indicator of phylogenetic identity. Whole-genome sequencing provides more rigorous tools for assessing the distributions of these genes.

5.2.2.1. Expected outcomes

Focusing on *Salmonella* survival on flaxseed, we found that survival rate of Agona is significantly higher than that in Enteritidis and Montevideo. I hypothesize that if we compare genome of these serovars, Agona has additional genetic features which make this serovar different from Enteritidis and Montevideo. The same for *S.* Agona FSL S10-1759 which has a significantly lower *kmax* than *S.* Enteritidis FSL S10-1644. It could be alterations in the accessory genome, or variation in promoter and regulatory element sequences allowing for differential expression of factors allowing increased survival. So, if Agona use the other

mechanisms to combat low moisture condition, scientists should consider it when they are developing new inhibitory techniques.

Worley et al. used Whole Genome Sequence (WGS) data of 445 *Salmonella* isolates from 266 distinct serovars to create phylogenetic tree (Worley *et al.*, 2018). They recognized two new clades. Based on their finding although the concept of serovars continues to be useful but WGS will improve the accuracy of molecular descriptions, including serovars. Serovars as a method of classification may have more significant limitations than previously understood (Worley *et al.*, 2018). Using WGS will improve the accuracy of molecular descriptions, including serovars, I hypothesis that if we add more strains in our study and compare WGS of a population of *Salmonell*a in different serovars we can find a new clade within serovars as we had response variability between strains. Having higher resolution of *Salmonella* classification will help to select better set of strains for study which results in reliable outcome to design next studies or control measures.

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APPENDIX

Table A1. Average D-values for the 8 strains at high and low inoculum levels

Strain	Serovar	Inoculum level	rep	Slope	D-value
ATCC BAA-1045	Enteritidis	H	1	0.7043	1.419849
ATCC BAA-1045	Enteritidis	H	2	0.8108	1.23335
ATCC BAA-1045	Enteritidis	L	1	1.3479	0.741895
ATCC BAA-1045	Enteritidis	L	2	1.4687	0.680874
FSL S10-1646	Enteritidis	H	1	1.166	0.857633
FSL S10-1646	Enteritidis	H	2	1.2685	0.788333
FSL S10-1646	Enteritidis	L	1	1.2588	0.794407
FSL S10-1646	Enteritidis	L	2	1.9176	0.521485
FSL R8-8615	Agona	H	1	0.80498	1.242267
FSL R8-8615	Agona	H	2	1.20054	0.832959
FSL R8-8615	Agona	L	1	0.9121	1.096371
FSL R8-8615	Agona	L	2	0.87952	1.136984
FSL M8-0485	Agona	H	1	0.99149	1.008583
FSL M8-0485	Agona	H	2	0.70187	1.424765
FSL M8-0485	Agona	L	1	1.72267	0.580494
FSL M8-0485	Agona	L	2	0.99149	1.008583
FSL R9-2434	Tennessee	H	1	0.89183	1.12129
FSL R9-2434	Tennessee	H	2	0.83063	1.203905
FSL R9-2434	Tennessee	L	1	1.00579	0.994243
FSL R9-2434	Tennessee	L	2	0.87952	1.136984
FSL S10-1757	Tennessee	H	1	1.3102	0.763242
FSL S10-1757	Tennessee	H	2	1.31048	0.763079
FSL S10-1757	Tennessee	L	1	1.604	0.623441
FSL S10-1757	Tennessee	L	2	1.469	0.680735
FSL R8-3706	Montevideo	H	1	1.16973	0.854898
FSL R8-3706	Montevideo	H	2	0.95429	1.047899
FSL R8-3706	Montevideo	L	1	1.23089	0.81242
FSL R8-3706	Montevideo	L	2	1.60283	0.623896
FSL R8-2812	Montevideo	H	1	0.9831	1.017191
FSL R8-2812	Montevideo	H	2	0.91157	1.097008
FSL R8-2812	Montevideo	L	1	1.1129	0.898553
FSL R8-2812	Montevideo	L	2	1.4398	0.694541

H: High, L: Low