DID YOU JUST EAT THAT? NOVEL APPROACHES TO CONTROL LISTERIA MONOCYTOGENES ON READY-TO-EAT-FOODS

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The Gram-positive bacterium *Listeria monocytogenes* is an ubiquitous, intracellular pathogen which has been causative organism in several outbreaks of foodborne disease. Listeriosis has a mortality rate of about 24%, making it one of the leading causes of deaths associated with foodborne illness. Severe forms of listeriosis mainly affect pregnant women, their fetuses, newborns, and immunocompromised persons, with symptoms of abortion, neonatal death, septicemia, and meningitis.

L. monocytogenes can contaminate a variety of foods, with recent high incidences in dairy products. *L. monocytogenes* can survive and grow in a variety of environments and refrigeration, making it difficult to control and highlighting the importance of optimizing control strategies against this pathogen. The overall goal of the work presented here was to (i) investigate the role of the environment on *L. monocytogenes* sensitivity to two methods (i.e. nisin and bacteriophage) currently used on foods, to inhibit bacterial contamination and (ii) understand how changing environmental conditions can lead to transmission of *L. monocytogenes* to humans and subsequently cause disease.

Nisin and phage reduced *L. monocytogenes* counts on cheese in temperature- and pH-dependent manners. Nisin-mediated reduction of *L. monocytogenes* was more pronounced at lower storage temperatures, whereas phage was more effective at higher temperatures. However, both control strategies were able to

reduce *L. monocytogenes* numbers when cheese was formulated at higher pH. Furthermore, serotype was found to also affect the sensitivity of *L. monocytogenes* to both nisin and phage treatment. Serotype 1/2 strains showed significantly higher susceptibility to both treatments than serotype 4b strains.

L. monocytogenes must rapidly adapt to changes in the environment for survival and to cause disease. Previously, micro-array studies have been used to characterize virulence regulation in *L. monocytogenes*. Here, we use a bioinfromatics approach to expand our knowlege of virulence, the PrfA regulon, in *L. monocytogenes*. We identified a PrfA-dependent gene with a novel PrfA-box and putative sigA (σ^{A})-dependent promoter region.

Overall, this work provides experimental evidence that environment, as well as serotype affect *L. monocytogenes* sensitivity to control strategies, and that environmental conditions should be carefully considered when applying interventions against this important foodborne pathogen. Understanding the conditions that surround the interaction between pathogen and control strategy could prevent foodborne infections. Furthermore, better understanding of sigB (σ^{B}) and PrfA-dependent regulation of virulence related genes under different environmental conditions can allow for further improvement of *Listeria* control strategies.

BIOGRAPHICAL SKETCH

Lory Henderson was born in Phenix City, Alabama. As a child of a military family, discipline and determination were instilled in her at a young age. Lory earned Bachelor of Science degrees in Biology and Psychology at the University of New Mexico, where she completed an Honors thesis investigating the effects of nutrient availability and human impact on antimicrobial production in cave bacteria under the direction and mentorship of Dr. Diana Northup. From there, Lory began her graduate studies at Cornell University in Dr. Martin Wiedmanns lab in 2014. Throughout her graduate career, Lory was passionate about mentoring and was a strong advocate for engaging youth in science and higher education through her roles in outreach activities. Lory's PhD journey was filled with persevering through failures and celebrating both academic and personal achievements. Upon completion of her dissertation, Lory plans to pursue a career that allows her to combine her interests in science and policy, as well as creating opportunities for students from underrepresented communities in academia and beyond.

This dissertation is lovingly dedicated to my mother, Doris Henderson. Her support, encouragement, and constant love have sustained me throughout my life.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

In spite of continuous efforts to ensure the safety of foods, foodborne illness remains a significant concern in the United States and worldwide. Foodborne illness affects approximately 1 in 6 or 48 million people in the United States each year, accounting for an estimated 128,000 hospitalizations and 3,000 [1]. In addition to adverse health affects, economically, foodborne pathogens impose a burden of over $15 billion USD annually [2]. The foodborne pathogen *Listeria monocytogenes* is the third leading cause of death associated with foodborne illness in the United States [3]. *L. monocytogenes* is not only a human pathogen, but also, causes disease in animals. Additionally, *L. monocytogenes* is found in both natural and food-processing environments [4], and its ability to rapidly adapt to changing environments (from soil, to food, to human intestinal tract) enables its survival. Thus, understanding how the environment could change *L. monocytogenes*' sensitivity to control strategies, subsequently leading to transmission to humans and causing disease represents a key component in understanding the nature of listeriosis, in order to reduce morbidity and mortality associated with this foodborne pathogen. The overall goal of this dissertation was to assess the effects of temperature and pH on *L. monocytogenes* sensitivity to two control strategies (i) nisin, a bacteriocin used in a variety of foods to prevent *L. monocytogenes* contamination of foods and (ii) bacteriophage (phage), as well as to improve our understanding of virulence regulation in *L. monocytogenes*.

1.0.1 Signifcance of Listeriosis

In the United States foodborne infections associated with *L. monocytogenes* account for an estimated 1600 illnesses, including 260 deaths annually [1, 5]. Listeriosis primarily affects pregnant women, newborns, older adults, and people with weakened immune systemes [6]. Pregnant women usually get a mild illness, but listeriosis causes severe disease in newborn babies and can cause abortion of a fetus [6]. Older adults (65 years or older) and those who are immunocompromised can develop severe infections of the bloodstream (sepsis) or brain (meningitis or encephalitis) [7].

1.0.2 *L. monocytogenes* - Foodborne Pathogen

L. monocytogenes is a Gram-positive, non-spore-forming, facultatively anaerobic, rod-shaped bacterium. The genus *Listeria* currently includes at least 17 species [8]. Only two of these species are considered pathogens, *L. monocytogenes* and *L. ivanovii*; however, *L. ivanovii* is mainly an animal pathogen, most common in ruminants [8].

Many phylogenetic and molecular subtyping studies have discovered at least four lineages (I, II, III, and IV) of *L. monocytogenes* [9]. Additionally, characterization of the somatic (O) and flagellar (H) antigens (called serotyping) was adopted as a classification method among *L. monocytogenes* strains [9]. Although, more than 14 serotypes of *L. monocytogenes* have been described, only three serotypes (1/2a, 1/2b, and 4b) cause the majority of clinical listeriosis cases [10] and belong to lineages I (1/2b and 4b) and II (1/2a) [11, 12, 13, 14, 15]. Interestingly, lineage II strains are commonly isolated from

food products [16, 17], but most human listeriosis outbreaks are associated with lineage I isolates [18]. Lineage III and IV strains are rare and predominately isolated from animal sources [13, 17].

L. monocytogenes is a concern in the food industry as it can contaminate many food products, including but not limited to produce, ready-to-eat (RTE) deli meats and hot dogs, unpasteurized milk and dairy products, smoked meats, and soft cheese [19]. *L. monocytogenes* is of particular concern due to its ability to grow in a wide range of temperatures (0 to 45°C), pH (4.4 to 9.4), and high salt concentrations (13-14 w/v%) [20]. *L. monocytogenes* ability to rapidly adapt to changing environmental conditions enables it to survive the harsh conditions during food-processing. The *L. monocytogenes* contamination has continuously challenged the dairy industry, especially given the increase in number of listeriosis outbreaks in the United States associated with cheese, specifically the Hispanic-style fresh cheese "queso fresco" [4]. Hispanic-style fresh cheese is characterized by high water activity (a_w), low salt content, and near neutral pH, which creates an ideal environment for survival and growth of a number of foodborne pathogens [21], including *L. monocytogenes* [4].

1.0.3 Control of *L. monocytogenes* on Foods

Possible strategies to prevent problems associated with *L. monocytogenes* contamination in food and food-processing environments include (i) nisin and (ii) lytic phages [22]. Nisin and other bacteriocins are ribosomally synthesized, bacterially produced, cationic antimicrobial peptides (CAMPs) that inhibit the growth of a broad spectrum of pathogens [23], but are degraded by intestinal

proteases, making them safe for human consumption. Phages recognize and kill specific target bacteria; therefore, phage-based interventions can target specific foodborne pathogens without disrupting the normal and beneficial microflora of foods [24].

1.0.4 Virulence in *L. monocytogenes*

In most cases, *L. monocytogenes* infections are caused by ingestion of contaminated food. For *L. monocytogenes* survival in the stomach and intestine, the bacterium must use a number of protective mechanisms. The transition from soil-dwelling saprophyte to pathogen is mediated through complex regulatory pathways that modulate the expression of multiple genes, including virulence factors [25]. One of the virulence genes, *prfA*, encodes PrfA (positive regulatory factor A). PrfA plays a central role in regulating the expression of many virulence genes that are essential for *L. monocytogenes* pathogenicity, entry into and survival in host cells and its cell-to-cell spread into neighboring cells [26, 27, 28, 29].

The ultimate goal of this dissertation was to understand the effects of storage temperature and pH of a lab-scale cheese model on *L. monocytogenes*' sensitivity to nisin and phage treatment. The following chapters address key knowledge gaps in our understanding of *L. monocytogenes* response to these control strategies. Namely, this dissertation (i) provides a review of *L. monocytogenes* resistance mechanisms to bacteriocins, mainly nisin, (ii) describes the effect of environmental conditions on *L. monocytogenes*' sensitivity to nisin and phage, and (iii) expands our understanding of virulence regulation in *L. monocytogenes*.

CHAPTER 2

MECHANISMS FOR GRAM POSITIVE PATHOGEN SURVIVAL ON FOODS IN THE PRESENCE OF BACTERIOCINS: ROLE OF ADAPTATION, ACCLIMATION AND PHYSIOLOGICAL REFUGE

2.1 ABSTRACT

Bacteriocins are antimicrobial peptides produced by bacteria, most of which exhibit a broad spectrum of activity against many bacterial genera and species, including bacterial pathogens that contaminate food products and subsequently cause disease. Therefore, these substances have been used in or have potential for food applications in either food preservation or prevention and control of bacterial contamination. However, there is concern that continuous exposure of bacteria to bacteriocins, similar to conventional antibiotics, can select for resistant cells. In this review, we discuss molecular mechanisms involved in resistance to bacteriocins commonly used in foods, produced by Gram-positive bacteria; specifically the role of (i) adaptation, (ii) acclimation, and (iii) physiological refuge.

2.2 INTRODUCTION

Many cationic antimicrobial peptides (CAMPs) have been useful in agriculture. The most relevant CAMPs used in the food industry are bacteriocins, par-

tially due to the fact that many bacteria that produce bacteriocins are considered food grade by the U.S. Food and Drug Administration (FDA) [30]. Bacteriocins are defined as ribosomally synthesized, bacterially produced peptides that inhibit the growth of a broad spectrum of Gram-positive pathogens [31, 32, 23].

Traditionally, bacteriocins have been used as biopreservatives to extend the shelf-life and improve the quality of food products by reducing the number of foodborne pathogens or spoilage organisms [33]. Bacteriocins are also sensitive to human intestinal proteases and can therefore, be considered as safe [32]. Furthermore, the use of bacteriocins as natural food preservatives meets consumer demands for high quality and safe foods without the use of traditional food preservatives. However, limited effectiveness of pathogen elimination and cost hinder broader use of bacteriocins in foods [34]. Nevertheless, there is an ongoing search for new and more effective bacteriocins as well as research to optimize existing bacteriocins to address efficacy and economic concerns.

In addition to enhancing food safety and quality, bacteriocins have been investigated for their potential use in plant, animal, and human health to control drug-resistant pathogens [35, 33, 36]. However, continuous exposure of bacteria to bacteriocins could result in bacteria developing resistance to bacteriocins [37, 38]. In the present review, we discuss the role of (i) adaptation, (ii) acclimation, and (iii) physiological refuge in Gram-positive bacteria resistance to bacteriocins used in foods.

2.2.1 Classification of Bacteriocins

Gram-positive bacteria produce the majority of bacteriocins investigated for biotechnological applications [35, 33, 36]. The bacteriocins produced by lactic acid bacteria (LAB) have been intensely studied as most bacteriocin producers belong to this group, which naturally occur in foods and have a long history of safe use in the food industry [39]. Specifically, bacteriocins produced by LAB are studied for their ability to inhibit undesirable microorganisms and extend product shelf-life [40, 41]. In 1993, Klaenhammer classified bacteriocins produced by LAB according to their structure and biochemical characteristics [42]. Klaenhammer proposed four major classes: Class Ia and b, Class IIa, b, and c, Class III, and Class IV [42]. Class I are lantibotics (from lanthionine-containing antibiotic), small ($<$ 5 kDa), heat-stable peptides, containing the unusual amino acids lanthionine, alpha-methyllanthionine, dehydroalanine, and dehydrobutyrine [34]. This class is derived by post-translational modifications [42, 43]. Class I bacteriocins can be further divided into subclasses, class Ia and Ib based on structure [42, 44]. Class Ia bacteriocins (e.g. nisin) are cationic and hydrophobic peptides that have a flexible structure in comparison to the globular and more rigid class Ib [45, 37]. Class II bacteriocins are small ($<$ 10 kDa), heat-stable, non-lanthionine-containing membrane active peptides. This class can also be divided into subgroups based on the type of peptide it contains [23]. Class IIa include pediocin-like peptides, which have the N-terminal consensus sequence: -Tyr-Gly-Asn-Val-Xaa-Cys and have been studied due to their anti-*Listeria* activity [34, 42]. Class IIb bacteriocins are characterized by their ability to form pore complexes consisting of two peptides for full activity [42, 37], and class IIc are thiol activated peptides, which require reduced cysteine residues for activity [32]. Class III bacteriocins are large ($>$ 30 kDa) and heat-labile. Class

IV are complex bacteriocins composed of protein in addition to lipid or carbohydrate moieties required for activity [42, 32]. As the discovery of novel molecules increases, the classification of bacteriocins is continuously revised [46, 47]. In the most recent classification, which will be used for this review, bacteriocins produced by lactic acid bacteria (LAB) are divided into three main classes: Class I, II, and II (Table 2.1). Class IV bacteriocins have been removed as bacteriocins in this class have not been adaquately characterized at the biochemical level [34]. This review will focus on class I bacteriocins as these bacteriocins or the bacteria responsible for their production are most commonly studied and used in foods [48].

2.2.2 Bacteriocin Modes of Action

Bacteriocins use diverse mechanisms to kill their bacterial target cells. Most peptide antimicrobials are cationic, which suggests that interactions with anionic bacterial cell surface components are critical in bacteriocin activity [49, 50]. In general, bacteriocins are bacteriocidal, causing cell death by membrane permeablization and pore formation [51, 43] due to their cationic properties. Additionally, some bacteriocins interfere with cell wall synthesis [50].

Class I Class I bacteriocins or lantibiotics, such as nisin, have two modes of action. The first involves lipid II, which is located in the cytoplasmic membrane and is necessary to transport peptidoglycan monomers from inside to outside of the cell to build the cell wall [52]. Nisin is able to bind to lipid II, and thus prevents cell wall synthesis [53, 54]. The second mode of action (barrel stave mechanism) involves binding of nisin monomers (staves), inserting and aggre-

Table 2.1: Classification and examples of bacteriocins produced by Gram-positive bacteria

Classification	Features	Bacteriocin	Producer organism
Class I (lantibiotics)	Small (< 5 kDa), linear peptides, modified amino acids (lanthionine, β-methyl lanthionine, and dehydrated amino acids	Ia. Nisin (linear)	*Lactococcus lactis*
		Ib. Mersacidin (globular)	*Bacillus spp.*
Class II	Small (< 10 kDa), linear peptides, heat stable	IIa. Pediocin PA-1	*Pediococcus acidilactici*
		IIb. Lactococcin G (two-components)	*Lactococcus lactis*
		IIc. Acidocin B	*Lactobacillus acidophilus*
Class III (Bacteriolysins)	Large (> 30 kDa), heat-labile proteins	Lysostaphin	*Lactobacillus spp.*

gating with the membrane to form a water-filled pore (barrel) [55, 54, 56].

Class II Similar to class I bacteriocins, class II bacteriocins, such as pediocins use a membrane molecule, upon which it docks [57]. In this case, the docking molecule is the mannose-phosphotransferase system (Man-PTS). After docking, the membrane is permeabilized, the proton motive force is disrupted, causing ATP depletion, resulting in inhibition of cellular biosynthesis [58, 59].

Class III Class III bacteriocins, also called bacteriolysins have a lytic mode of action. These bacteriocins cleave the cross-bridges of peptidoglycan, which contain glycine residues, using a glycine endopeptidase [35].

2.2.3 Bacteriocins Used in Foods

As a way to extend shelf-life, food preservatives (e.g. sorbic acid, benzoic acid, and nitrite) are incorporated into foods to inhibit or decrease bacterial growth [60]. Although most commercial food preservation methods are either chemical (i.e. chemical compounds) or physical (i.e. refrigeration or drying), bacteriocins are natural preservatives that can be used in food products. One such antimicrobial that is the most widely used as a biopreservative is nisin (Table 1). Nisin, a bacteriocin produced by *Lactococcus lactis*, is a 34-amino acid CAMP, used commercially against a broad spectrum of Gram-positive organisms [50]. It is one of the two bacteriocins (nisin and pediocin PA-1) approved for use as a preservative in a variety of foods (beverages, canned foods, dairy products, meat products, and seafood products) by the U.S. FDA [61]. Another

commercially available bacteriocin is pediocin PA-1 (Table 1), which inhibits the growth of *L. monocytogenes* in dairy and meat products [61]. Although it is not commercially available, Lacticin 3147 is a two-peptide lantibiotic which is bactericidal against many pathogens (Table 1) [62] and has been proposed as a food preservative to control food-spoilage bacteria as well as pathogens in dairy products [63].

2.3 MECHANISMS OF RESISTANCE TO BACTERIOCINS

A major concern with the use of antimicrobial agents is the frequency at which susceptible organisms can develop resistance to treatments. Therefore, it is essential to understand the potential for bacteria to develop resistance to bacteriocins, specifically those used in foods. Here, we discuss three strategies that pathogens capable of surviving exposure to antimicrobial peptides can use to evade bacteriocins: (i) adaptation, (ii) acclimation, and/or (iii) physiological refuge (Table 2.2). In this review, we define mechanisms of adaption as innate properties of an organism that confer resistance [64]. This type of resistance is generally constitutive and characterized by mutations that are present even in the absence of antimicrobial exposure. It is, however, important to note that mutations have been selected for in the presence of an antimicrobial. For example, passaging an organism in the presence of increasing concentrations of an antimicrobial can select for a mutation, which confers resistance to the antimicrobial [65]. Here, we describe acclimation mechanisms of resistance as triggered in response to an antimicrobial. This type of resistance is usually characterized by a change in gene expression resulting in a physiological change, which can be reversible in the absence of the treatment. Lastly, we use the term physio-

logical refuge in reference to the ability of bacteria to show reduced sensitivity to a bacteriocin under specific conditions that are different from exposure to the treatment. The use of adaptation, acclimation, and/or physiological refuge can provide pathogens with the greatest likelihood of survival in environments containing antimicrobial peptides [49]. Below we provide clear examples of these resistance mechanisms. We focus on the ability of Gram-positive bacteria to develop resistance to nisin, given that it has been widely studied and is approved for use in foods [33, 66].

2.3.1 Resistance due to Adaptation

In the following section we describe how pathogens use adaptation to acquire resistance to nisin. This type of resistance involves spontaneous mutations in genes associated with regulatory elements [67, 68, 69, 70].

Mutations in Regulatory Elements

Enhanced nisin resistance has been associated with increased expression of three genes, *pbp229*, *hpk1021*, and *lmo2487*,which encode a penicillin-binding protein, a histidine kinase, and a protein of unknown function, respectively [69]. Inactivation of these genes via plasmid integration eliminated the nisin resistant phenotype. Furthermore, when *hpk1021* was inactivated, it also decreased expression of *pbp2229*, indicating that the nisin resistance mechanism is a result of pbp2229 expression [69].

The LisRK two-component system (TCS) of *L. monocytogenes* is also involved

Table 2.2: Classification of Gram-positive bacteria mechanisms of reduced sensitiviy to bacteriocins.

	Adaptation	**Acclimation**	**Physiological refuge**
Heritability	Heritable (mutations)	Non-heritable	Ability to gain refuge is heritable, regulation of expression is not
Distribution among species/clonal group (innate vs acquired)	Innate: mutation found in a particular genera or species	Innate: all have the ability to acclimate	Ability of a bacterium to become transiently resistant under specific conditions (different from exposure to the intervention)
	Acquired: developed by a formerly susceptible strain	Note: this could also be considered "acquired" as the change occurs in respons to exposure	
Identification method	Detectd through WGS of "survivors"	Identified through studies of gene expression (e.g. RNA-seq) under exposure conditions	Identified through studies of gene expression (e.g. RNA-seq) under physiological refuge conditions
	Confirmed via complementation or site directed mutagenesis	Confirmed through over-expression or gene knock-outs	
Examples	Passaging bacteria in the presence of increasing concentrations of nisin can select for mutations that change membrane composition, resulting in nisin resistance in *L. monocytogenes*.	In response to nisin exposure, *dltA* and *mprF* are "turned on" and can change the net negative charge on the cell wall, inhibiting binding of cationic peptides.	Under high salt conditions, *L. monocytogenes* shows nisin resistance.

in listerial susceptibility to nisin [68]. A *L. monocytogenes* mutant, lacking the histidine kinase sensor component (LisK) is less sensitive than the wild type. The LisRK TCS regulates the LiaFSR 3CS (three-component system). LiaFSR is induced in response to CAMPs that target the cell membrane and controls listerial response to environmental stressors [71]. In the presence of CAMPs, a wild type LisRK strain carrying a fully functional LisFSR is able to remodel the protein composition of the cytoplasmic membrane, ultimately decreasing expression of genes encoding for a protein similar to penicillin binding protein (PBP), PBP2229 [67, 72]. Conversely, the LisRK deletion mutant strain, has increased expression of PBP2229, which leads to nisin resistance, presumably by enhancing the incorporation of the disaccharide-pentapeptide moiety of lipid II into the growing peptidoglycan chain, resulting in PBP2229 potentially shielding lipid II (nisins target) [69].

2.3.2 Resistance due to Acclimation

Previous studies have shown that many Gram-positive organisms, such as *L. monocytogenes*, have mechanisms that enable resistance to bacteriocins via acclimation [36]. A few key mechanisms include resistance associated with (i) bacteriocin degradation [73, 74, 75] and (ii) cell envelope changes [2, 76, 77].

Bacteriocin Degradation

Previous studies have shown that some nisin-resistant *Bacillus* spp. (*Bacillus cereus* and *Paenibacillus polymyxa*) up-regulate production of an enzyme, nisinase, which degrades nisin by breaking its C-terminal lanthionine ring [73, 74].

In *Bacillus subtilis*, an extracytoplasmic function (ECF) sigma factor, σ^W, up-regulates activity of the nisin resistance gene *sppA* in the presence of nisin, which encodes a signal peptide peptidase, SppA. SppA is proposed to bind and cleave peptide antimicrobials that insert into the cell membrane [75]. Nisin degradation is also observed in *L. lactis* (non-nisin producing strains). When nisin is present, this organism, the nisin resistance membrane protein NSR acts as a protease and removes the nisin C-terminal tail [78].

Cell Envelope Changes

Bacteriocin resistance due to cell envelope modifications can be characterized by, but not limited to, the following mechanisms of regulation: (i) cell membrane changes (ii) cell envelope genes, (ii) TCS, and (iii) sigma factors.

Cell Membrane Changes A previous study shows that after several passages of *L. monocytogenes* Scott A in increasing concentrations of nisin, the strain acclimated to the presence of nisin by producing less diphosphatidylglycerol and more phosphatidylglycerol [65]. Decreasing the amount of diphosphatidylglycerol reduces sensitivity to nisin as nisin molecules are unable to penetrate as deep into the lipid monolayers when they are comprised of phosphatidylglycerol rather than diphosphatidylglycerol [65]. In additon to phospholipid compositions, acclimation to nisin has been correlated to alterations in fatty acid composition [79]. Specifically, a lower ratio of C15:C17 fatty acids and the presence of more phosphatidylethanolamine and less phosphatidylglycerol and cardiolipin in the cell membrane increase resistance of *L. monocytogenes* to nisin [79]. As a result of the aforementioned cell membrane changes, the membrane

is less fluid (i.e. rigid) and the net negative charge is decreased. Thus, these changes in cell membrane composition can prevent nisin from inserting into the cell membrane and weaken the ability of nisin to bind to lipid II [79].

Cell Envelope Genes Wall teichoic acids (WTAs) and LTAs are a main component of the Gram-positive bacterial cell wall and are highly negatively charged due to deprotonized phosphate groups. It has been shown experimentally, that Gram-positive bacteria, such as *S. aureus* [77], *C. difficile* [76], *B. cereus* [2], and *L. monocytogenes* [80] can resist interactions with bacteriocins and other CAMPs by up-regulating the *dltABCD* operon, which encodes proteins that incorporate D-alanine residues onto teichoic acids, reducing the net negative charge of the cell wall [81, 82]. Additionally, the *mprF* gene encodes the MprF protein, which reduces the net negative charge of the cell membrane by synthesizing lysylphosphatidylglycerols by adding L-lysine to phosphatidylglycerol, which results in a positive charge of the cell membrane [83, 84, 85].

TCS Regulation Evidence suggests that TCS are activated by CAMPs and other cell envelope-acting antimicrobials [86]. TCS are signal transduction devices, which are engaged in many gene regulatory systems that respond to changing growth conditions, leading to the ability of many pathogenic bacteria to efficiently acclimate to different niches inside and outside of their host organisms [87]. Sensing of a specific input signal leads to autophosphorylation of a conserved histidine residue on a sensor histidine kinase. The phosphoryl group is then transferred to an aspartic acid residue of the response regulator, which functions as a transcriptional activator [87]. The TCS VirRS controls the expression of both *dltA* and *mprF* in *L. monocytogenes* [88]. In *S. aureus*, GraRS

is responsible for this regulation [89, 90]. Inactivation of the response regulator component of the TCS, VirR or GraR results in increased bacterial susceptibility to bacteriocins due to the organisms inability to modify its cell envelope. In other Gram-positive organisms, instead of regulating the charge of the bacterial cell envelope, the TCS inhibits the CAMPs from binding to the cell membrane [90]. For example, the TCS NsrRS found to be involved in nisin resistance in *Streptococcus mutans*, upregulates the expression of NsrX, a putative membrane-bound protein that binds to nisin and inhibits its binding to lipid II, which prevents nisin activity [90].

Sigma Factor Regulation Alternative sigma factors also play a role in bacterial acclimation to bacteriocins. For example, in *B. subtilis*, the ECF sigma factors σ^X and σ^M also contribute to bacteriocin resistance. σ^M regulates expression of *ltaSa*, encoding a stress-activated LTA synthase [75], which produces longer and denser LTAs. More LTAs seemingly limit nisins ability to gain access to its target in the cell membrane. σ^X activates the synthesis of phosphatidylethanolamine and the *dlt* operon, which decreases the net negative charge of the cell wall [75]. Together, σ^X and σ^M regulate the *B. subtilis* cell envelope structure such that nisin cannot access lipid II.

2.3.3 Resistance due to Physiological Refuge

Here, we define physiological refuge as the ability of a bacterium to become transiently resistant under specific conditions. This could also be termed cross-protection (Table 2.2). Nisin resistance in *L. monocytogenes* is shown to vary depending on the conditions surrounding the interaction. Resistance to

bacteriocins depends on various factors, such as growth phase and environmental stressors. Stationary-phase cells exhibit higher resistance to nisin than exponential-phase cells [91]. Temperature also influences nisin resistance. *L. monocytogenes* strain Scott A resistance to nisin is higher when grown at 10°C compared to growth at 37°C [65]. Furthermore, the addition of NaCl provided a protective effect and increased nisin resistance at low temperature [92]. Additionally, prior exposure of *L. monocytogenes* to salt stress has also been shown to lead to nisin resistance in a LiaR-dependent manner [93]. LiaR is the response regulator of the three-component system LiaFSR, which regulates the expression of genes associated with nisin resistance in *L. monocytogenes* [71]. In acid stress environments, *L. monocytogenes* uses a glutamate decarboxylase system to withstand the low pH. GadD1 catalyzes the breakdown of glutamate into γ-aminobutyrate and carbon dioxide, forming ATP, which could restore intracellular levels of ATP that are depleted by nisin activity on the cell, and thus leading to nisin resistance [94, 95]. In each example above, specific conditions (i.e. growth phase, temperature, salt and acid stress) cause a temporary change in the bacteria that allows for survival in the presence of nisin. In other words, the bacteria gain physiological refuge or cross-protection from nisin under each condition.

2.4 PRACTICAL IMPLICATIONS FOR RESISTANCE TO BACTERIOCINS USED TO CONTROL GRAM POSITIVE PATHOGENS

Given the importance of bacteriocins in controlling foodborne pathogens and spoilage bacteria, it is essential to understand mechanisms that can lead to bacterial resistance to bacteriocins. In addition to use as food additives, research efforts have also investigated the use of different antimicrobial combinations to get more effective responses (e.g. inhibiting pathogen growth). This process is called hurdle technology [96]. In a hurdle technology application, two or more bacteriocins are combined with other types of natural antimicrobials, chemicals, or physical treatments [96]. Using a combination of antimicrobials might be an effective strategy; however, research shows that sensitivity to bacteriocins can depend not only on the bacteriocins, but also on the environmental conditions that surround the interaction between the bacteriocins and the organism [93, 92, 91]. Therefore, understanding how bacteria use adaptation, acclimation, and/or physiological refuge to resist bacteriocins is increasingly essential. Insights on how foodborne pathogens evade bacteriocin activity will allow for optimization of existing and development of new interventions that are effective against foodborne pathogens and spoilage bacteria under conditions encountered in foods.

2.5 CONCLUSIONS

The continued use of bacteriocins in food applications could eventually result in bacterial pathogen resistance. Given the importance of bacteriocins in preventing and controlling spoilage organisms and foodborne pathogens, it is necessary to understand ways in which bacteria become resistant. Furthermore, it is known that intrinsic factors of food systems can affect bacteriocin activity and the development of resistance to bacteriocins [33, 97], making it essential to investigate these conditions and how they affect bacteria susceptibility to bacteriocins. Understanding the conditions surrounding the interaction between bacteria and bacteriocins can facilitate the optimization of current control mechanisms and the development of new strategies to combat bacterial resistance to bacteriocins.

CHAPTER 3

ENVIRONMENTAL CONDITIONS AND SEROTYPE AFFECT *LISTERIA MONOCYTOGENES* SENSITIVITY TO NISIN TREATMENT IN A LABORATORY CHEESE MODEL

3.1 ABSTRACT

The growth of *Listeria monocytogenes* on refrigerated, ready-to-eat food products is a major concern for the dairy industry. Previous work has demonstrated that *Listeria monocytogenes* is sensitive to cell-wall acting control strategies (i.e. bacteriophage) in a temperature and pH-dependent manner on a lab-scale fresh cheese. Natural antimicrobials, such as nisin, also target the bacterial cell wall and are commonly used to inhibit *Listeria monocytogenes* growth on cheese. The goal of this study was to determine the effect of a range of pH and temperatures on the effectiveness of nisin against several strains of *Listeria monocytogenes* in a lab-scale, cheese model. Cheese was made with or without the addition of nisin at different pH and then inoculated with *Listeria monocytogenes*; after incubation at 6, 14, or 22°C, *Listeria monocytogenes* numbers were quantified on days 1, 7, and 14. Our data show that nisin treatment is able to reduce *Listeria monocytogenes* numbers at each temperature tested; however, it is more effective when cheese is stored at lower temperatures. Additionally, nisin treatment was more effective at inhibiting *Listeria monocytogenes* numbers on cheese made at higher pH (6 and 6.5) compared to cheese made at pH 5.5; this effect was at least partially due to the activity of nisin resistance genes *dltA* and *mprF*. Serotype was found to also affect the susceptibility of *Listeria monocytogenes* to nisin treatment; serotype 4b strains showed lower susceptibility to nisin treatment compared to

serotype 1/2 strains. Overall, our results highlight the importance of considering environmental conditions specific to a food matrix when developing and applying nisin-based intervention strategies against *Listeria monocytogenes*.

3.2 INTRODUCTION

Controlling *L. monocytogenes* in foods is essential to food safety due to the high mortality rate associated with listeriosis, especially among susceptible populations, such as pregnant women, newborns, the elderly, and those with compromised immune systems [98, 99]. *L. monocytogenes* can also cause disease in animals and is found in both natural and food-processing environments [100]. Different serotypes of *L. monocytogenes* are associated with specific environments and disease cases [8]. Each serotype belongs to at least one of four lineages: I, II, III, and IV [11, 12, 13, 101, 15]; however, the majority of *L. monocytogenes* isolates commonly associated with human clinical cases belong to lineages I (serotype 1/2b and 4b) [8], whereas lineage II (serotypes 1/2a) isolates are more prevalent in food products and natural and farm environments [16, 17].

L. monocytogenes is of particular concern in ready-to-eat (RTE) dairy foods that allow its growth, such as Hispanic-style fresh cheese [99]. Hispanic-style fresh cheese is characterized by high water activity, low salt content, and near neutral pH, which creates an ideal environment for survival and growth of a number of foodborne pathogens [21], including *L. monocytogenes* [102, 4]. Furthermore, *L. monocytogenes* can tolerate a number of environmental stressors associated with foods, including a wide range of temperatures (0 to 45°C), pH (4.4 to 9.4), and high salt concentrations (13-14 w/v%) [20]. The ability of *L. mono-*

cytogenes to survive adverse environmental conditions increases the likelihood of transmission from the environment to humans via contaminated food products. A critical point in controlling *L. monocytogenes* in the food supply focuses on prevention of post-processing contamination and/or reformulation of RTE foods using antimicrobials [103].

One control strategy currently used for *L. monocytogenes* on RTE foods is the addition of bacteriocins. Bacteriocins are ribosomally synthesized cationic antimicrobial peptides (CAMPs) produced by bacteria that inhibit the growth of a broad spectrum of pathogens [23]. Some bacteria that produce bacteriocins are considered food grade by the U.S. Food and Drug Administration (FDA) [34], and bacteriocins are sensitive to human intestinal proteases, making them useful for food preservation [32]. Although, there are a number of bacteriocins that have been studied for their antimicrobial properties, nisin and pediocin are commercially available for use in a variety of food products [34, 104]. Of these two bacteriocins, nisin is the most widely used [105]. Nisin is produced by *Lactococcus lactis* and is active against a broad number of Gram-positive bacteria [106]. Nisin has two modes of action: (i) binding to lipid II, a precursor molecule in cell-wall synthesis, and thus preventing synthesis of the cell-wall component peptidoglycan [53, 54] and (ii) aggregation of nisin molecules to form complexes to create a pores in the bacterial cell membrane, subsequently causing cell lysis [55, 54].

The bacterial cell envelope provides structural integrity to the cell, but also protects these organisms from unpredictable or sometimes hostile environments [107]. Maintaining the integrity and function of the cell envelope under fluctuating environmental conditions is essential for bacterial survival. Bacteria can

sense and respond to cell envelope stressors through alternative sigma factors and/or two-component systems (TCS) [108]. *L. monocytogenes* harbors at least 15 TCS, four of which have been reported to play a critical role in regulating the cell envelope stress response (*liaRS*, *lisRK*, *cesRK*, and *virRS*) [109, 86]. A number of genes associated with resistance to nisin and other cationic antimicrobial peptides (CAMPs) are a part of TCS regulons, including *dltABCD* [110, 76] and *mprF* [84, 85, 111]. For example, in some Gram-positive bacteria harboring mutation in the *dlt* operon, the strain is more sensitive to nisin and other CAMPs such as polymyxin B and gallidermin due to no D-alanine on the lipoteichoic acids (LTAs), making the cell wall more positively charged [110, 76]. In addition to genetic regulation, the environment (food-related stressors) can also affect the efficacy of CAMPs [108, 109, 112, 113, 86]. Previous studies have shown that nisin can inhibit growth of *L. monocytogenes* on cheese [3], hotdogs [114], and smoked meats [115], suggesting that the use of nisin or other bacteriocins can reduce pathogen prevalence or the incidence of foodborne outbreaks. Furthermore, the antimicrobial effect of nisin varies with the food matrix and under different environmental conditions [116, 117, 34, 118].

Given that the characteristics of fresh-style cheese allow for*L. monocytogenes* growth, the objective of this study was to investigate the effects of temperature and pH on *L. monocytogenes* sensitivity to nisin in a lab-scale cheese model. Understanding the effects of the environment on *L. monocytogenes* sensitivity to antimicrobial treatments will allow for development and application of new strategies or optimization of current control strategies to prevent *Listeria*-related foodborne outbreaks and infections.

3.3 MATERIALS AND METHODS

3.3.1 Bacterial Strains and Growth Conditions

All *L. monocytogenes* strains used in this study are listed in Table 3.1. A non-polar, in-frame deletion mutation of mprF was constructed from the *L. monocytogenes* 10403S parent strain using the splicing by overlap extension (SOE) method as previously described [119, 120]. Additionally, the Δ*dltAmprF* mutant was constructed by deleting the *mprF* open reading frame (ORF) from the Δ*dltA* mutant strain using SOE. All mutations were confirmed by PCR and subsequent sequencing of the chromosomal copy of the deletion allele. As previously described by Henderson et al. (2019), all strains were streaked from frozen brain heart infusion (BHI; Difco, Becton Dickinson and Co., Sparks, MD) stocks, stored at -80°C in 15% glycerol, onto a BHI agar plate, followed by incubation at 37°C for 24 h. A single colony was subsequently inoculated into 5 mL of BHI broth in 16 mm tubes, followed by incubation at 37°C with shaking (230 rpm) for 16 h (Series 25 Incubator, New Brunswick Scientific, Edison, NJ). After 16 h, 50 μL BHI culture was inoculated into fresh 5 mL BHI broth and grown to OD_{600} = 1.0 at 30°C.

3.3.2 Growth of *L. monocytogenes* in a Lab-scale Cheese Model Containing Nisin

We used the method previously described [121] to make approximately 10 g miniature cheese at different pH (6.5, 6.0 or 5.5). To account for bacteria present

Table 3.1: Strains and plasmids used in this study

Strain or plasmid	Strain/genotype	Serotype	Reference
Strain			
FSL X1-0001	10403S	1/2a	Bishop and Hinrichs (1987)
FSL R9-5621	5621	1/2a	Henderson et al. (2019)
FSL R9-5623	5623	4b	Henderson et al. (2019)
FSL R9-5624	5624	1/2b	Henderson et al. (2019)
FSL R9-5625	5625	4b	Henderson et al. (2019)
FSL D4-0041	Δ*dltA*	1/2a	Gift from Helene Marquis
FSL B2-0451	Δ*mprF*	1/2a	This study
FSL B2-0445	Δ*dltAmprF*	1/2a	This study
N/A	*P. cerevisiae* E66	N/A	Gift from Randy Worobo
Plasmid			
pBMB100	Δ*mprF* on pKSV7	N/A	This study
pKSV7	Integrative shuttle vector (Amp^r)	N/A	Wiedmann et al. (1998)

and to ensure that no *L. monocytogenes* was in the milk before cheese was made, milk samples were plated on plate count agar (PCA; Difco, Becton Dickinson and Co.) and on *L. monocytogenes* plating medium (LMPM; Difco, Becton Dickinson and Co.). After 48 h of incubation at 32°C for PCA and 24 h of incubation at 30°C for LMPM, colonies were counted using a Q Count Colony Counter (Advanced Instruments, Norwood, MA).

For treated cheese, 50 mg Nisaplin (Danisco) (equivalent to 2 μg/mL or 2 ppm nisin, since Nisaplin contains 2.5% nisin) was added to 600 mL of pasteurized, whole milk, prior to any acidification, to ensure even distribution throughout the final product [122]. Although 2 ppm nisin was able to inhibit *L. monocytogenes* growth during cheese experiments, this amount was not sufficient to extract from cheese. Therein, for nisin extraction experiments, we tested a range of concentrations that would allow us to extract nisin and observe inhibition, which resulted in the addition of 25 ppm nisin to milk used to make cheese at each pH (6.5, 6.0, or 5.5).

Immediately after cheese was made, it was surface inoculated with 100 μL of a stationary phase (OD_{600} = 1.0) culture of *L. monocytogenes* at a level of approximately 10^7 cfu/g. For pH and temperature experiments, four selected *L. monocytogenes* outbreak strains were used (Table 3.1). For cell wall mutant experiments, three deletion mutants (Δ*dltA*, Δ*mprF*, and Δ*dltAmprF*) were used (Table 3.1). Plates of six cheese per plate were covered and incubated at 6, 14 or 22°C for 1, 7, or 14 days. For cell wall deletion mutant experiments, cheese was incubated at 6°C for 1 day. All plates included a lab strain (10403S), as well as a un-inoculated cheese control.

On the day of sampling, cheese was diluted 1/10 with PBS and homogenized

using a Stomacher (Seward., Worthing, UK). Homogenates were then serially diluted and plated on modified Oxford agar (MOX; Difco, Becton Dickinson and Co.) using an Autoplate spiral plating system (Advanced Instruments., Norwood, MA) for *L. monocytogenes* quantification. After 48 h of incubation at 30°C [123], colonies were counted using a Q Count Colony Counter (Advanced Instruments). Experiments were performed in biological triplicate.

3.3.3 Nisin Extraction and Activity Assay

We modified a previously described method for acid extraction of nisin [124]. While 2 ppm nisin was enough to inhibit *L. monocytogenes* growth on our cheese experiments, it was an insufficient amount to extract from cheese; therefore, for nisin extraction experiments, cheese was made with 25 ppm nisin at each pH (6.5, 6.0, or 5.5). To extract nisin, aach 10 g cheese was added to 40 mL 0.02 N HCl. The pH was then adjusted to 2.0 with 6 N HCl and the samples were heated to 100°C for 5 min. Samples were then cooled to 20°C and the volume was adjusted to 40 mL with 0.02 N HCl. Samples were centrifuged for 20 min at 4000 x g at 4°C. The supernatant was held at 4°C for 30 min and then filtered through a 0.22 μm sterile filter. The extracts were adjusted to pH 5.5 using 6 N NaOH to ensure high nisin solubility and stored at 4°C until nisin activity testing.

For nisin activity assessment, a well agar diffusion method [125] was used in which wells (8.8 mm in diameter) were cut out of MRS (Difco, Becton Dickinson and Co.) agar plates, and a total volume of 500 μL of nisin extracted from the cheese was added to each well. A control well was made using 500 μL of a

25 ppm nisin stock preparation. The nisin was allowed to diffuse into the agar, then 6 mL of MRS soft agar (0.75%) seeded with approximately 10^5 cfu/mL of *Pediococcus cerevisiae* E66 (nisin sensitive strain) was overlaid onto the plates. After the agar solidified, the plates were incubated at 30°C for 24 h. The diameter of zones of inhibition were measured to determine nisin activity. Experiments were performed as two independent biological replicates.

3.3.4 Statistical Analysis

All statistical analyses were carried out in R Statistical Programming Environment [126]. As previously described [121], we constructed individual mixed effects models for temperature and pH using lmer function in the lme4 package [127]. For each model, the response was the log cfu/g of the number of *L. monocytognes*, defined as log count and random effects were (i) replicate and (ii) plate nested within milk batch. Fixed effects were (i) temperature or pH, (ii) day, (iii) nisin, (iv) strain, (v) age of the milk (based on a 21-day code date), (vi) log of the aerobic plate counts (bacterial counts in the milk before cheese was made; milk apc), and (vii) inoculum (log cfu/g of *L. monocytogenes* inoculated on each cheese). We also included interactions between (i) temperature or pH and nisin, (ii) nisin and strain, and (iv) nisin and day. Post-hoc pairwise comparisons of means were performed using lmerTest [127].

We also constructed a linear mixed effects model for the *L. monocytogenes* mutant strains using lmer function in the lme4 package [127]. For this model, the response was log reduction (cfu/g) of *L. monocytogenes* and the fixed effects were (i) pH and (ii) strain.

A two-way analysis of variance (ANOVA) was calculated for the effect of strain and pH on *L. monocytogenes* log reduction for mutant strains, whereas a one-way ANOVA was calculated for the effect of pH on nisin extracted from the whey. Post-hoc estimated marginal means tests were performed with the emmeans package [128]. The cut-off for significance for all statistics was set at $p < 0.05$. Raw data and the R code used for all statistical analyses are available on GitHub at https://github.com/lohenderson/.

3.4 RESULTS

3.4.1 Temperature Affects *L. monocytogenes* Susceptibility to Nisin

To assess the effect of different cheese incubation temperatures on the ability of nisin to reduce *L. monocytogenes* numbers on cheese, nisin (2 ppm) was added to pasteurized milk prior to production of a lab-scale cheese model (pH 6.5). After cheese was made, it was surface inoculated with one of four recent *L. monocytogenes* outbreak strains or reference strain 10403S. Cheese was then incubated at either 6, 14, or 22°C and *L. monocytogenes* numbers were quantified at day 1, 7, and 14 (Figure 3.1). *L. monocytogenes* was not detected in the milk nor the uninoculated controls (data not shown). A linear mixed effects model was used to specifically determine whether (i) temperature, (ii) day of incubation, (iii) presence or absence of nisin, and (iv) strain as well as interactions between (v) temperature and nisin, (vi) nisin and strain, and (vii) nisin and day showed significant effects on log transformed bacterial numbers (Table 3.2). While Fig-

ure 3.1 represents the actual observed data, least square means of estimated *L. monocytogenes* counts averaged for all strains from the temperature model are shown in Figure 3.2 and estimated for each strain individually in Figure 3.3.

Presence of nisin showed a significant effect ($p < 0.001$) on *L. monocytogenes* numbers with a model-estimated effect size of -3.44, indicating 3.44 log cfu/g lower *L. monocytogenes* numbers in the presence of nisin (Table 3.2). The significance of the factor nisin supports that nisin significantly reduces *L. monocytogenes* numbers in the cheese model, as evident by the data shown in Figure 3.1. For cheese incubated at 6°C, average *L. monocytogenes* numbers (across all sampling days and strains) were 6.69 and 8.95 log cfu/g for cheese made with and without nisin, respectively (Figure 3.4). Nisin-treated cheese also consistently showed lower bacterial numbers at day 1 as compared to untreated cheese (2.26, 2.78, and 2.03 log cfu/g lower average *L. monocytogenes* numbers [across the 5 strains tested] for 6, 14, and 22°C) (Figure 3.4).

The interaction between nisin and day 7 and 14 also had significant effects on *L. monocytogenes* numbers with higher numbers at both days as compared to the reference (i.e., day 1) (Table 3.2). The significance of this interaction is not surprising considering that *L. monocytogenes* showed growth over time in the presence of nisin (average log counts (cfu/g) across strains were 6.4 and 7.5 for day 1 and 14, respectively, at 6°C; Figure 3.4).

We also found a significant interaction effect between nisin and storage at 14 and 22°C with an effect size of 0.97 and 1.39, respectively, indicating 0.97 and 1.39 log cfu/g higher *L. monocytogenes* numbers relative to nisin treatment at 6°C (Table 3.2). While on average, nisin-treated cheese showed lower *L. monocytogenes* numbers across all temperatures, the difference between *L. monocy-*

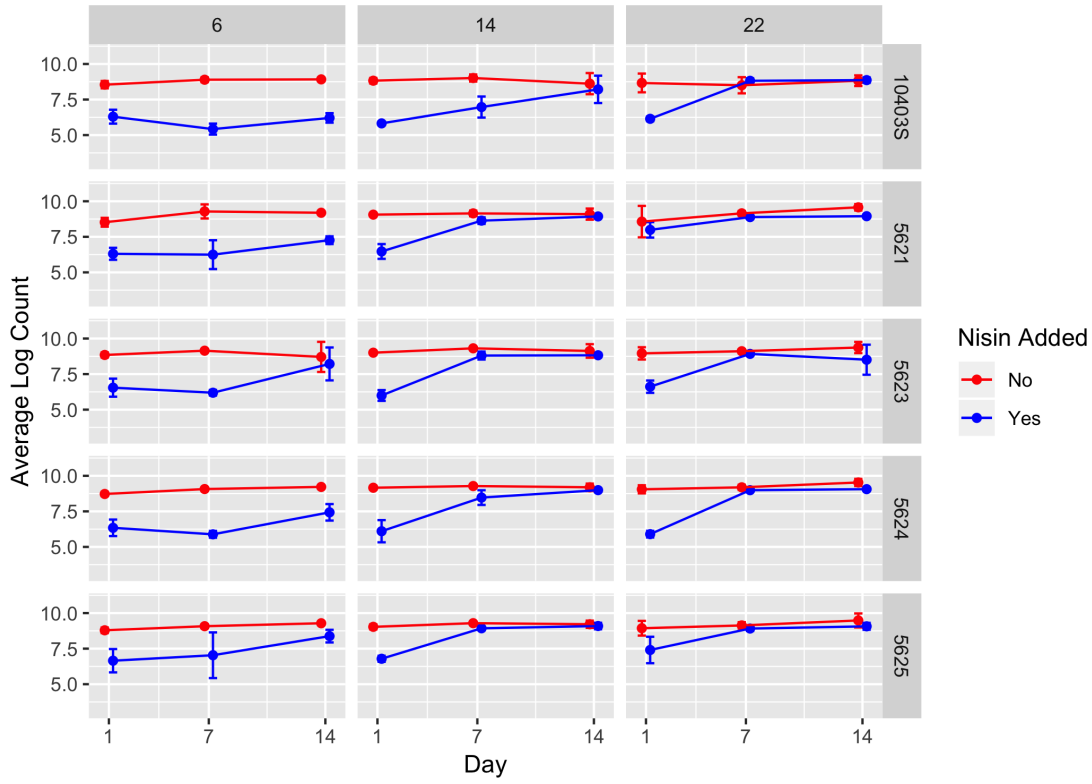

Figure 3.1: Average counts (log cfu/g) of *L. monocytogenes* in the presence (blue line) and absence (red line) of nisin in a lab-scale cheese model. Each cheese was inoculated with a single strain of *L. monocytogenes* (10403S, FSL R9-5621, FSL R9-5623, FSL R9-5624, or FSL R9-5625) to a level of approximately 7 log cfu/g. These results represent the effect of temperature (6, 14, and 22°C) on *L. monocytogenes* sensitivity to nisin. The data points are slightly offset such that the reader can clearly see each point; however, the points still correspond to 1, 7, and 14 days. All values are the arithmetic mean of three independent experiments, and error bars denote standard error. For some data points, error bars are not visible because standard error was too low to yield a visible error bar. All cheese was made at pH 6.5.

Table 3.2: Model parameters for all fixed effects in the temperature model for *L. monocytogenes* counts

Response variable	Fixed effects	Levels	Estimate	Standard error	p-value	Significance
L. monocytogenes count (log cfu/g)	Temperature (°C)	6	Ref[1]			
		14	0.09	0.22	0.685	
		22	0.15	0.23	0.510	
	Nisin	N[2]	Ref			
		Y[3]	-3.44	0.29	< 0.001	***
	Day	1	Ref			
		7	0.26	0.21	0.226	
		14	0.31	0.21	0.150	
	Strain	10403S	Ref			
		5621	0.31	0.12	0.008	**
		5623	0.3	0.12	0.011	*
		5624	0.39	0.12	< 0.001	***
		5625	0.38	0.12	< 0.001	***
	Inoculum[4] (log cfu/g)		0.06	0.11	0.605	
	Milk age[5]		-0.04	0.03	0.282	
	Milk apc[6] (log cfu/mL)		-0.05	0.10	0.644	
	Temperature:Nisin Y	6:Nisin Y	Ref			
		14:Nisin Y	0.97	0.30	0.002	**
		22:Nisin Y	1.39	0.30	< 0.001	***
	Nisin Y:Day	Nisin Y:1	Ref			
		Nisin Y:7	1.05	0.30	0.001	**
		Nisin Y:14	1.59	0.30	< 0.001	***
	Nisin Y:Strain	Nisin Y:Strain 10403S	Ref			
		Nisin Y:Strain 5621	0.45	0.16	0.007	**
		Nisin Y:Strain 5623	0.33	0.16	0.043	*
		Nisin Y:Strain 5624	0.08	0.16	0.630	
		Nisin Y:Strain 5625	0.66	0.16	< 0.001	***

[1]Ref indicates reference; therefore, estimates, standard error, and p-values are not calculated
[2]N denotes the absence of phage
[3]Y denotes the presence of phage
[4]Avererage number (log cfu/g) of *L. monocytogenes* inoculated onto each cheese
[5]Age of milk when cheese was made, based on a 21-day code date
[6]Bacterial aerobic plate counts (apc; log cfu/mL) in milk before cheese was made
***$p < 0.001$; **$p < 0.01$; *$p < 0.05$

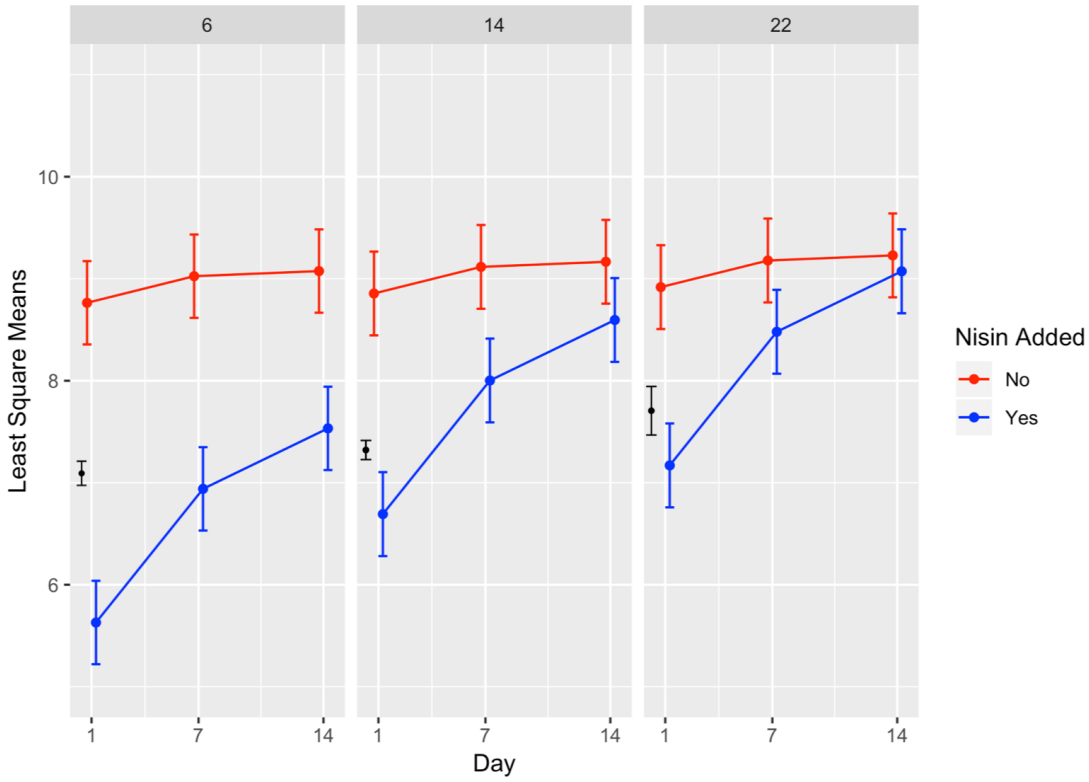

Figure 3.2: Least square means of estimated *L. monocytogenes* numbers (log cfu/g) calculated from the temperature model, which are based on data shown in Figure 3.1. Calculated initial (day 0) *L. monocytogenes* number based on the average inoculum level (approximately 7 log cfu/g) are shown in black. Predicted numbers for cheese made with nisin are shown in blue, while predicted numbers for cheese made without nisin are shown in red. These results represent the predicted effect of temperature (6, 14, and 22°C) on *L. monocytogenes* sensitivity to nisin across strains (10403S, FSL R9-5621, FSL R9-5623, FSL R9-5624, and FSL R9-5625). The data points are slightly offset such that the reader can clearly see each point; however, the points still correspond to 1, 7, and 14 days. Error bars denote the 95% confidence interval. All cheese for these predictions was made at pH 6.5.

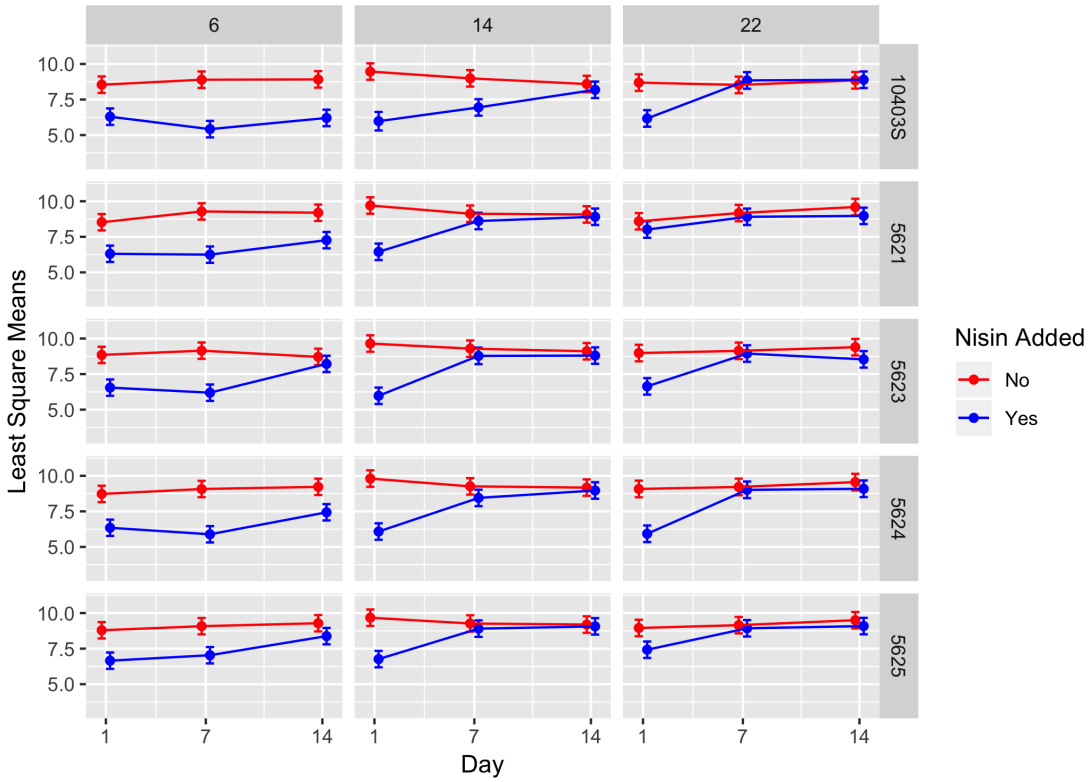

Figure 3.3: Least square means of estimated *L. monocytogenes* numbers (log cfu/g) calculated from the temperature model, which is based on data shown in Figure 3.1. Predicted numbers for cheese made with nisin are shown in blue, while predicted numbers for cheese made without nisin are shown in red. These results represent the predicted effect of temperature (6, 14, and 22°C) on *L. monocytogenes* (10403S, FSL R9-5621, FSL R9-5623, FSL R9-5624, and FSL R9-5625) sensitivity to nisin. The data points are slightly offset such that the reader can clearly see each point; however, the points still correspond to 1, 7, and 14 days. Error bars denote the 95% confidence interval. All cheese for these predictions was made at pH 6.5.

Temperature (°C)	pH	Strain	Nisin	Day	Rep[1] 1	Rep 2	Rep 3	Average[2]	Std Dev[3]	Log Reduction (N-Y)
6	6.5	10403S	N	1	8.50	8.79	8.35	8.55	0.22	2.25
6	6.5	5621	N	1	8.38	8.83	8.36	8.52	0.27	2.22
6	6.5	5623	N	1	8.92	8.95	8.66	8.85	0.16	2.30
6	6.5	5624	N	1	8.79	8.81	8.57	8.72	0.13	2.38
6	6.5	5625	N	1	8.89	8.89	8.59	8.79	0.17	2.14
6	6.5	10403S	Y	1	6.24	5.91	6.74	6.30	0.42	
6	6.5	5621	Y	1	6.02	6.17	6.72	6.31	0.37	
6	6.5	5623	Y	1	6.02	6.49	7.12	6.55	0.55	
6	6.5	5624	Y	1	5.79	6.77	6.47	6.34	0.50	
6	6.5	5625	Y	1	5.84	6.96	7.16	6.65	0.71	
6	6.5	10403S	N	7	8.77	8.85	9.06	8.89	0.15	3.47
6	6.5	5621	N	7	9.78	9.02	9.05	9.28	0.43	3.04
6	6.5	5623	N	7	9.08	9.12	9.24	9.15	0.08	2.96
6	6.5	5624	N	7	9.06	9.10	9.04	9.07	0.03	3.18
6	6.5	5625	N	7	8.99	9.21	9.03	9.08	0.12	2.04
6	6.5	10403S	Y	7	5.12	5.78	5.36	5.42	0.33	
6	6.5	5621	Y	7	5.57	5.92	7.24	6.25	0.88	
6	6.5	5623	Y	7	6.00	6.23	6.34	6.19	0.17	
6	6.5	5624	Y	7	5.69	6.11	5.86	5.89	0.21	
6	6.5	5625	Y	7	6.38	6.10	8.63	7.04	1.39	
6	6.5	10403S	N	14	8.95	8.80	9.00	8.92	0.10	2.71
6	6.5	5621	N	14	9.12	9.26	9.19	9.19	0.07	1.93
6	6.5	5623	N	14	9.22	7.65	9.26	8.71	0.92	0.49
6	6.5	5624	N	14	9.22	9.19	9.25	9.22	0.03	1.79
6	6.5	5625	N	14	9.20	9.36	9.31	9.29	0.08	0.91
6	6.5	10403S	Y	14	6.03	6.54	6.05	6.21	0.29	
6	6.5	5621	Y	14	7.54	7.13	7.12	7.26	0.24	
6	6.5	5623	Y	14	7.92	9.33	7.40	8.21	1.00	
6	6.5	5624	Y	14	7.21	8.01	7.08	7.43	0.50	
6	6.5	5625	Y	14	7.95	8.51	8.69	8.38	0.38	
6	6	10403S	N	1	8.14	8.10	8.28	8.83	0.17	3.00
6	6	5621	N	1	8.11	8.25	8.36	9.06	0.08	2.59
6	6	5623	N	1	8.15	8.34	5.23	9.00	0.03	3.01
6	6	5624	N	1	8.61	8.32	8.02	9.16	0.11	3.06
6	6	5625	N	1	8.58	8.24	7.40	9.03	0.16	2.24
6	6	10403S	Y	1	6.68	6.84	7.30	5.82	0.08	
6	6	5621	Y	1	6.55	7.12	6.87	6.47	0.45	
6	6	5623	Y	1	6.51	7.14	7.08	6.00	0.33	

Figure 3.4: Subset of observed *L. monocytogenes* counts on cheese in the presence and absence of nisin. Full table is available on GitHub at https://github.com/lohenderson/

togenes numbers on treated and untreated cheese varied considerably by temperature. For example, the lowest log difference between nisin-treated and untreated cheese for day 1 (0.58 log) was found for strain 5621 grwon on cheese incubated at 22°C, with a higher corresponding log difference of 2.22 and 2.59 for 6 and 14°C, respectively (Figure 3.1; Figure 3.4). Importantly, however, growth of *L. monocytogenes* was still observed in nisin-treated cheese, particularly those stored at 14 and 22°C (Figures 3.1 and 3.2) where, for a number of strains, by day 7 and 14, *L. monocytogenes* numbers were similar for nisin-treated and untreated cheese and differed by < 0.5 log. For example, at 6°C, only strains 10403S, 5621, and 5624 (both day 7 and 14) showed > 0.5 log difference between nisin-treated and untreated cheese.

Finally, we found a significant interaction effect between presence of nisin and strains 5621 (serotype 1/2a), 5623 (serotype 4b), and 5625 (serotype 4b) ($p = 0.007$; effect size of 0.45, $p = 0.043$; effect size of 0.33 and $p < 0.001$; effect size of 0.66, respectively). This indicates that these strains have reduced sensitivity to nisin with 0.45, 0.33, and 0.66 log cfu/g higher *L. monocytogenes* numbers, respectively, in the presence of nisin as compared to the reference strain 10403S.

3.4.2 pH Affects *L. monocytogenes* Susceptibility to Nisin Treatment

To assess the effect of different pH on nisin inhibition of *L. monocytogenes* numbers, nisin (2 ppm) was added to the milk, and cheese was made at pH 5.5, 6.0, and 6.5 prior to *L. monocytogenes* surface inoculation. Bacterial numbers were quantified after storage at 6°C for 1, 7, and 14 days (Figure 3.5). In gen-

eral, nisin-treated cheese showed lower numbers of *L. monocytogenes* compared to untreated cheese when cheese is made at each pH tested; however, higher *L. monocytogenes* numbers were observed in nisin-treated cheese made at pH 5.5 compared to nisin-treated cheese made at pH 6.0 and 6.5. A linear mixed effects model was used to determine whether (i) pH, (ii) day of incubation, (iii) presence or absence of nisin, and (iv) strain as well as interactions between (v) pH and nisin, (vi) nisin and strain, and (vii) nisin and day showed significant effects on log transformed bacterial numbers (Table 3.3). While Figure 3.5 represents the actual observed data, least square means of estimated *L. monocytogenes* counts averaged for all strains from the pH model are shown in Figure 3.6 and estimated for each strain individually in Figure 3.7.

Presence of nisin showed a significant effect ($p < 0.001$) with an effect size of -1.87, which indicates *L. monocytogenes* numbers are 1.87 log cfu/g lower in the presence of nisin treatment (Table 3.3). For cheese made at pH 6.0, average bacterial numbers (across all sampling days and strains) were 6.14 and 8.61 log cfu/g for cheese made with and without nisin, respectively (Figure 3.4). Although nisin-treated cheese showed lower *L. monocytogenes* numbers across pH, the difference between *L. monocytogenes* numbers on treated and untreated cheese varied considerably by pH; for example, the lowest log difference between nisin-treated and untreated cheese for day 1 (0.14 log) was found for strain 5621 on cheese made at pH 5.5, with higher corresponding log differences of 1.39 and 2.22 for pH 6.0 and 6.5, respectively (Figure 3.4). Differences between *L. monocytogenes* numbers on treated and untreated cheese made at pH 5.5 were minimal and ranged from 0.14 to 1.73 log cfu/g, based on sobserved data across strains as compared to 0.33 to 3.6 log cfu/g and 0.49 to 3.47 log cfu/g for pH 6.0 and pH 6.5, respectively (Figure 3.4); Figure 3.5). Similar to results ob-

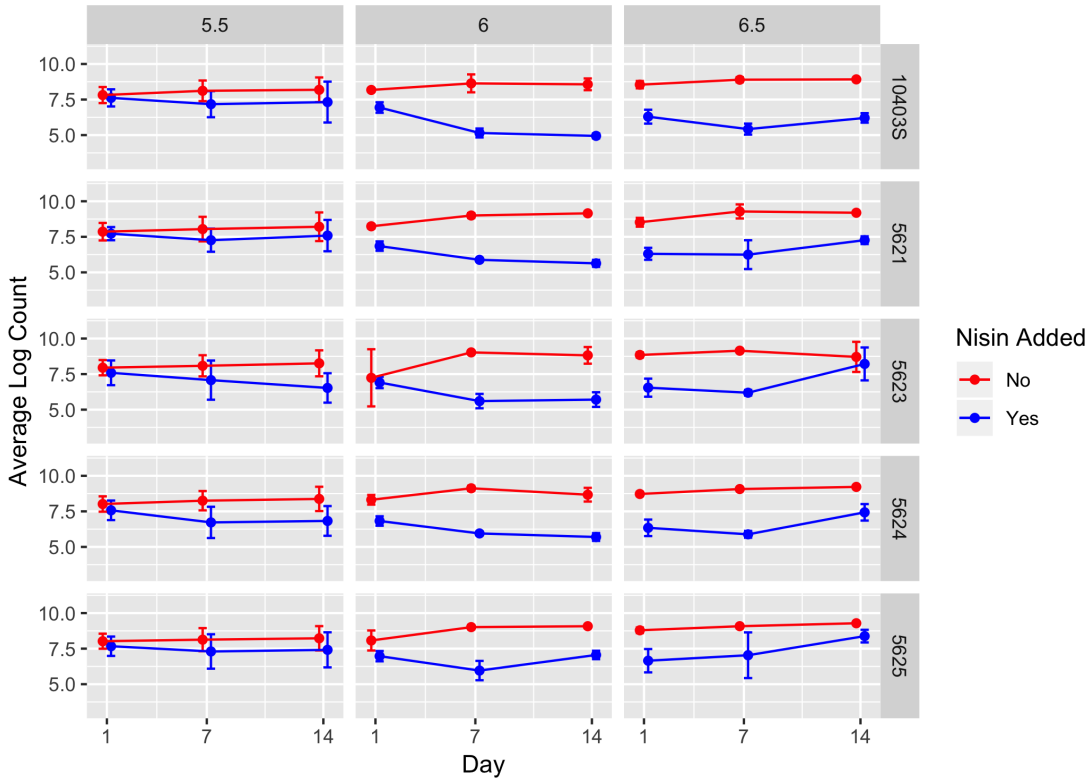

Figure 3.5: Average counts (log cfu/g) of *L. monocytogenes* in the presence (blue line) and absence (red line) of nisin in a lab-scale cheese model. Each cheese was inoculated with a single strain of *L. monocytogenes* (10403S, FSL R9-5621, FSL R9-5623, FSL R9-5624, or FSL R9-5625) to a level of approximately 7 log cfu/g. These results represent the effect of pH (5.5, 6.0, and 6.5) on *L. monocytogenes* sensitivity to nisin. The data points are slightly offset such that the reader can clearly see each point; however, the points still correspond to 1, 7, and 14 days. All values are the arithmetic mean of three independent experiments, and error bars denote standard error. For some data points, error bars are not visible because standard error was too low to yield a visible error bar. All cheese was stored at 6°C.

Table 3.3: Model parameters for all fixed effects in the pH model for *L. monocytogenes* counts

Response variable	Fixed effects	Levels	Estimate	Standard error	p-value	Significance
L. monocytogenes count (log cfu/g)	pH	6.5	Ref[1]			
		6	1.45	0.71	0.053	
		5.5	-2.19	0.56	< 0.001	***
	Nisin	N[2]	Ref			
		Y[3]	-1.87	0.32	< 0.001	***
	Day	1	Ref			
		7	0.52	0.23	0.031	*
		14	0.52	0.23	0.031	*
	Strain	10403S	Ref			
		5621	0.18	0.12	0.127	
		5623	0.02	0.12	0.899	
		5624	0.21	0.12	0.083	
		5625	0.2	0.12	0.092	
	Inoculum[4] (log cfu/g)		0.02	0.09	0.817	
	Milk age[5]		-0.49	0.18	0.015	*
	Milk apc[6] (log cfu/mL)		-0.42	0.2	0.038	*
	pH:Nisin Y	6.5:Nisin Y	Ref			
		6.0:Nisin Y	-0.22	0.33	0.511	
		5.5:Nisin Y	1.44	0.33	< 0.001	***
	Nisin Y:Day	Nisin Y:1	Ref			
		Nisin Y:7	-1.18	0.33	< 0.001	***
		Nisiin Y:14	-0.69	0.33	0.042	*
	Nisin Y:Strain	Nisin Y:10403S	Ref			
		Nisin Y:5621	0.23	0.16	0.163	
		Nisin Y:5623	0.34	0.16	0.035	*
		Nisin Y:5624	0.03	0.16	0.848	
		Nisin Y:5625	0.61	0.16	< 0.001	***

[1]Ref indicates reference; therefore, estimates, standard error, and p-values are not calculated
[2]N denotes the absence of phage
[3]Y denotes the presence of phage
[4]Avererage number (log cfu/g) of *L. monocytogenes* inoculated onto each cheese
[5]Age of milk when cheese was made, based on a 21-day code date
[6]Bacterial aerobic plate counts (apc; log cfu/mL) in milk before cheese was made
***$p < 0.001$; **$p < 0.01$; *$p < 0.05$

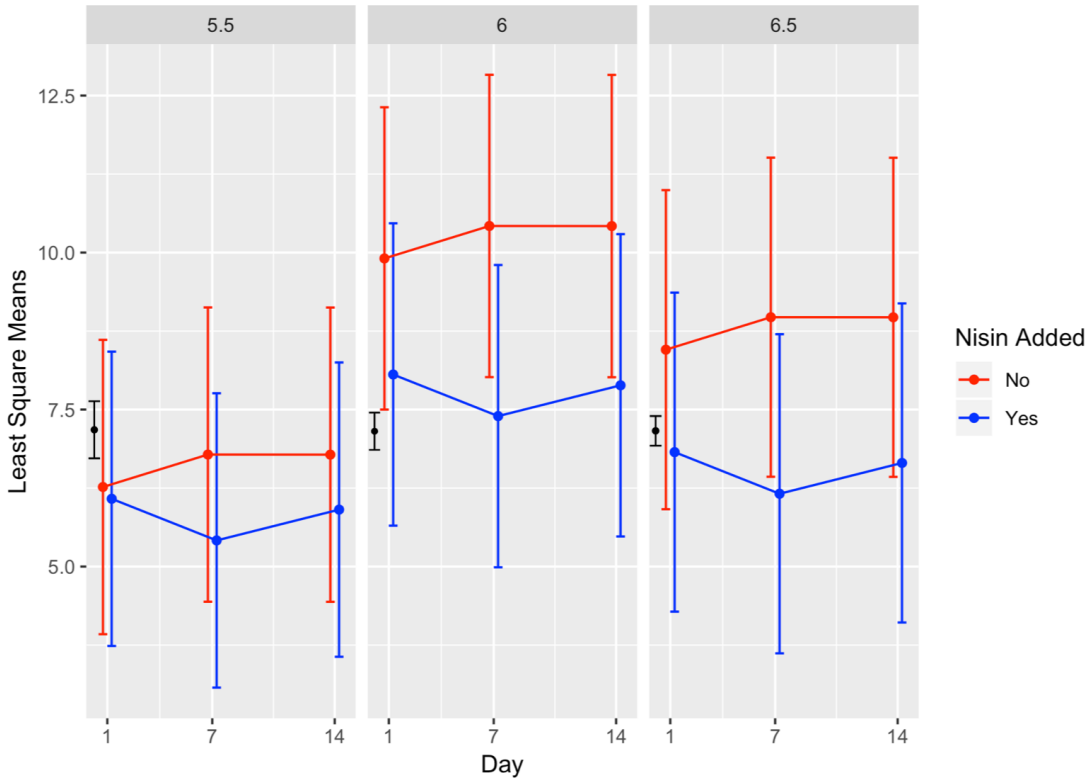

Figure 3.6: Least square means of estimated *L. monocytogenes* numbers (log cfu/g) calculated from the pH model, which are based on data shown in Figure 3.5. Calculated initial (day 0) *L. monocytogenes* number based on the average inoculum level (approximately 7 log cfu/g) are shown in black. Predicted numbers for cheese made with nisin are shown in blue, while predicted numbers for cheese made without nisin are shown in red. These results represent the predicted effect of pH (5.5, 6.0, and 6.5) on *L. monocytogenes* sensitivity to nisin across strains (10403S, FSL R9-5621, FSL R9-5623, FSL R9-5624, and FSL R9-5625). The data points are slightly offset such that the reader can clearly see each point; however, the points still correspond to 1, 7, and 14 days. Error bars denote the 95% confidence interval. All cheese for these predictions was stored at 6°C.

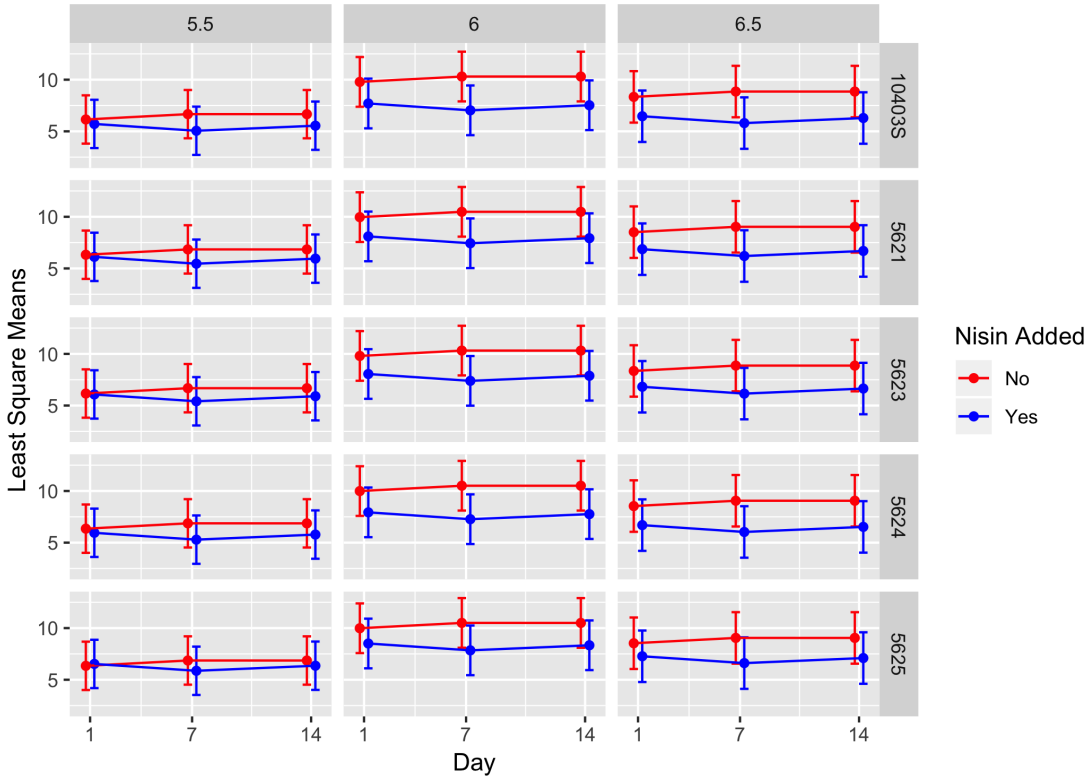

Figure 3.7: Least square means of estimated *L. monocytogenes* numbers (log cfu/g) calculated from the pH model, which is based on data shown in Figure 3.5. Predicted numbers for cheese made with nisin are shown in blue, while predicted numbers for cheese made without nisin are shown in red. These results represent the predicted effect of pH (5.5, 6.0, and 6.5) on *L. monocytogenes* (10403S, FSL R9-5621, FSL R9-5623, FSL R9-5624, and FSL R9-5625) sensitivity to nisin. The data points are slightly offset such that the reader can clearly see each point; however, the points still correspond to 1, 7, and 14 days. Error bars denote the 95% confidence interval. All cheese for these predictions was stored at 6°C.

tained from the temperature model, *L. monocytogenes* still grew on nisin-treated cheese, but only on cheese made at pH 6.5 (Figure 3.5) where, by day 14, all *L. monocytogenes* strains had higher numbers for nisin-treated cheese compared to day 7.

We found a significant interaction effect between nisin and pH 5.5 with an effect size of 1.44, indicating 1.44 log cfu/g higher *L. monocytogenes* numbers relative to nisin treatment at pH 6.5. The fact that nisin seemed less effective against *L. monocytogenes* on cheese made at pH 5.5 is surprising considering that nisin is more stable at lower pH [129]; thus, one could have hypothesized that nisin would have effectively killed *L. monocytogenes* at this pH.

We also found significant interaction effects between presence of nisin and strains 5623 and 5625, the two 4b strains used here ($p = 0.035$; effect size of 0.34 and < 0.001; effect size 0.61, respectively); this indicates that these strains show 0.34 and 0.61 log cfu/g higher numbers in the presence of nisin as compared to the reference strain 10403S.

Presence of bacteria in the milk ($p = 0.038$) and the age of the milk ($p = 0.015$) also showed a significant effect on *L. monocytogenes* numbers with an effect size of -0.42 and -0.49, respectively, indicating that older milk could have more native bacteria present, which can reduce *L. monocytogenes* numbers by 0.43 to 0.49 log cfu/g.

3.4.3 *L. monocytogenes* Reduced Sensitivity to Nisin on Lab-scale Cheese Made at pH 5.5 is at least Partially *dltA*- and *mprF*-dependent

As mentioned before, the reduced nisin sensitivity effect observed for strains growing in cheese made at pH 5.5 was surprising given the fact that nisin should be stable at this pH [129]. We hypothesized that this effect could be due to (i) a more pronounced loss of nisin in the whey for cheese made at pH 5.5 compared to pH 6.5 as nisin is more soluble at lower pH [130] or (ii) a response in cells grown at pH 5.5 that involves the nisin resistance genes *dltA* and *mprF*.

In order to test whether nisin loss was responsible for the reduced sensitivity of *L. monocytogenes* to nisin on cheese made at pH 5.5, 25 ppm nisin was added to the milk, and cheese was made at pH 5.5, 6.0, and 6.5. After extracting nisin from each cheese, soft agar diffusion assays were performed using the nisin sensitive strain *P. cerevisiae* E66 and the diameter of the zones of inhibition caused by the extracted nisin were measured (Figure 3.8). A one-way ANOVA was used to determine differences in zones of inhibition between the nisin-extracted samples; the results show there was no significant difference among diameters of the zones of inhibition ($p = 0.31$; Table 3.4), indicating that nisin loss in the whey is similar regardless of the pH at which the cheese was made, therein nisin loss does not explain the decreased ability of nisin to kill *L. monocytogenes* observed for cheese made at pH 5.5.

Table 3.4: Observed variance of *L. monocytogenes* killed by nisin extracted from cheese made at different pH

	Df	Sum Sq	Mean Sq	P-value
pH	1	3.79	3.79	0.31
Residuals	4	11.2	2.81	

Df - degrees of freedom
Sum sq - sum of squares
Mean sq - mean squares

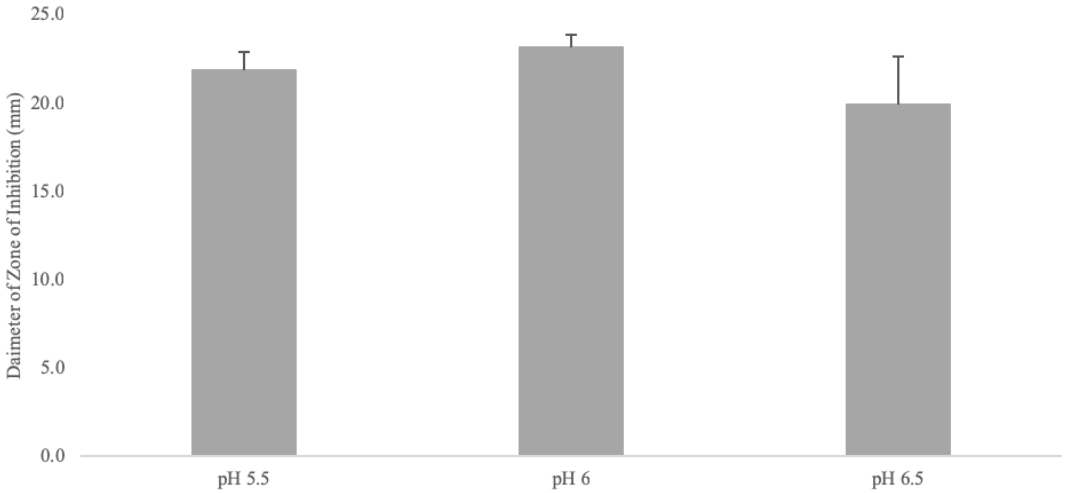

Figure 3.8: Average size (mm) of zone of inhibitions of a 25 ppm nisin control and nisin extracted from cheese made at pH 5.5, 6.0, and 6.5 against *P. cerevisiae*. Bars that do not share any letters represent values that are significantly different. Results are of two biological replicates. Error bars denote standard error.

To determine whether the significant interaction effect between nisin and pH 5.5 involves the activity of nisin resistance genes (*dltA* and *mprF*), nisin (2 ppm) was added to the milk, and cheese was made at pH 5.5, 6.0, and 6.5 prior to *L. monocytogenes* (10403S, Δ*dltA*, Δ*mprF*, and Δ*dltAmprF*) surface inoculation. Bacterial numbers were quantified after storage at 6°C for 1 day and the log reduction (between untreated cheese and nisin-treated cheese) was calculated (Figure 3.9). A two-way ANOVA was used to determine differences in log reduction of *L. monocytogenes* between (i) strain, (ii) pH, and (iii) the interaction between strain and pH. Estimated marginal means were used to determine if the log reduction at each pH differs within strain.

Strain showed an expected significant effect ($p < 0.001$) on *L. monocytogenes* log reduction in the presence of nisin with larger log reductions (1.75 to 5.39 log cfu/g) in deletion mutant strains, especially Δ*mprF* and the double mutant Δ*dltAmprF* compared to the reference strain 10403S (0.31 to 1.23 log cfu/g) (Figure 3.9). In addition, pH 5.5 had a significant overall effect on the activity of nisin, with a more pronounced effect for Δ*dltA* and Δ*mprF* (Figure 3.9). While pH 5.5 significantly reduces nisin sensitivity in the Δ*dltA* and Δ*mprF* mutants, pH 5.5 does not reduce nisin sensitivity in the double mutant, suggesting that the pH effect observed at pH 5.5 involves both *dltA* and *mprF* (Figure 3.9).

3.5 DISCUSSION

While this study indicates that on average nisin reduces *L. monocytogenes* numbers on the lab-scale cheese model, we also show that pH, temperature, and strain have significant effects on the efficacy of nisin treatment in cheese.

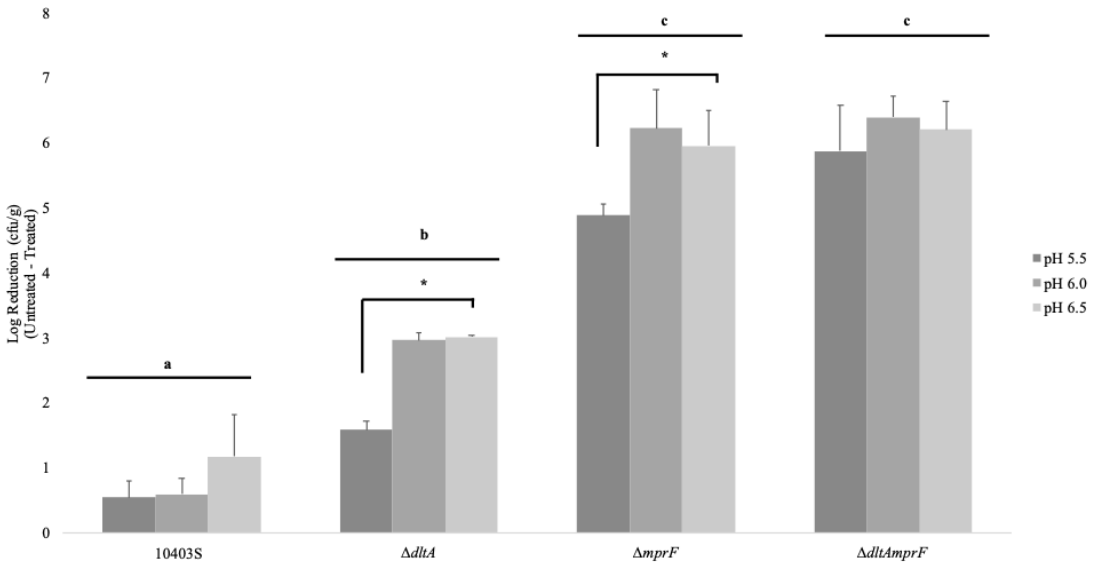

Figure 3.9: Log reduction of *L. monocytogenes* strains on cheese made at pH 5.5, 6.0, and 6.5 between untreated and treated cheese. These results represent the effect of pH on *L. monocytogenes* (10403S, Δ*dltA*, Δ*mprF*, and Δ*dltAmprF*) sensitivity to nisin. Bar groups that do not share any letters represent values that are significantly different between strains. Asterisks denote a significant difference between pH 5.5 and 6.5 within a strain. Results are an average of three biological replicates. Error bars denote standard error.

These findings are increasingly important as they suggest the need to consider environmental conditions to optimize the effectiveness of nisin treatment on foods.

3.5.1 The Effectiveness of Nisin is Enhanced at Lower Storage Temperatures

While our data support that nisin can significantly decrease *L. monocytogenes* numbers in cheese, we also found that (i) the effectiveness of nisin appears to be enhanced when cheese is stored at lower temperatures and (ii) *L. monocytogenes* growth can occur in the presence of nisin and more rapid growth is seen when cheese is stored at higher temperatures.

We found that *L. monocytogenes* grew approximately 1 log higher on nisin-treated cheese stored at 14 and 22°C compared with nisin-treated cheese stored at 6°C (Figure 3.2), consistent with previous observations that *L. monocytogenes* can grow in the presence of nisin on cheese [131, 132].

Contrary to our results, previous studies have shown that nisin is more effective at killing *L. monocytogenes* at higher temperatures. For example, a study showed that *L. monocytogenes* grown in BHI broth at 10°C was more sensitive to nisin than cells grown at 30°C [133]. The differences observed between the effect of temperature on nisin-mediated *L. monocytogenes* killing in our study compared to others could be due to a decreased ability of nisin to bind to *L. monocytogenes* grown on a food matrix relative to *L. monocytogenes* grown in liquid media.

In addition to the temperature effect observed on nisin efficiency, our data also shows that *L. monocytogenes* growth occurs, even in the presence of nisin, and more rapid growth is seen when cheese is stored at higher temperatures. This finding is consistent with other studies that have reported a transient bactericidal effect against *L. monocytogenes*, followed by regrowth of cells in food matrices and laboratory media supplemented with nisin [134]. Overall, our data as well as previous data indicate the potential for regrowth of *L. monocytogenes* during long-term refrigerated storage, even if nisin treatment provided an initial *L. monocytogenes* reduction. Hence, studies over product shelf-life that consider storage time and temperature before consumption are essential to appropriately assess the effectiveness of nisin treatment and provide a more accurate evaluation of the usefulness of nisin applications. There are a number of possible reasons for re-growth of *L. monocytogenes* after initial significant reduction, including, but not limited to, (i) too low a concentration of nisin to kill all cells; (ii) emergence and subsequent growth of nisin resistant mutants [135]; and (iii) binding of nisin to food matrix components, such as fat [116], which decrease nisin action and therefore, its ability to kill *L. monocytogenes*

3.5.2 The effect of pH 5.5 to Decrease Nisin Efficiency Against *L. monocytogenes* is Partially due to the Activity of *dltA* and *mprF*

Our data showed that when cheese is formulated at pH 5.5, nisin is less effective at killing *L. monocytogenes* compared to when cheese is made at pH 6.5. *L. monocytogenes* encounters many stresses in a food environment, and tol-

erance to a stress condition could lead to cross-protection against a subsequent stress [95, 86, 93]. For example, osmotic stress has been reported to induce cross-protection against nisn in *L. monocytogenes* [93]. Previous work by our group indicated that LiaR, the response regulator of the LiaFSR three-component system contributes to nisin resistance in *L. monocytogenes* upon exposure to salt stress Δ*liaR* strains were more senstive to nisin than wild-type strains [93].

Reduced sensitivity to nisin, independent of *dltA* and *mprF*, has also been associated with changes in cell membrane fatty acid composition, resulting in more rigid membrane fluidity [95, 136]. Changes in cell membrane composition can be attributed to acid tolerance in *L. monocytogenes*, in which diffusion of fatty acids across the membrane is inhibited resulting in more rigid cell membranes [137], partially decreasing sensitivity to nisin. Futhermore, it has been shown that *L. monocytogenes* uses a glutamate decarboxylase (gad) [138, 94, 139] system to survive acid stress. GadD1 catalyzes the breakdown of glutamate into γ-aminobutyrate and carbon dioxide, forming ATP, which could restore intracellular levels of ATP that are depleted by nisin activity on the cell [95], and thus lead to reduced sensitivity to nisin. Given that *gad* plays a role in *L. monocytogenes* survival in acidic conditions, our results indicate that *L. monocytogenes* reduced sensitivity to nisin observed at pH 5.5 could only be partially due to the activity of *dltA* and *mprF*.

We also show that the decreased effect of nisin against *L. monocytogenes* in cheese formulated at pH 5.5 is at least partially dependent on the presence of nisin-resistance genes *dltA* and *mprF*. While our results show a greater sensitivity to nisin in *dltA* and *mprF* mutant strains compared to 10403S, pH 5.5 still showed a protective effect that was more pronounced for the single mu-

tants compared to the double mutant. Therefore, we can conclude that reduced sensitivity to nisin at pH 5.5 could be partially due to the activity of *dltA* and *mprF*, which confer nisin resistance in *L. monocytogenes*. Wall teichoic acids (WTAs) are a main component of the Gram-positive bacterial cell wall and are highly negatively charged due to deprotonized phosphate groups. It has been shown experimentally, that Gram-positive bacteria, such as *Staphylococcus aureus* [77], *Clostridium difficile* [76], *Bacillus cereus* [2], and *L. monocytogenes* (Reichmann et al., 2013) can resist interactions with bacteriocins by upregulating the *dlt* operon, which encodes proteins that incorporate D-alanine residues onto teichoic acids, reducing the net negative charge of the cell wall [81]. Additionally, the *mprF* gene facilitated nisin resistance by encoding the MprF protein, which reduces the net negative charge of the cell membrane by synthesizing lysylphosphatidylglycerols and adding L-lysine to phosphatidylglycerol, which results in a positive charge [83, 85]. As bacteriocins are positively charged, the activity of *dltA* and *mprF*, will result in inhibition of nisin action on the cell wall; therefore, resulting in a reduction in *L. monocytogenes* sensitivity to nisin.

3.5.3 *L. monocytogenes* Serotype Affects the Efficiency of Nisin

In addition to the effects of environmental conditions, our data also indicate that serotype 4b strains showed reduced sensitivity to nisin across the temperature and pH conditions tested. These findings are consistent with previous work that supports that serotype 4b strains are more resistant to nisin compared to other *L. monocytogenes* serotypes [93, 140]. For example, a study reported that the effec of nisin was strain dependent as the Scott A (4b) strain appeared to be more resistant compared to other serotypes [141]. It would be interesting to test

other serotype 1/2a and 4b strains (from other sources in addition to those associated with cheese outbreaks) to compare their sensitivity to nisin to the strains tested here.

Our data is also consistent with a previous study that found that serotype also affects the effectiveness of phage treatment against *L. monocytogenes* in a lab-scale cheese model, although the overall phage-dependent reduction was limited [121]. Given that both phage and nisin target the bacterial cell envelope, both findings suggest that serotype 4b strains might have adapted resistance to cell envelope-acting antimicrobials. Although, recently serotypes 1/2a, 1/2b, and 4b strains have been linked to a number of Hispanic-style cheese outbreaks [142, 143, 144, 19], historically, isolates of serotype 4b strains have caused the greatest proportion of listeriosis outbreaks and the largest number of outbreak-associated cases compared to serotype 1/2a and 1/2b strains [145]. Differences in nisin sensitivity among *L. monocytogenes* serotypes highlight the importance of validating nisin-based treatment strategies using different *L. monocytogenes* serotypes and environmental conditions relevant to a given application.

3.6 CONCLUSIONS

This study shows the critical role of temperature, pH, and *L. monocytogenes* serotype in the effectiveness of antimicrobials intended for food preservation. Improved understanding of how environmental conditions affect antimicrobial efficiency could facilitate the development and/or effective implementation of control strategies. Additionally, the potential for cross-protection induced by food-relevant stress (e.g. pH) should be considered to avoid overestimation of

antimicrobial strength in a food product. This is important as our data suggest that nisin-based control strategies could have limited impact on reducing public health risks if cheese is made at pH 5.5 or stored at 14 or 22°C or higher and consumed after 14 days, while the impact of nisin treatment could be substantial if cheese is typically stored at refrigeration temperatures and consumed within a few days after production.

CHAPTER 4

ENVIRONMENTAL CONDITIONS AND SEROTYPE AFFECT *LISTERIA MONOCYTOGENES* SUSCEPTIBILITY TO PHAGE TREATMENT IN A LABORATORY CHEESE MODEL

4.1 ABSTRACT

Listeria monocytogenes can survive and grow in a variety of environments, including refrigeration, making it difficult to control and highlighting the importance of optimizing control strategies against this pathogen. *Listeria*-phages are attractive biocontrol agents because phages bind to specific wall teichoic acids (WTA) on the bacterial cell wall, enabling inhibition of pathogens without disrupting the normal microbiota or structure of the food. Common stresses found on dairy products can affect cell wall composition and structure and subsequently affect the efficiency of control strategies that target the cell wall. The goal of this study was to determine the effect of a range of pH and temperatures on the effectiveness of a commercial phage cocktail treatment against several strains of *L. monocytogenes* in a cheese matrix. We developed a lab-scale, cheese model that was made at different pH, treated with phage, and then inoculated with *L. monocytogenes*. Cheese was incubated at 6, 14, or 22∘C for 14 days, and bacterial numbers were quantified on days 1, 7, and 14. Our data show that phage treatment has a limited ability to reduce *L. monocytogenes* numbers at each temperature tested; however, it is more effective on specific strains of *L.*

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monocytogenes when cheese is stored at higher temperatures. More specifically, the average *L. monocytogenes* numbers on phage-treated cheese stored at 22∘C were significantly lower than numbers on phage-treated cheese stored at 6 or 14∘C. Similarly, phage treatment was significantly more effective at inhibiting *L. monocytogenes* numbers on cheese made at higher pH (6 and 6.5) compared to numbers observed on cheese made at pH 5.5 where *L. monocytogenes* did not grow. Furthermore, serotype was found to also affect the susceptibility of *L. monocytogenes* to phage treatment; serotype 1/2 strains showed significantly higher susceptibility to phage treatment than serotype 4b strains. Overall, our results suggest the importance of considering the efficacy of phage under conditions (i.e. temperature and pH) specific to a given food matrix when applying interventions against this important foodborne pathogen.

4.2 INTRODUCTION

L. monocytogenes is a Gram positive foodborne pathogen that can cause severe listeriosis in susceptible populations, such as immunocompromised individuals, pregnant women, newborns, and elderly adults [15]. This bacterium is the third leading cause of death related to foodborne illness in the United States [146], and the Centers for Disease Control and Prevention (CDC) estimate that*L. monocytogenes* causes approximately 1600 cases annually, including 260 deaths [1, 19]. *L. monocytogenes* also causes disease in animals and can be isolated from both natural and food-processing environments [4].

Different serotypes of *L. monocytogenes* are associated with specific environments and disease cases [8]. *L. monocytogenes* consists of at least four lineages:

I, II, III, and IV [11, 12, 13, 14, 15]. Most *L. monocytogenes* isolates belong to lineages I and II, which contain serotypes more commonly associated with human clinical cases, including serotype 1/2a (lineage II) and serotypes 1/2b and 4b (lineage I) [8]. Lineage II strains are common in food products, widespread in natural and farm environments, and are less frequently isolated from human clinical cases [16, 17]. Most human listeriosis outbreaks are associated with lineage I isolates [18]. Lineage III and IV strains are rare and predominately isolated from animal sources [13, 17] and therefore, were not included among the strains used in this study.

L. monocytogenes contamination is a concern in dairy processing environments based on its ability to grow in a wide range of temperatures (0 to 45°C), pH (4.4 to 9.4), and high salt concentrations (13-14 w/v%) [20]. *L. monocytogenes* ability to rapidly adapt to changing environmental conditions enables it to survive harsh environments during food-processing. Since 2006, the number of listeriosis outbreaks in the United States associated with cheese, two-thirds of which were Hispanic-style cheese, has increased [4], which continues to raise concerns regarding *L. monocytogenes* contamination of fresh cheese and increases the need for precise intervention strategies. Soft-ripened or unripened cheese made from raw or improperly pasteurized milk is most commonly associated with disease outbreaks in cheese in the United States [4]. More specifically, the majority of dairy-associated foodborne outbreaks in the United States are linked to the Hispanic-style fresh cheese, queso fresco [4]. Hispanic-style fresh cheese is characterized by high water activity (a_w), low salt content, and near neutral pH, which creates an ideal environment for survival and growth of a number of foodborne pathogens [21], specifically *L. monocytogenes* [4, 102].

One possible strategy to prevent problems associated with *L. monocytogenes* contamination in food and food-processing environments is the use of lytic bacteriophages or phages [22]. Phages are the most abundant organisms on Earth, can be isolated from various environments, including food, and are also components of the human gut microbiota [147]. Lytic phage infects bacteria, resulting in lysis of the bacterial cell. Phage recognizes and kills specific target bacteria; therefore, phage-based interventions can target specific foodborne pathogens without disrupting the normal and beneficial microflora of foods [24]. A number of phage-based preparations have been approved for food applications in the United States and Europe such as ListShield™, Listex P-100™, EcoShield™, SalmoFresh™, and Salmonelex™. While some studies have shown that these phage preparations can decrease *L. monocytogenes* [148, 149], *E. coli* [150], and *Salmonella* [151] numbers on produce, dairy, and meat products, whether they can reduce pathogen prevalence or outbreak incidence remains to be determined. Furthermore, the efficacy of phage has been shown to vary in different food matrices and under different environmental conditions [152].

The objective of this study was to investigate the effect of environmental conditions (e.g. temperature and pH) on *L. monocytogenes* sensitivity to a commercial phage cocktail in a lab-scale cheese model. Effective and improved prevention of *L. monocytogenes*-related foodborne outbreaks and infections will require thorough understanding of the effects of the environment on *L. monocytogenes* sensitivity to treatments in order to allow for appropriate development and application of control strategies.

4.3 MATERIALS AND METHODS

4.3.1 Bacterial Strains and Growth Conditions

We obtained four recent cheese outbreak strains that encompass the most common *L. monocytogenes* serotypes (4b, 1/2a and 1/2b) (Table 4.1). *L. monocytogenes* strain 10403S (serotype 1/2a) was used as a reference in this study. For all experiments, strains were streaked from frozen brain heart infusion (BHI; Difco, Becton Dickinson and Co., Sparks, MD) stocks, stored at -80°C in 15% glycerol, onto a BHI agar plate, followed by incubation at 37°C for 24 h. A single colony was subsequently inoculated into 5 mL of BHI broth in 16 mm tubes, followed by incubation at 37°C with shaking (230 rpm) for 16 h (Series 25 Incubator, New Brunswick Scientific, Edison, NJ). After 16 h, 50 μL BHI culture was inoculated into fresh 5 mL BHI broth and grown to OD_{600} = 1.0 at 30°C.

4.3.2 Lab-scale Cheese Model

We modified a previously described method [3] to make approximately 10 g miniature cheese in 6-well plates. Briefly, using aseptic technique, 600 mL of pasteurized, non-homogenized, whole milk (Trinity Valley Dairy, purchased at retail in 1 gallon containers) was warmed to 35°C. $CaCl_2$ (Dairy Connection Inc., Madison, WI) was added to milk to a final concentration of 1 mg/mL and then the milk was combined with 6 mL rennet solution (90 μL double strength vegetable rennet (Chr. Hansen, CHY-Max, Milwaukee, WI) diluted in 5910 μL of sterile water). To account for bacteria present and to ensure that no *L. monocytogenes* was in the milk before cheese was made, milk samples were plated on

Table 4.1: *L. monocytogenes* strains used in this study

FSL ID	Previous ID	Outbreak	Source Type	Source Site	Serotype	Reference
FSL X1-0001	-	Lab strain 10403S	Human	Skin lesion	1/2a	Bishop and Hinrichs (1987)
FSL R9-5621	2021L-5324	2012 Ricotta cheese	Food	Cheese	1/2a	CDC (2012)
FSL R9-5623	2013L-5223	2013 Semi-soft fresh-style cheese	Human	Placenta	4b	CDC (2013)
FSL R9-5624	2014L-6028	2013 Queso fresco	Human	Blood	1/2b	CDC (2014)
FSL R9-5625	2014L-6388	2014 Soft cheese	Human	Blood	4b	CDC (2015)

FSL: Food Safety Laboratory, Cornell University

plate count agar (PCA; Difco, Becton Dickinson and Co.) and on *L. monocytogenes* plating medium (LMPM; Difco, Becton Dickinson and Co.). After 48 h of incubation at 32°C for PCA and 24 h of incubation at 30°C for LMPM, colonies were counted using a Q Count Colony Counter (Advanced Instruments, Norwood, MA).

To make cheese at different pH, at a range characteristic of different cheese types, milk was acidified with the addition of vinegar (white, distilled; Wegmans) to reduce the pH from 6.5 to 6.0 or 5.5 prior to the addition of $CaCl_2$ and rennet. While acetic (vinegar), citric, and lactic acid are commonly used when making fresh cheese [153], the textural properties of queso blanco-type cheese were rated higher by consumers for cheese made with acetic acid [154, 153]. To ensure the desired pH was achieved, the pH of the milk was measured using a surface pH meter (Mettler Toledo). The milk was then poured into mini vats and incubated at 35°C for 45 min in a water bath. The curds were then cut using modified cheese harps. After cutting, curds were returned to the water bath and heated, while the temperature of the water bath was increased progressively until reaching 40°C (approximately 30 min). Whey (60 mL) was removed and replaced with 60 mL of NaCl solution (0.16 g/mL). The curds were stirred gently with a plastic sterile loop and returned to the water bath for an additional 20 min incubation at 40°C. After incubation, the remaining whey was drained, and 15 g of curd were scooped into each well of a 6-well plate. Plates were covered and pressed with 30 mm caps for 2 h at room temperature. Cheese was subjected to analyses to determine pH and a_w (Aqualab Series 4TE, FF Instrumentation, Christchurch, NZ) throughout storage (Table 4.2). All samples fell within the range of compositional values expected in commercial fresh cheese [155, 156].

Table 4.2: Proximate data of the lab-scale model cheese and a commercial cheese

Cheese	Temperature (°C)	Day	pH	a_w	Reference
Lab-scale (pH 6.5)	6	1	6.49 ± 0.03	0.9893	This study
		7	6.53 ± 0.01	0.9893	
		14	6.49 ± 0.00	0.9872	
	14	1	6.46 ± 0.19	0.9921	
		7	6.40 ± 0.22	0.9937	
		14	5.81 ± 0.03	0.991	
	22	1	6.36 ± 0.19	0.9903	
		7	6.45 ± 0.24	0.9904	
		14	5.95 ± 0.30	0.9920	
Lab-scale (pH 6.0)	6	1	5.99 ± 0.00	0.9947	This study
		7	5.92 ± 0.15	0.9920	
		14	5.70 ± 0.17	0.9891	
Lab-scale (pH 5.5)	6	1	5.69 ± 0.02	0.9973	This study
		7	5.63 ± 0.00	0.9936	
		14	5.62 ± 0.02	0.9862	
Queso fresco	-	-	5.30 to 6.50	0.9900	Hnosko et al. 2008 and Trmcic et al. 2016

4.3.3 Growth of *L. monocytogenes* in the Lab-scale Cheese Model Containing a Commercial *Listeria*-phage Cocktail

Immediately after cheese was made, phage was applied to the surface of cheese by evenly pipetting 200 μL of Listshield^{TM}(Intralytix, Baltimore, MD), an FDA-approved commercial *Listeria*-phage cocktail, consisting of a mixture of equal proportions of six lytic bacteriophages, specifically effective against *L. monocytogenes* serotypes, to cover the surface of the 10 g cheese for a target final concentration of approximately 8 x 10^6 pfu/g or 9 x 10^6 pfu/cm^2 (the recommended dose by the manufacturer is between 1-4 mL/lb of a working stock that has been diluted to 1 x 10^9 pfu/mL). Following 30 min of incubation at room temperature, each of the cheese samples were then surface inoculated by spotting 100 μL of one of the 5 selected *L. monocytogenes* strains at a level of approximately 10^5 cfu/g, with an additional un-inoculated cheese control. Plates of six cheese per plate were covered and incubated at 6, 14 or 22°C for 1, 7, or 14 days, the typical shelf-life for a fresh cheese [157].

At 1, 7, or 14 days post inoculation, cheese was diluted 1/10 with PBS and homogenized using a Stomacher (Seward., Worthing, UK). Homogenates were then serially diluted and plated on modified Oxford agar (MOX; Difco, Becton Dickinson and Co.) using an Autoplate spiral plating system (Advanced Instruments.) for *L. monocytogenes* quantification. After 48 h of incubation at 30°C [123], colonies were counted using a Q Count Colony Counter (Advanced Instruments). Experiments were performed in at least biological triplicate.

4.3.4 Statistical Analyses

All statistical analyses were carried out in R Statistical Programming Environment [126]. We constructed individual linear mixed effects models for temperature and pH using the ”lmer” function in the ”lme4” package [127]. For each model, the response was log cfu/g of the number of *L. monocytognes* (log count) and random effects were (i) replicates and (ii) plate nested within milk batch. Fixed effects were (i) temperature or pH, (ii) day of incubation, (iii) presence or absence of phage, (iv) strain, (v) age of the milk (based on a 21-day code date), and (vi) the log cfu/mL of the aerobic plate counts (apc; bacterial counts in the milk before cheese was made). We also included interactions between (i) phage and temperature or pH, (ii) phage and strain, (iii) phage and day, and (iv) day and temperature or pH. Post-hoc pairwise comparisons of means were performed using lsmeans [158]. The cut-off for significance was set at $P < 0.05$. Raw data and the R code used for statistical analyses are available on GitHub at https://github.com/lohenderson/.

4.4 RESULTS

4.4.1 Temperature Affects *L. monocytogenes* Susceptibility to Phage Treatment

To assess the effect of different cheese incubation temperatures on the ability of phage to reduce *L. monocytogenes* loads on cheese, the commercial phage cocktail was added on the surface of cheese made at pH 6.5 from pasteurized

milk prior to surface inoculation of 5 different *L. monocytogenes* strains. *L. monocytogenes* was not detected in the milk or the uninoculated controls (data not shown). *L. monocytogenes* numbers were quantified at day 1, 7, and 14 for cheese incubated at 6, 14, or 22°C (Figure 4.1).

A linear mixed effects model was used to specifically determine whether (i) temperature, (ii) day of incubation, (iii) presence or absence of phage, and (iv) strain as well as interactions between (v) temperature and presence of phage and (vi) temperature and day of incubation, and (vii) strain and presence of phage showed significant effects on log transformed bacterial numbers (Table 4.3). While Figure 4.1 represents the actual observed data, least square means of estimated *L. monocytogenes* counts from the temperature model are shown across all strains in Figure 4.2 and calculated for each strain individually in Figure 4.3.

Presence of phage showed a significant effect ($p < 0.001$) on *L. monocytogenes* numbers with a model-estimated effect size of -1.34 (Table 4.3), indicating 1.34 log lower *L. monocytogenes* numbers in the presence of phage. This supports that phage significantly reduces *L. monocytogenes* numbers in the model cheese, as clearly evident by the data shown in Figure 4.1; for cheese incubated at 6°C, average *L. monocytogenes* numbers (across the three time points) were 6.33 and 7.13 log cfu/g for cheese with and without phage, respectively. Phage-treated cheese also consistently showed lower *L. monocytogenes* numbers at day 1 as compared to untreated cheese (0.76, 1.39, and 2.18 log cfu/g lower average *L. monocytogenes* numbers [across the 5 strains tested] for 6, 14, and 22°C, respectively).

Not surprisingly, both day 7 and 14 also had significant effects on *L. monocytogenes* numbers with higher numbers at both days as compared to the reference

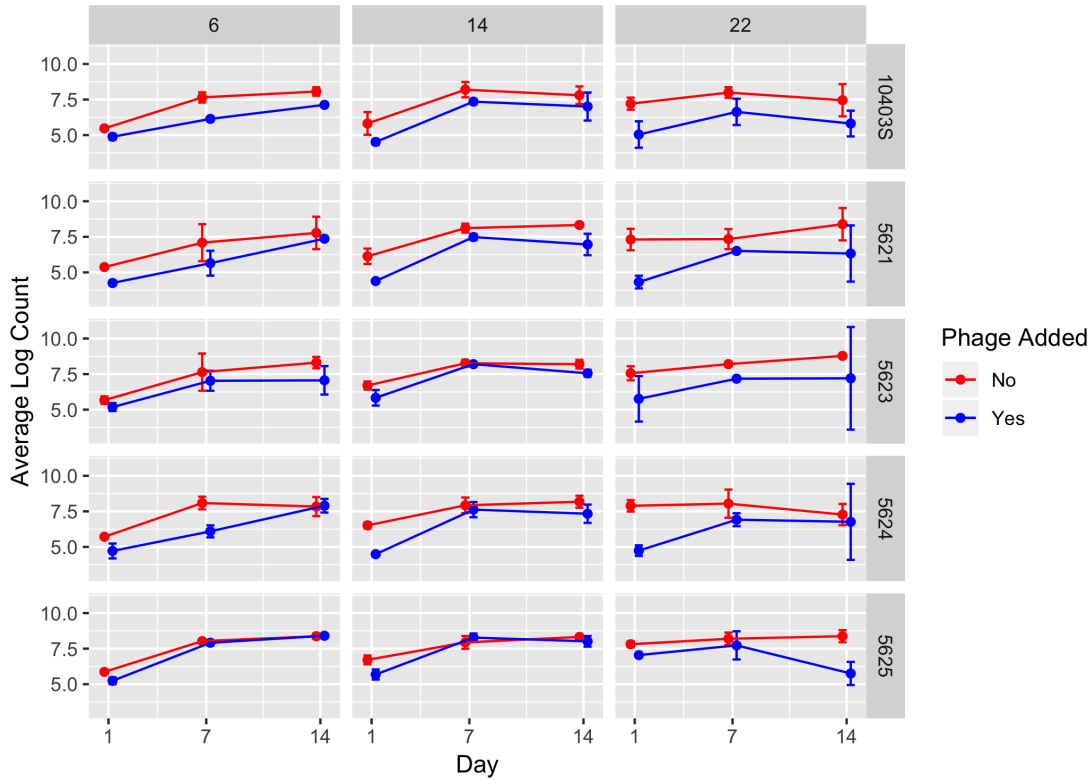


Figure 4.1: Average counts (log cfu/g) of *L. monocytogenes* in the presence (blue line) and absence (red line) of phage treatment in a lab-scale cheese model. Each cheese was inoculated with a single strain of *L. monocytogenes* (10403S, FSL R9-5621, FSL R9-5623, FSL R9-5624, or FSL R9-5625) to a level of approximately 5 log cfu/g. These results represent the effect of temperature (6, 14, and 22°C) on *L. monocytogenes* sensitivity to a commercial phage cocktail. The data points are slightly offset such that the reader can clearly see each point; however, the points still correspond to 1, 7, and 14 days. All values are the arithmetic mean of three independent experiments, and error bars denote standard error. For some data points, error bars are not visible because standard error was too low to yield a visible error bar. All cheese was made at pH 6.5.

Table 4.3: Model parameters for all fixed effects in the temperature model for *L. monocytogenes* counts

Response Variable	Fixed Effects	Levels	Estimate	Standard Error	p-value	Significance
L. monocytogenes count (log cfu/g)	Temperature (°C)	6	Ref[1]			
		14	0.46	0.28	0.110	
		22	1.48	0.32	< 0.001	***
	Phage	N[2]	Ref			
		Y[3]	-1.34	0.27	< 0.001	***
	Day	1	Ref			
		7	1.57	0.26	< 0.001	***
		14	2.35	0.26	< 0.001	***
	Strain	10403S	Ref			
		5621	-0.01	0.14	0.921	
		5623	0.37	0.14	0.011	*
		5624	0.22	0.14	0.131	
		5625	0.42	0.14	0.003	**
	Milk age[4]		0.1	0.07	0.202	
	Milk apc[5] (log cfu/mL)		-0.07	0.10	0.559	
	Temperature:Phage Y	6:Phage Y	Ref			
		14:Phage Y	-0.03	0.26	0.911	
		22:Phage Y	-0.79	0.27	0.006	**
	Phage Y:Day	Phage Y:1	Ref			
		Phage Y:7	0.65	0.26	0.018	*
		Phage Y:14	0.48	0.27	0.085	
	Temperature:Day	6:Day 1	Ref			
		14:Day 7	0.37	0.32	0.257	
		14:Day 14	-0.49	0.32	0.131	
		22:Day 7	-0.89	0.32	0.008	**
		22:Day14	-1.84	0.34	< 0.001	***
	Phage Y:Strain	PhageY:Strain 10403S	Ref			
		Phage Y:Strain 5621	-0.15	0.20	0.456	
		Phage Y:Strain 5623	0.33	0.20	0.107	
		Phage Y:Strain 5624	-0.02	0.20	0.94	
		Phage Y:Strain 5625	0.68	0.20	< 0.001	***

[1]Ref indicates reference; therefore, estimates, standard error, and P-values are not calculated
[2]N denotes the absence of phage
[3]Y denotes the presence of phage
[4]Age of milk when cheese was made, based on a 21-day code date
[5]Bacterial aerobic plate counts (apc; log cfu/mL) in milk before cheese was made
***$p < 0.001$; **$p < 0.01$; *$p < 0.05$

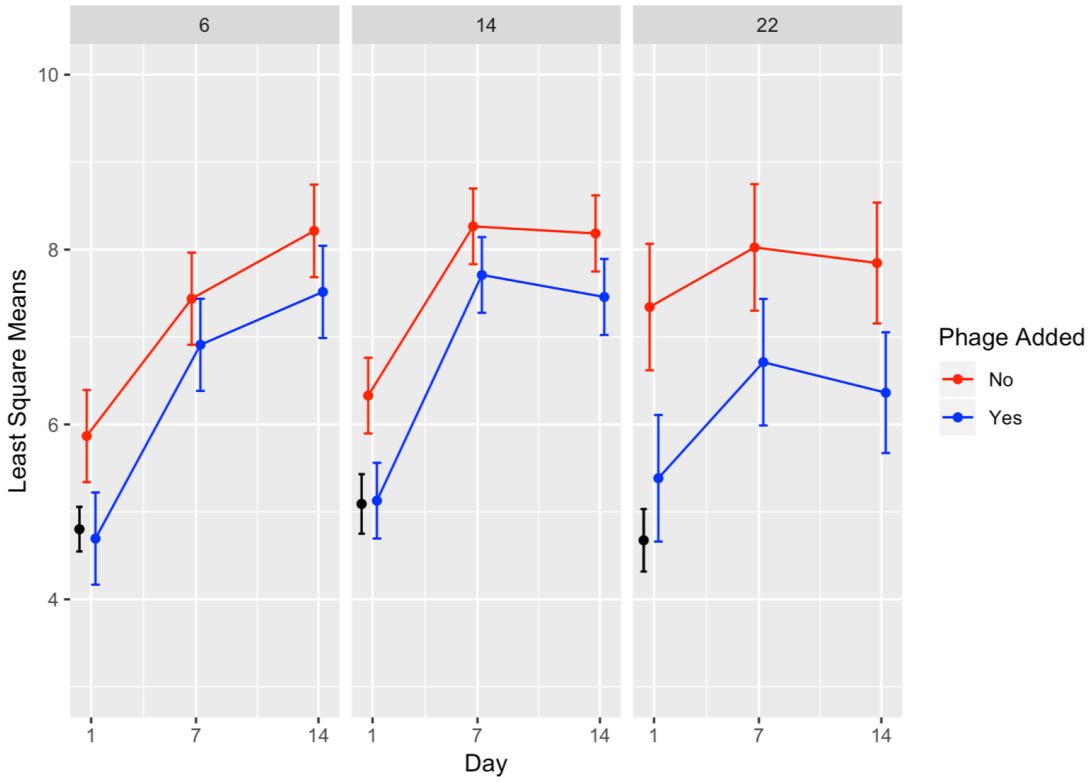


Figure 4.2: Least square means of estimated *L. monocytogenes* numbers (log cfu/g) calculated from the temperature model, which are based on data shown in Figure 4.1. Calculated initial (day 0) *L. monocytogenes* number based on the average inoculum level (approximately 5 log cfu/g) are shown in black. Predicted numbers for cheese treated with phage is shown in blue, while predicted numbers for cheese without phage treatment are shown in red. These results represent the predicted effect of temperature (6, 14, and 22°C) on *L. monocytogenes* sensitivity to a commercial phage cocktail across strains (10403S, FSL R9-5621, FSL R9-5623, FSL R9-5624, and FSL R9-5625). The data points are slightly offset such that the reader can clearly see each point; however, the points still correspond to 1, 7, and 14 days. Error bars denote the 95% confidence interval. All cheese for these predictions was made at pH 6.5.

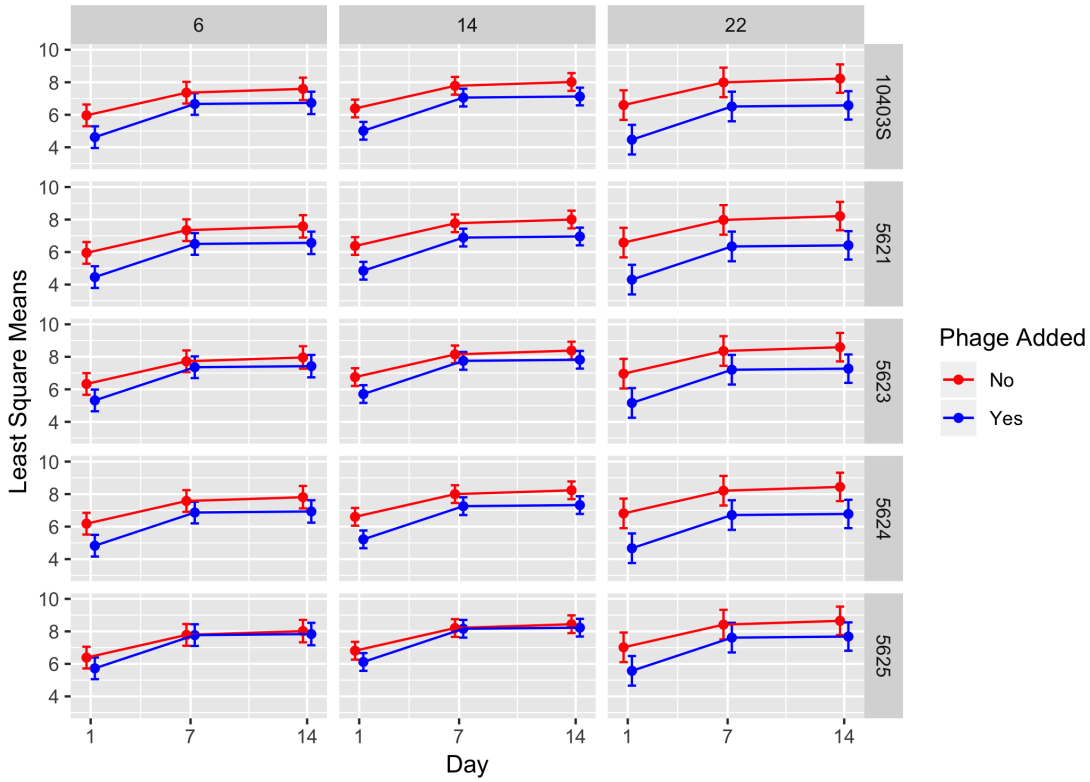


Figure 4.3: Least square means of estimated *L. monocytogenes* numbers (log cfu/g) calculated from the temperature model, which is based on data shown in Figure 4.1. Predicted numbers for cheese treated with phage is shown in blue, while predicted numbers for cheese without phage treatment are shown in red. These results represent the predicted effect of temperature (6, 14, and 22°C) on *L. monocytogenes* (10403S, FSL R9-5621, FSL R9-5623, FSL R9-5624, and FSL R9-5625) sensitivity to a commercial phage cocktail. The data points are slightly offset such that the reader can clearly see each point; however, the points still correspond to 1, 7, and 14 days. Error bars denote the 95% confidence interval. All cheese for these predictions was made at pH 6.5.

(i.e., day 1) (Table 4.3). The significance of day is not surprising considering that *L. monocytogenes* showed growth over time (average counts across strains were 4.85 and 7.57 log cfu/g for day 1 and 14 at 6°C, respectively), consistent with previous observations that *L. monocytogenes* grows at refrigeration temperatures on soft cheeses with pH $>$ 5.9 [159, 148].

Importantly, we found a significant interaction effect (p = 0.006) between phage and storage at 22°C with an effect size -0.79, indicating 0.79 log lower *L. monocytogenes* numbers relative to phage treatment at 6°C. While phage-treated cheese showed lower *L. monocytogenes* numbers across temperatures, the difference between *L. monocytogenes* numbers on treated and untreated cheese varied considerably by temperature; the lowest difference between phage-treated and untreated cheese for day 1 (0.49 log) was found for cheese incubated at 6°C, with higher corresponding differences of 0.86 and 1.80 log for 14 and 22°C, respectively (Figure 4.1). Importantly, however, growth of *L. monocytogenes* was still observed in phage-treated cheese (Figure 4.1) where, for a number of strains, by day 7 and 14 the difference between *L. monocytogenes* numbers on phage-treated and untreated cheese was $<$ 0.5 log. For example, at 14°C, only strain 10403S (both day 7 and 14) and strain 5621 (only day 14) showed $>$ 0.5 log difference between phage-treated and untreated cheese. For example, at 14°C, only strain 10403S (both day 7 and 14) and strain 5621 (only day 14) showed $>$ 0.5 log difference between phage-treated and untreated cheese. Interesting, at higher temperatures (i.e., 14 and 22∘C), we found a few instances where day 14 *L. monocytogenes* numbers in phage-treated cheese were lower than day 7 numbers; this pattern was most pronounced for the serotype 4b strain 5625 (Figure 4.1).

Finally, we also found a significant interaction effect between presence of phage and strain 5625, one of the two 4b strains used here ($p < 0.001$; effect size of 0.68); this indicates that this strain shows 0.68 log higher numbers in the presence of phage as compared to the reference strain 10403S. For strain 5623, the other serotype 4b strains used here, the interaction effect between strain and temperature did not meet the $p < 0.05$ cut-off ($p = 0.107$), but showed the second highest positive effect size (0.33) for the different strain-phage interaction analyses.

4.4.2 pH Affects *L. monocytogenes* Susceptibility to Phage Treatment

To assess the effect of different pH on phage inhibition of *L. monocytogenes* numbers, the commercial phage cocktail was added on the surface of cheese made at pH 5.5, 6.0, and 6.5 prior to *L. monocytogenes* inoculation. *L. monocytogenes* numbers were quantified after storage at 6°C for 1, 7, and 14 days. A linear mixed effects model was used to determine whether (i) pH, (ii) day of incubation, (iii) presence or absence of phage, and (iv) strain as well as interactions between (v) pH and presence of phage, (v) strain and presence of phage, and (vi) pH and day showed significant effects on log transformed *L. monocytogenes* numbers (Table 4.4). Data obtained (Figure 4.4) clearly show *L. monocytogenes* growth at pH 6.0 and 6.5, as well as a reduced effect of phage on *L. monocytogenes* numbers at pH 5.5, relative to pH 6.0 and 6.5. Least square means of estimated *L. monocytogenes* numbers calculated from the pH model across strains (Figure 4.5) and for each strain individually (Figure 4.6) also clearly showed no

Table 4.4: Model parameters for all fixed effects in the pH model for *L. monocytogenes* counts

Response Variable	Fixed effects	Levels	Estimate	Standard Error	p-value	Significance
L. monocytogenes count (log cfu/g)	pH	pH 6.5	Ref[1]			
		pH 6.0	-0.27	0.22	0.245	
		pH 5.5	-0.44	0.37	0.288	
	Phage	N[2]	Ref			
		Y[3]	-1	0.17	< 0.001	***
	Day	1	Ref			
		7	2.04	0.14	< 0.001	***
		14	2.49	0.14	< 0.001	***
	Strain	10403S	Ref			
		5621	-0.06	0.12	0.618	
		5623	0.10	0.12	0.413	
		5624	0.22	0.12	0.066	
		5625	0.33	0.12	0.006	**
	Milk age[4]		0.01	0.14	0.947	
	Milk apc[5] (log cfu/mL)		0.07	0.06	0.361	
	pH:Phage Y	pH 6.5:Phage Y	Ref			
		pH 6.0:Phage Y	0.13	0.14	0.362	
		pH 5.5:Phage Y	0.38	0.15	0.014	*
	Phage:Day	Phage Y:1	Ref			
		Phage Y:7	-0.30	0.14	0.044	*
		Phage Y:14	0.17	0.15	0.246	
	pH:Day	pH 6.5:Day 1	Ref			
		pH 6.0:Day 7	-0.39	0.17	0.031	*
		pH 6.0: Day 14	-0.43	0.17	0.019	*
		pH5.5:Day 7	-2.00	0.17	< 0.001	***
		pH5.5: Day 14	-2.77	0.19	< 0.001	***
	Phage Y:Strain	Phage Y:Strain 10403S	Ref			
		Phage Y:Strain 5621	-0.02	0.17	0.881	
		Phage Y:Strain 5623	0.43	0.17	0.011	*
		Phage Y:Strain 5624	0.15	0.17	0.376	
		Phage Y Strain 5625	0.66	0.17	< 0.001	***

[1]Ref indicates reference; therefore, estimates, standard error, and P-values are not calculated
[2]N denotes the absence of phage
[3]Y denotes the presence of phage
[4]Age of milk when cheese was made, based on a 21-day code date
[5]Bacterial aerobic plate counts (apc; log cfu/mL) in milk before cheese was made
***$p < 0.001$; **$p < 0.01$; *$p < 0.05$

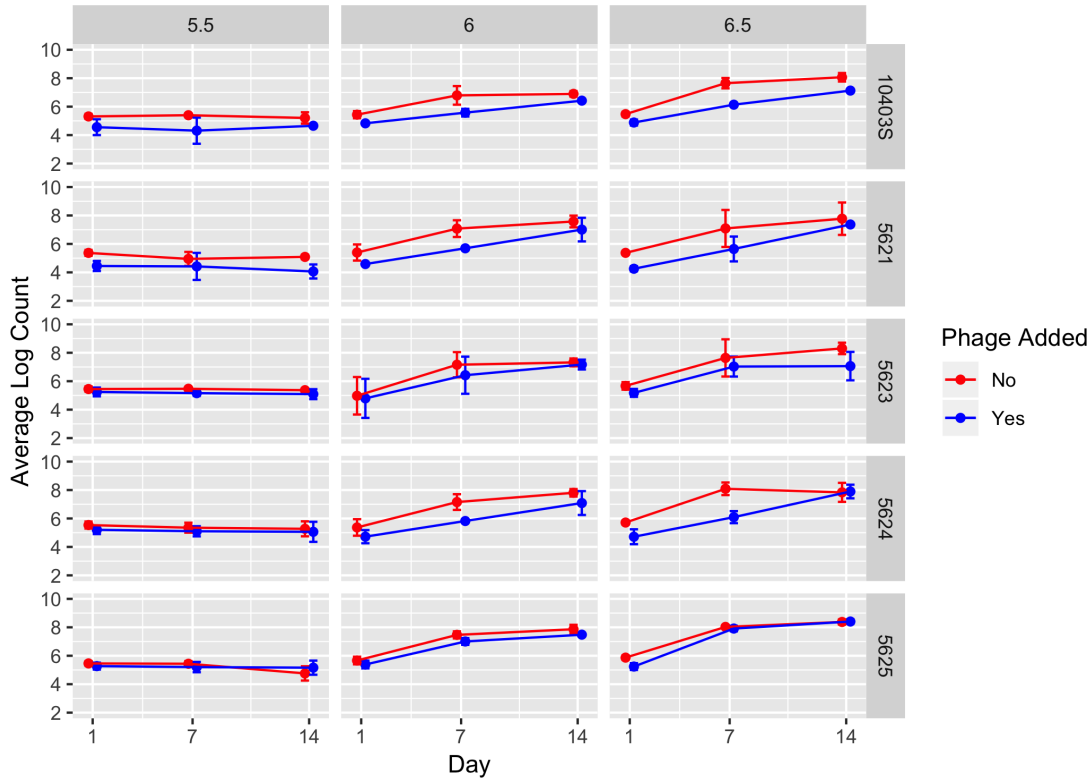


Figure 4.4: Average counts (log cfu/g) of *L. monocytogenes* in the presence (blue line) and absence (red line) of phage treatment in a lab-scale cheese model. Each cheese was inoculated with a single strain of *L. monocytogenes* (10403S, FSL R9-5621, FSL R9-5623, FSL R9-5624, or FSL R9-5625) to a level of approximately 5 log cfu/g. These results represent the effect of pH (5.5, 6.0, and 6.5) on *L. monocytogenes* sensitivity to a commercial phage cocktail. The data points are slightly offset such that the reader can clearly see each point; however, the points still correspond to 1, 7, and 14 days. All values are the arithmetic mean of at least three independent experiments, and error bars denote standard error. For some data points, error bars are not visible because standard error was too low to yield a visible error bar. All cheese was made at pH 6.5.

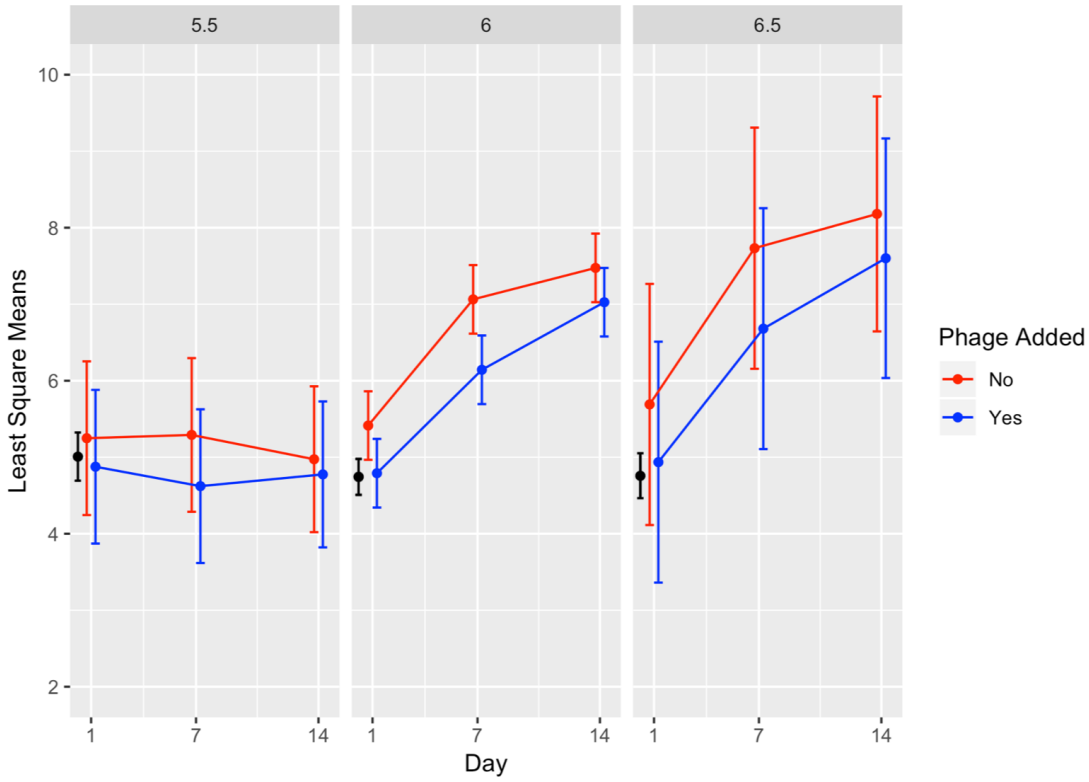


Figure 4.5: Least square means of estimated *L. monocytogenes* numbers (log cfu/g) calculated from the pH model, which are based on data shown in Figure 4.4. Calculated initial (day 0) *L. monocytogenes* number based on the average inoculum level (approximately 5 log cfu/g) are shown in black. Predicted numbers for cheese treated with phage is shown in blue, while predicted numbers for cheese without phage treatment are shown in red. These results represent the predicted effect of pH (5.5, 6.0, and 6.5) on *L. monocytogenes* sensitivity to a commercial phage cocktail across strains (10403S, FSL R9-5621, FSL R9-5623, FSL R9-5624, and FSL R9-5625). The data points are slightly offset such that the reader can clearly see each point; however, the points still correspond to 1, 7, and 14 days. Error bars denote the 95% confidence interval. All cheese for these predictions was made at pH 6.5.

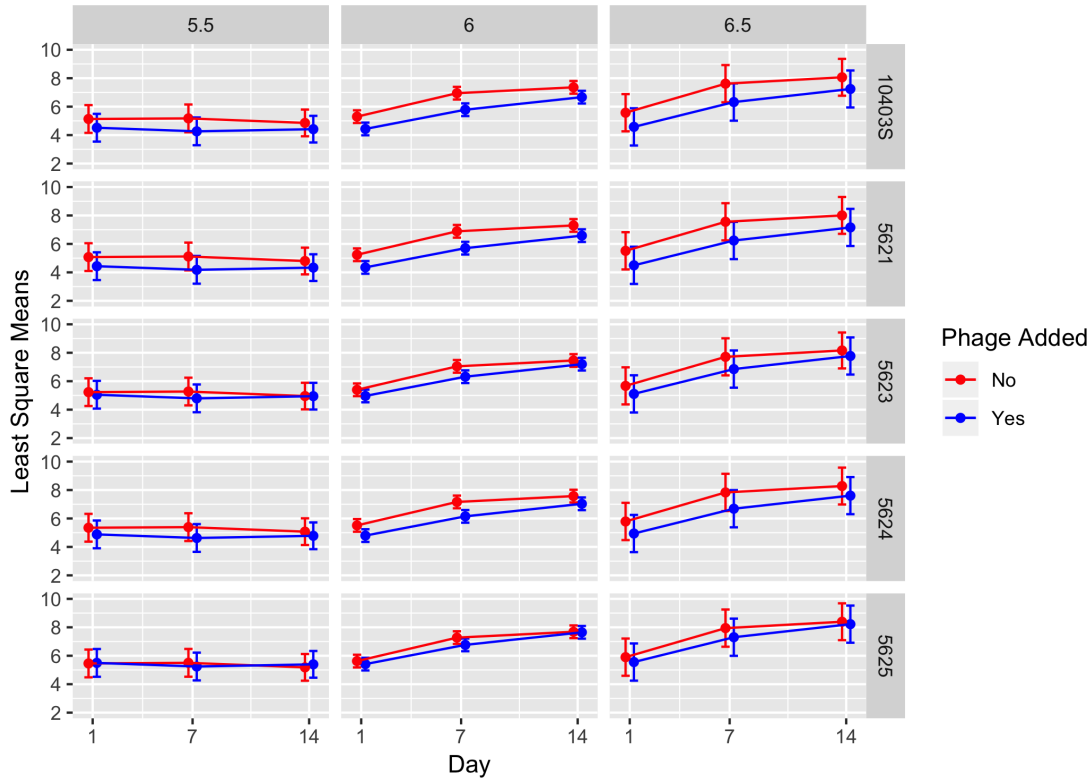


Figure 4.6: Least square means of estimated *L. monocytogenes* numbers (log cfu/g) calculated from the pH model, which is based on data shown in Figure 4.4. Predicted numbers for cheese treated with phage is shown in blue, while predicted numbers for cheese without phage treatment are shown in red. These results represent the predicted effect of pH (5.5, 6.0, and 6.5) on *L. monocytogenes* (10403S, FSL R9-5621, FSL R9-5623, FSL R9-5624, and FSL R9-5625) sensitivity to a commercial phage cocktail. The data points are slightly offset such that the reader can clearly see each point; however, the points still correspond to 1, 7, and 14 days. Error bars denote the 95% confidence interval. All cheese for these predictions was made at pH 6.5.

L. monocytogenes growth at pH 5.5, regardless of treatment.

Presence of phage showed a significant effect ($p < 0.001$) with an effect size of -1.00, which indicates *L. monocytogenes* numbers are 1.00 log lower in the presence of phage treatment (Table 4.4). For cheese made at pH 6.0, average *L. monocytogenes* numbers (across strains and the three time points) were 6.00 and 6.66 log cfu/g for cheese with and without phage, respectively. The average observed *L. monocytogenes* numbers across strains (Figure 4.6) also indicated that phage-treated cheese showed consistently lower *L. monocytogenes* numbers (4.58 to 7.48 log cfu/g) at all days as compared to untreated cheese 4.97 to 7.86 log cfu/g. Although phage-treated cheese showed lower *L. monocytogenes* numbers across pH, the difference between *L. monocytogenes* numbers on treated and untreated cheese varied considerably by pH; for example, the lowest difference between phage-treated and untreated cheese for day 1 (0.23 log) was found for cheese made at pH 5.5, with numerically higher corresponding differences of 0.28 and 0.63 log for pH 6 and 6.5, respectively. Differences between *L. monocytogenes* numbers on treated and untreated cheese made at pH 5.5 were minimal and ranged from 0.10 to 0.97 log, as compared to 0.15 to 1.38 and 0.11 to 2.00 log for pH 6.0 and pH 6.5, respectively (Figure 4.4).

Similar to data obtained from the temperature model, day 7 and 14 also had significant effects on *L. monocytogenes* numbers with higher numbers at both days as compared to the reference (i.e. day 1) (Table 4.4). Again, the significance of day is not surprising considering that *L. monocytogenes* showed growth over time for cheese formulated at pH 6.0 and 6.5. For example, average counts across strains were 4.86 and 7.03 log cfu/g for day 1 and 14, respectively, on cheese made at pH 6.0. Similar to the observations for pH 6.5 cheese incubated

at 6 and 14°C, pH 6.0 cheese incubated at 6°C also showed growth of *L. monocytogenes* for phage-treated cheese (Figure 4.4); least square means data showed only a 0.46 log difference between phage-treated and untreated pH 6.0 cheese at day 14 (Figure 4.5).

Finally, we also found significant interaction effects between presence of phage and strains 5623 and 5625, the two 4b strains used here ($p = 0.012$; effect size of 0.43 and $p < 0.001$; effect size 0.66, respectively); this indicates that these strains show 0.43 and 0.66 log cfu/g higher numbers in the presence of phage as compared to the reference strain 10403S. These findings are consistent with an initial plaque size screen of the five *L. monocytogenes* strains used here against the phage cocktail. Briefly, two independent replicates of a plaque assay on LB-MOPS medium both showed weaker lysis of the two serotype 4b strains as compared to the three other strains (Table 4.5).

4.5 DISCUSSION

While this study clearly indicates that the commercial phage cocktail tested here on average reduces *L. monocytogenes* numbers on cheese (with an overall effect size of around 1 log), we also show that both environmental conditions (especially temperature) as well as strain have significant effects on the efficacy of phage treatment in cheese. These findings have important implications as they (i) suggest that optimization of environmental conditions could be used to enhance the effectiveness of phage treatment and (ii) provide initial data that could be used in a formal risk assessment to quantify the effects of phage treatment with regard to reducing the risk of human listeriosis cases.

4.5.1 While Phage Reduction of *L. monocytogenes* on Lab-scale Cheese is Limited, Effectiveness of Phage is Enhanced at Higher Storage Temperatures

While our data support that phage can significantly decrease *L. monocytogenes* numbers in cheese, we also found that (i) the effectiveness of phage seems to be enhanced when cheese is stored at higher temperatures and (ii) considerable *L. monocytogenes* growth can occur even in the presence of the phage cocktail. Our data showing considerable *L. monocytogenes* growth on cheese treated with phage are consistent with a study that found that while surface treatment of queso fresco with phage P100 (7.7 log pfu/ cm^2) lead to an initial decrease (reduced to an undetectable limit, < 5 cfu/cm^2) in *L. monocytogenes* numbers on cheese stored at 4°C, there was subsequent regrowth (back to inoculum level, approximately 4 log cfu/cm^2) of *L. monocytogenes* by the end of the 28 days of storage [160]. Similarly, another study reported that when soft cheese was inoculated with approximately 10^5 cfu/g of *L. monocytogenes* and treated with 8.3 x 10^7 pfu/g of phage P100, there was an initial 2 log reduction (at 30 min after treatment); however after 7 days of storage at 10°C, they only found an approximate 1 log reduction of *L. monocytogenes* numbers on phage-treated cheese compared to untreated controls [148]. Studies in tryptic soy broth (TSB) also showed re-growth of *L. monocytogenes* after 2 weeks, even though phage treatment at 2 x 10^9 or 2 x 10^{10} pfu/mL resulted in a 4 to 7 log reduction at different temperatures (4, 10, and 20°C) within the first 2 weeks [161]. Conversely, it has been reported that there was complete eradication (below detection limit) and therefore, no regrowth of *L. monocytogenes* on a ripened soft cheese stored at 14°C when the washing solution was inoculated with 2 x 10^1 cfu/cm^2 *L. monocytogenes* at the

Table 4.5: *L. monocytogenes* sensitivity to phage cocktail using a phage plaquing assay

		Phage Concentration (pfu/mL)		
Strain	Serotype	1×10^9	4×10^8	1×10^8
10403S	1/2a	++	++	++
5621	1/2a	++	++	++
5623	4b	+	+/-	+/-
5624	1/2b	++	++	+
5625	4b	+	+/-	+/-

++ Strong lysis, + Lysis, +/- Weak lysis, - No lysis
Results are an average of two phage plaquing assays

beginning of the ripening period, and 2 x 10^6 pfu/cm^2 P100 was subsequently applied to the washing/smearing solution during the rind washings [162]. The observation that our data does not show an initial reduction of the magnitude of that observed in previous studies [160, 148] or an undetectable level of *L. monocytogenes* [162], this could be due to differences in experimental set up. For example, in some cases, the cheese was inoculated and then homogenized prior to phage treatment [148], which could increase the efficacy of phage treatment.

Overall, our data as well as some previous data indicate the potential for regrowth of *L. monocytogenes* during long-term storage, even if phage treatment provided for an initial *L. monocytogenes* reduction. Hence, studies over product shelf-life are essential to appropriately evaluate the effectiveness of phage treatment; use of these data in risk assessments (which would consider, among other parameters, storage time before consumption) can then provide a more accurate assessment of the public health impact of phage applications. There are a number of possible reasons for re-growth of *L. monocytogenes* after initial significant reduction, including, but not limited to, (i) emergence and subsequent growth of phage resistant mutants [163]; (ii) transient resistance of *L. monocytogenes* to phage cocktail; (iii) degradation of phage or binding of phage to food matrix components, inhibiting ability of phage to infect *L. monocytogenes*.

Importantly, while we specifically found a significant interaction effect between phage treatment and cheese incubation at 22°C, storage or incubation at this temperature is neither feasible nor realistic for Hispanic-style fresh cheese as incubation at this temperature can allow growth of molds and other spoilage or pathogenic microorganisms, which could possibly reduce *L. monocytogenes* numbers due to competition. Consistent with our findings, it has been reported

that 10^6 to 10^7 pfu/mL of lytic phage (LMP7) inhibited *L. monocytogenes* growth in skim milk stored at 4, 10, and 30°C; however, phage treatment was most effective at 30°C, resulting in a nearly 1 log difference between treated and untreated skim milk after 1 day compared to ¡ 0.5 log difference at 10°C [164]. Preliminary evidence for enhanced effectiveness of phage in foods incubated at higher temperatures has also been reported for non-dairy foods. For example, a study showed that *Listeria* phage A511 was able to reduce *L. monocytogenes* numbers on hot dogs, chocolate milk, and cabbage by 2.3 to 5 log cfu/cm^2 when stored at 6°C compared to untreated controls, whereas at 20°C, phage was able to reduce *L. monocytogenes* numbers by 3.8 to 6.4 log cfu/cm^2 [165].

Previous studies have shown that native bacteria present in the milk can grow on cheese, especially when stored at higher temperatures [166, 167] and subsequently outcompete *Listeria*, resulting in limited *L. monocytogenes* growth [168, 169, 170]. However, our data show similar *L. monocytogenes* numbers at all temperatures in non-treated cheese (Figure 4.1 and 4.2), suggesting that the enhanced effectiveness of phage-mediated *L. monocytogenes* reduction at higher temperatures is due to temperature affecting the phage-*Listeria* interaction and not due to an effect of the native bacteria on *Listeria*. Other possible explanations for increased phage-mediated *L. monocytogenes* reduction at higher temperatures include (i) enhanced sensitivity of metabolically more active *L. monocytogene*s and (ii) enhanced expression of phage receptors in *L. monocytogenes* grown at 22°C as compared to lower temperatures (such as 6°C). A number of studies have indicated that metabolically active bacteria are more effectively killed by phage [171, 172, 173]. Prior studies have theorized that metabolically active bacteria are required for successful use of phage because these control strategies rely on host cell metabolism for phage replication and consequently

bacterial cell lysis, leading to the release of progeny phage. Specific studies supporting this theory include a study, which found that conditions that reduced metabolism and growth rate of the host cell decreased the effectiveness of phage treatment [161]. Previous studies also support that temperature can affect the physiological state of *L. monocytogenes* in a way that enhances the accessibility of phage receptors on the host. For example, previous studies have shown that phage adsorption of P100-like *Listeria* phage is more efficient at 30°C than at 37°C, potentially due to more accessible WTA receptors [163, 174]. Finally, temperatures (and other environmental conditions) facilitating more rapid growth of *L. monocytogenes* also will lead to larger *L. monocytogenes* populations, which will enhance the likelihood of phage-bacteria contact and also will lead to a more rapid phage propagation, further enhancing the effectiveness of phage treatment. This mechanism could in particular contribute to enhanced die-off seen here in at least one strain at day 14.

4.5.2 The Effectiveness of Phage is Enhanced by Lower pH and *L. monocytogenes* Growth on Lab-scale Cheese

While our data indicate that pH can play a role in the effectiveness of phage treatment, the predominant effect of pH observed here was a lack of *L. monocytogenes* growth on the lab-scale cheese formulated at pH 5.5, along with a reduced ability of phage to kill *L. monocytogenes* in cheese at that pH. The lack of *L. monocytogenes* growth at pH 5.5 is consistent with results a previous study, which reported that growth of *L. monocytogenes* was supported on various cheese types, characterized by higher pH (5.9 to 7.7), whereas cheese with low pH (4.9 to 5.7)

did not support *L. monocytogenes* growth [175]. Similarly, it has been shown that *L. monocytogenes* grew better on cheese when the pH rose above 6.0 [165]. Others have also shown that the acidity of the environment has an effect on the efficiency of phage infection or the success of phage treatment. For example, research has shown that phage MS2 formed 1.1 and 3.0 log pfu/mL fewer plaques on plaquing media with pH 3.9 and 2.5, respectively, as compared to plaquing media with pH of 6.7 [176]. Additionally, studies on different, low pH food matrices, such as hard cheese or apples, have reported little to no *L. monocytogenes* reduction in the presence of phage. For example, a previous study reported a 0.7 log reduction of *L. monocytogenes* when hard cheese (typically pH 5.1 to 5.4) samples were treated with 1 x 10^8 pfu/g of a commercial phage cocktail [149], whereas others reported a 2 log reduction of *L. monocytogenes* on soft cheese (typically pH 6.2 to 6.5) when treated with 8.3 x 10^7 pfu/g of phage P100 [148]. Furthermore, it has also been shown that no significant difference of *L. monocytogenes* numbers between control and phage-treated samples of fresh-cut apple slices (pH 3.76) [177].

Importantly, our data support that the effectiveness of phage seems to be enhanced when cheese is made at higher pH. Although we did not find a significant effect at pH 6.0 (relative to pH 6.5), cheese formulated at pH 6.0 showed the greatest phage-mediated reduction of *L. monocytogenes* numbers compared to non-treated cheese. However, as observed with temperature, *L. monocytogenes* is still able to grow in the presence of the phage cocktail. Consistent with our findings, a study reported that in two types of Brazilian soft cheese, which have near neutral pH, bacteriophage P100 (applied at approximately 10^7 pfu/g) initially reduced *L. monocytogenes* numbers by approximately 2 log compared to the untreated control, at 30 minutes post-infection; however, the difference

between treated and untreated was only 1 log after 7 days of storage [148]. Previous work also found that *Listeria* phage A511 (approximately 10^8 pfu/ cm^2) was able to reduce *L. monocytogenes* inoculum levels below detectable levels (approximately 10^3 cfu/cm^2) in cheese that had a pH of 7.6 after ripening [165].

Possible explanations for an enhanced effectiveness of phage-mediated *L. monocytogenes* reduction at higher pH (Figure 4.5) include but are not limited to (i) enhanced phage attachment to the host and (ii) increased sensitivity of *L. monocytogenes* not grown under multiple stress conditions. Reduced phage stability would also result in a decrease of overall efficacy of phage treatment; however, prior data suggest that reduced phage stability at the lower pH used here is not an issue; a study reported that phage P100 was stable when incubated in TSB adjusted to a pH range of 4 to 10, and phage P100 numbers decreased by less than 0.5 log pfu/mL after 24 h and between 1 to 2 log pfu/mL after 1 month, respectively [161]. These data suggest that phage is stable under the pH conditions tested in our study and the phage cocktail is most likely able to remain effective over shelf-life of cheese. Besides phage stability, attachment of the phage to its bacterial host is essential to the success of phage treatment and can also be affected by pH. Previous studies suggest that environmental factors can change *L. monocytogenes* cell envelope physiology, subsequently influencing the effectiveness of cell envelope acting bactericidal treatments [178]. For example, a previous study has shown that low pH changes the cell membrane composition, leading to tolerance of *Listeria* to other antilisterial compounds such as the cationic antimicrobial, nisin [65]. Thus, one could hypothesize that a similar mechanism could contribute to an enhanced effect of phage (which also bind to cell envelope components) at higher pH.

4.5.3 *L. monocytogenes* Serotype Affects the Efficiency of the Phage Cocktail

In addition to the effects of environmental conditions, our data also indicate that serotype 4b strains showed reduced sensitivity to the phage cocktail used, across pH and temperature conditions tested. These findings are consistent with a considerable body of work that supports that host range of *Listeria* phage corresponds to host serotype [179, 180, 181]. Differences among *L. monocytogenes* serotypes can be attributed to the composition of their WTAs, cell surface polysaccharides. For example, serotype 1/2 strains have terminal rhamnose and N-acetylglucosamine (GlcNAc) residues, whereas 4b strains are decorated with terminal glucose and galactose residues [182]. As an example of *Listeria*-phage host specificity patterns, previous work reported that *Listeria*-phage A118, a temperate Siphovirus, attacks 1/2 serotypes and Siphoviral phage A500 primarily lyses serotype 4b, whereas the broad host range Myovirus phage A511 lyses the majority of *L. monocytogenes* strains [179]. Importantly, however, a putative CRISPR system has been identified in *Listeria*, which potentially provides defense against bacteriophage infection. While locus I is conserved in both serotype 1/2a and 4b strains, locus II is only present in 4b strains. Furthermore, only locus II seems to be functional [183]. While the phage cocktail used in this study included both *Siphoviridae* and *Myoviridae* phage, which should have a broad host range against *L. monocytogenes* serotypes, our data suggest that at least some phage in this cocktail could have reduced or limited ability to lyse either serotype 4b strains in general or the specific serotype 4b strains included here. Our findings are also consistent with a previous study that indicates that at least some wildtype *L. monocytogenes* strains might be resistant to commer-

cially used phage cocktails [181]. In addition, or alternatively, the serotype 4b strains tested here might have a specific ability to adapt to the Hispanic-style fresh cheese environments, making them less susceptible to phage treatment in this food matrix. Overall, our findings, along with previous studies, further support the importance of validating phage-based treatment strategies with both *L. monocytogenes* serotypes and environmental conditions relevant to a given application.

While our data suggests that pH, temperature, and serotype affect the effectiveness of phage treatment against *L. monocytogenes* in a lab-scale cheese model, the overall phage-dependent reduction was limited. Additionally, except for cheese made at pH 5.5, all treated samples allowed growth of *L. monocytogenes* after a 14-day incubation. Our results highlight a need for fine-tuning control strategies against *L. monocytogenes*. In comparison, the bacteriocin nisin can be added to cheese to control for *L. monocytogenes*, and it has been proven to reduce numbers in a dose-dependent way [3]. Whether environmental conditions affect the effectiveness of nisin treatment in cheese is the focus of our follow up study. In addition to nisin, plant extracts such as ferulic acid have also been used to inhibit the growth of *L. monocytogenes* with some success on cheese [3]. Importantly, *L. monocytogenes* seems less likely to develop resistance to ferulic acid [184] than to nisin or phage; therein, a combined treatment approach could be an appropriate strategy to reduce *L. monocytogenes* numbers in a food matrix and decrease the likelihood of resistance, which perhaps could be effective regardless of environmental factors.

4.6 CONCLUSIONS

Overall, our data suggest that phage-based control strategies can reduce *L. monocytogenes* populations in cheese, and that the effectiveness of phage-based control strategies is affected by *L. monocytogenes* strains and environmental conditions. Challenge studies, typically conducted by industry, provide only limited insights on the actual value of using phage-based control strategies either for public health or for an individual business that tries to reduce the risk of an outbreak or a recall associated with its products. Future work is needed to develop a formal risk assessment that can be used to predict the actual benefits of using phage-based control strategies to not only reduce the risk of contamination, but also decrease the number of human listeriosis cases, taking into account the expected distributions of storage temperatures and times between production and consumption, as well as the likelihood of contamination with different strain and serotypes. This is important as our data suggest that phage-based control strategies could have limited impact on reducing public health risks if, for example, cheese made at pH 6.5 is typically stored at 6°C or below and consumed after 14 days, while the impact of phage treatment could be substantial if cheese is typically stored at higher temperatures and consumed within a few days after production.

CHAPTER 5

RNA-SEQUENCING BASED REFINEMENT OF THE PRFA REGULON IN *LISTERIA MONOCYTOGENES*

5.1 ABSTRACT

PrfA is a transcriptional activator that regulates virulence in the food-borne pathogen *Listeria monocytogenes*. To gain further insight into PrfA-dependent regulation in *L. monocytogenes*, we grew the *L. monocytogenes* parent strain 10403S, a constitutively active *prfA** mutant, and Δ*prfA* under conditions demonstrated to induce PrfA activity (glucose-1-phosphate and pre-treated with 0.2% activated charcoal). We then used RNA-sequencing (RNA-seq) to characterize the PrfA regulon by comparing the transcript level between (i) wt (10403S) and *prfA**, (ii) wt and Δ*prfA*, and (iii) *prfA** and ΔprfA. A total of 16 genes and 2 ncRNAs were significantly, positively regulated by PrfA at the transcriptomic level in at least two of the three comparisons. While our data largely confirmed the known PrfA regulon, using sequencing data and *in silico* methods, we identified a novel PrfA-dependent gene along with a putative PrfA box and predicted σ^{A}-dependent -35 and -10 promoter elements within an open reading frame. Our data illustrate that while the core PrfA regulon has been determined, continued efforts are needed to fully elucidate the complex PrfA regulatory network in *L. monocytogenes*.

5.2 INTRODUCTION

Listeria monocytogenes is a Gram-positive bacterial pathogen that can cause listeriosis in humans, mainly pregnant women, newborns, elderly individuals, and immunocompromised people [99]. Listeriosis can be severe with a mortality rate between 20 and 30% [185]. In most cases, *L. monocytogenes* infections are caused by ingestion of contaminated food [186]. For *L. monocytogenes* survival in the stomach and intestine, the bacterium must use a number of protective mechanisms.

Once *L. monocytogenes* is inside the intestinal tract, it relies on a multitude of virulence factors for survival. Expression of the majority of virulence factors is controlled by the main virulence activator PrfA (positive regulatory factor A). The virulence gene, *prfA*, encodes PrfA, which is a 27 kDa protein, sharing structural and sequence similarities with the cAMP receptor protein (CRP) in Escherichia coli [187, 188, 189]. PrfA recognizes and binds to a 14-bp palindromic DNA sequence called the PrfA box, which is typically located approximately 41 bp upstream of the transcriptional start site of PrfA-regulated genes [190, 191]. Due to its importance for pathogenesis and expression, activity of PrfA are controlled at several levels (i.e. transcriptional, post-transcriptional and post-translational) to ensure that virulence genes are transcribed only when necessary within the host [185].

PrfA is transcriptionally controlled by two promoters (P1 and P2), located upstream of *prfA*. P1 is mediated by the housekeeping σ^{A} while P2 is regulated by σ^{A} and the regulator of the stress response, σ^{B} [192]. Together these sigma factors ensure constant basal levels of monocistronic *prfA* transcripts. Further

transcriptional control is mediated by a third promoter, P_{plcA}, which controls the expression of a bicistronic *plcA-prfA* transcript. As P_{plcA} is regulated by PrfA, a positive feedback loop ensues [193]. In addition, the *prfA* 5'-UTR contains a thermosensor structure that allows *prfA* mRNA translation at 37°C, but inhibits translation at 30°C by blocking the ribosomebinding site [193]. PrfA is also regulated posttranslationally. Because PrfA is a member of the CRP family of transcriptional regulators, of which many require the binding of a cofactor for full activity, it has been shown that upon invasion into host cells, the cofactor, glutathione. Upon invasion into host cells, glutathione binds PrfA, causing a conformational change and increases the binding affinity of PrfA to the PrfA box, subsequently increasing transcription of PrfA-dependent genes [194].

PrfA activation is also correlated with sugar metabolism, which could serve as another way for *L. monocytogenes* to sense the environment. It was shown that sugars transported by the phosphoenolpyruvate phosphotransferase system (PTS), such as glucose and cellobiose, repress PrfA activity, while the presence of nonPTS, hostderived sugars, such as glucose-1-phosphate (G1P) and glycerol, trigger *prfA* transcription and PrfA activity [195, 196]. Notably, the activation of PrfA in richmedia conditions was shown to require activated charcoal in the medium, though the reason for this effect has not been clearly determined [197].

Previous microarray studies of gene expression under PrfA-inducing conditions [198, 199] and studies investigating the mechanisms by which PrfA regulates gene expression [197, 200, 201, 202, 203] have identified 10 strongly PrfA-dependent genes, of which at least 6 are clustered together in *Listeria* Pathogenicity Island 1 (LIPI-1), a 9-kb gene cluster located between *prs* and *orfX*,

consisting of *prfA*, *plcA*, *hly*, *mpl*, *actA*, and *plcB*, which are essential for *Listeria* virulence [188, 29, 204]. *plcA* and *plcB* encode phosphatidylinositol phospholipase C (PI-PLC) and phoshatidylcholine phospholipase C (PC-PLC), respectively, which along with listeriolysin O (LLO, encoded by *hly*) allow vacuolar escape [205, 206, 207]. *mpl* encodes a zinc-metalloprotease necessary for PlcB maturation [208]. Lastly, *actA* is responsible for actin-based motility and cell-to-cell spread [209]. The remaining four PrfA-dependent genes (*hpt*, *inlC*, *inlA*, and *inlB*) have been reported to be either directly or indirectly regulated by PrfA or co-regulated by other transcriptional activators [201, 202]. For example, *inlAB* is controlled by both PrfA and σ^B [210]; therefore, these genes are not included in the core PrfA regulon. As many as 145 additional genes are weakly PrfA-dependent or associated with PrfA expression [198, 199], meaning under PrfA-inducing conditions, these genes are up-regulated; however, they have a σ^B promoter sequence and lack a PrfA box. Many of these genes encode regulators, transporters, metabolic enzymes, stress response mediator proteins among others, suggesting that in addition to virulence, PrfA could play a more general role in regulating *L. monocytogenes* homeostasis [198, 199].

While the PrfA regulon in *L. monocytogenes* has been investigated using microarray-based approaches, this is the first study that uses RNA-seq to characterize the PrfA regulon by comparing the transcriptomic data between wt and (i)Δ*prfA*, (ii) *prfA** (a constitutively active PrfA mutant), and (iii) Δ*prfA* under PrfA-inducing conditions. We expected that wt and *prfA** strains would have significantly higher expression of PrfA-dependent genes compared to Δ*prfA*, in which *prfA* is deleted; however, whether or not we would observe differences between wt and *prfA** strains were unclear. Although, *prfA** overexpresses PrfA-dependent genes, both strains were grown in PrfA-inducing conditions, which

could result in similar expression levels. The objective of the present study was to refine the PrfA regulatory network at the genome level using RNA-seq and advanced bioinformatics techniques to identify novel differentially regulated, PrfA-dependent genes and their associated PrfA boxes.

5.3 MATERIALS AND METHODS

5.3.1 Bacterial Strains and Growth Conditions

Three *L. monocytogenes* strains were used for this study: (i) wt (104033S), (ii) *prfA** mutant (G145S), and (iii) Δ*prfA* mutant. All strains and their isogenic mutants were maintained in frozen brain heart infusion (BHI; Difco, Becton Dickinson and Co., Sparks, MD) stocks, stored at -80°C in 15% glycerol. Strains were streaked from frozen glycerol stocks, onto BHI agar plates, followed by incubation at 37°C for 24 h. A single colony from these plates was subsequently inoculated into 5 mL of BHI broth in 16 mm tubes, followed by incubation at 37°C with shaking (230 rpm) for 16 h (Series 25 Incubator, New Brunswick Scientific, Edison, NJ). After 16 h, 100 μL BHI culture was inoculated into fresh 10 mL Luria Bertani (LB)-MOPS broth pre-treated with 0.2% activated charcoal, which was filtered prior to supplementation with 25 mM G1P to induce *prfA* transcription and PrfA activity [211] and grown to mid-log phase (approximately OD_{600} = 0.4) at 37°C, prior to RNA extraction.

5.3.2 RNA Isolation

RNA isolation was performed as previously described by our group [212, 213] with minor modifications. Briefly, for each sample, 10 mL of RNAprotect bacteria reagent (Qiagen, Valencia, CA) was added to 10 mL of bacterial culture. The mixture was then incubated at room temperature for 10 min to ensure that the bacterial RNA was stabilized. Cells were pelleted by centrifugation (4,637 x g, 30 min) at 4°C and suspended in nuclease free water with proteinase K (Thermo Fisher Scientific, Waltham, MA) (25 mg/mL) and lysozyme (Thermo Fisher Scientific) (50 mg/mL), followed by incubation at 37°C for 30 min. Cell lysates were subsequently mixed with 1 mL of Tri Reagent (Ambion, Austin, TX), transferred into bead-beating tubes that contained 3 mL of 0.1 mm zirconium beads, followed by adjustment (with Tri Reagent) to a final volume of 5 mL. The lysates were processed in the bead-beater at maximum speed for 4 min. After bead-beating, samples were centrifuged for 10 min at 4,637 g, 4°C. Supernatants were separated from the beads, transferred into a new tube, and mixed with 500 μL of bromo-chloro-propane (BCP). After incubation with BCP for 10 min, samples were centrifuged at 14,637 g for 15 min at 4°C. The aqueous layer was collected and nucleic acids were precipitated overnight at 80°C using 2.5 volumes of 100% ice-cold isopropanol. Nucleic acids were pelleted, washed with 75% ethanol, and resuspended in 100 μL nuclease free water. Total RNA was incubated with Turbo DNase (Life Technologies) to remove remaining DNA in the presence of RNasin (Promega). Subsequently, RNA was purified using phenol-chloroform/chloroform extractions, followed by precipitation and resuspension. Quality of RNA was assessed using a 2100 Bioanalyzer (Agilent Technology, Santa Clara, CA)(all samples had RNA integrity numbers > 5). Efficiency of DNase treatment was determined using qRT-PCR for the house-

keeping gene *rpoB* (all samples showed Ct $>$ 35, indicating absence of DNA contamination at relevant levels). All experiments were performed in biological triplicate.

5.3.3 cDNA Libraries and RNA-seq

Preparation of directional cDNA fragment libraries was performed using the ScriptSeq Complete Kit (Bacteria) (Illumina, San Diego, CA). 16S and 23S rRNA was removed from total RNA with Ribo-Zero rRNA Removal Reagents (Bacteria) and Magnetic Core Kit. rRNA-depleted samples were run on the 2100 Bioanalyzer (Agilent Technology) to confirm reduction of 16S and 23S rRNA and followed by purification using Agencourt RNAClean XP Kit (Beckman Coulter Inc, Brea, CA). The Cornell Core Facility for RNA-sequencing prepared and sequenced indexed RNA-seq libraries (ScriptSeq Complete Kit, Illumina). Sequencing was carried out on a NextSeq 500 (single-end, 75 bp per read).

5.3.4 RNA-seq alignment, coverage and differential expression analysis

Sequence reads were aligned to the *L. monocytogenes* 10403S genome using the BWA-MEM algorithm, version 0.7.3a [214]. The data for coverage per base on the sense and antisense strands were analyzed separately using SAMtools [215]. Differential expression of genes in each strain comparison (wt/Δ*prfA*, wt/*prfA**, and *prfA**/Δ*prfA*) were analyzed using the baySeq package for R version 2.2.0 [216]. Genes were considered differentially expressed if the FDR (False

Discovery Rate) was ≤ 0.05 and the FC (Fold Change) was ≥ 2.0 for up-regulated genes and ≤ 0.5 for down-regulated genes.

5.3.5 *In Silico* Identification of Putative PrfA Binding Sites

For putative PrfA-dependent genes found using baySeq, we used a Hidden Markov Model (HMM) search performed using HMMER [217] to identify putative PrfA binding sites. The training alignments included 14 PrfA boxes (10 previously confirmed and 4 likely PrfA-dependent). The model searched the complete *L. monocytogenes* 10403S genome. The output results were filtered and only hits with a score > 2 were kept. To confirm results of the HMM, *in silico* identification of putative PrfA binding sites were done using Virtual Footprint [218]. The search criteria included the use of custom position weight matrix based on all known PrfA boxes (5.1). The search was not restricted to non-coding regions, as per default, to allow detection of PrfA DNA-recognition sequence within ORFs.

5.3.6 Cluster Analysis of Gene-expression Data

Normalized FC of gene-expression was obtained from the Listeriomics sever [219] available at https://listeriomics.pasteur.fr/Listeriomics/. Conditions related to PrfA activity, specifically virulence genes expression, stress response and carbon source utilization were selected for analysis. Cluster analysis by Spearman correlation and data visualization were done using MeV tools [220], heat maps were constructed using Heatmapper [221] and tree visualization and

Table 5.1: PrfA-dependent genes and their associated PrfA box sequences used as search criteria for the HMM and Virtual Footprint.

LMRG gene	EGDe gene	Gene name	PrfA box[1] Canonical: **TTAACA**nn**TGTTAA**
LMRG_00126	*lmo0433*	*inlA*	a**TAACA**TAa**GTTAA**
LMRG_00127	*lmo0434*	*inlB*	
LMRG_01217	*lmo2067*	*bsh*	**TTAA**a**A**AT**T**t**TTAA**
LMRG_01301	*lmo2164*	*lapB*	**TTAACA**GA**TG**c**TAA**
LMRG_01613	*lmo2219*	*prsA*	**TT**t**ACA**CA**T**a**TTAA**
LMRG_02261	*lmo0838*	*hpt*	a**TAACA**AG**TGTTAA**
LMRG_02622	*lmo0200*	*prfA*	c**TAACA**AT**TGTTA**g
LMRG_02623	*lmo0201*	*plcA*	
LMRG_02624	*lmo0202*	*hly*	**TTAACA**AA**TGTTAA**
LMRG_02625	*lmo0203*	*mpl*	**TTAACA**AA**TGT**a**AA**
LMRG_02626	*lmo0204*	*actA*	
LMRG_02627	*lmo0205*	*plcB*	**TTAACA**AA**TGTTA**
LMRG_02628	*lmo0206*	*orfX*	
LMRG_02629	*lmo0207*	-	
LMRG_02825	*lmo1786*	*inlC*	**TTAAC**gCT**TGTTAA**

[1]Nucleotides conforming to the canonical PrfA box sequence are shown in bold and mismatches from the consensus are in lower case.

editing were done using Archaeopteryx [222].

5.3.7 Promoter Analysis

The -35 and -10 σ^{A}-dependent promoter elements were predicted using Promoter Hunter and matrices for -35 and -10 of *L. monocytogenes* σ^{70} recognition sequences [223] available at http://www.phisite.org/.

5.4 RESULTS AND DISCUSSION

The focus of this study was to refine the PrfA regulatory network by growing three strains of *L. monocytogenes* (wt, *prfA**, and Δ*prfA*) under PrfA-inducing conditions: a rich medium that was pre-treated with 0.2% activated charcoal and supplemented with G1P and then incubated at 37°C. We used RNA-seq to determine differential expression of PrfA-dependent genes by comparing normalized RNA-seq data between wt and Δ*prfA*, *prfA** and wt, and *prfA** and Δ*prfA* strains. Our data indicate that we (i) confirmed transcription of known PrfA-dependent genes and (ii) identified a novel PrfA box and its putative σ^{A}-dependent promoter region.

5.4.1 Under inducing conditions, PrfA differentially regulates approximately 1% of *L. monocytogenes* genes

To refine the PrfA regulon in *L. monocytogenes*, we collected RNA from each strain grown to mid-log phase under PrfA-inducing conditions and performed RNA-seq. Our analysis was performed by calculating the normalized RNA-seq coverage (NRC) for a given gene and comparing NRCs between strains with different genetic backgrounds (i.e. wt, *prfA**, and Δ*prfA*). Using this approach, we found 8 of the 2964 annotated protein-coding genes and non-protein-coding RNAs (ncRNAs) were identified as having significantly up-regulated (FDR ≤ 0.05 and FC ≥ 2.0) transcript levels under PrfA induction when comparing wt and Δ*prfA* (wt/Δ*prfA*) (Figure 5.1). No down-regulated genes were found for this comparison. Of the 8 up-regulated genes (*prfA*, *plcA*, *hly*, *mpl*, *actA*, *plcB*, *orfX*, and *LMRG_02629/lmo0207*) all are within the LIPI-1, and 6 (*prfA*, *plcA*, *hly*, *mpl*, *actA*, *plcB*) are members of the core PrfA regulon (Table 5.2).

When *prfA** was compared to wt (*prfA**/wt), we found 20 of the 2964 genes were significantly, differentially expressed (15 up-regulated, FDR ≤ 0.05 and FC ≥ 2.0 and 5 down-regulated, (FDR ≤ 0.05 and FC ≤ 0.5)) (Figure 5.2). The 15 up-regulated genes consisted of the 10 known PrfA-dependent transcripts identified by previous transcriptomic studies, except *hly* and the ncRNA rli57 and 5 putative PrfA-dependent genes, identified in this study (Table 5.2). Although, *hly* and rli57 had a FC ≥ 2.0 in *prfA**, compared to wt; they did not have an FDR ≤ 0.05, and therefore, were not considered significant in this comparison. Interestingly, we found from 3- to 85-fold induction of both known and putative PrfA-dependent genes and ncRNAs in the *prfA** mutant compared to wt, with higher induction for genes located within the LIPI-1 (Ta-

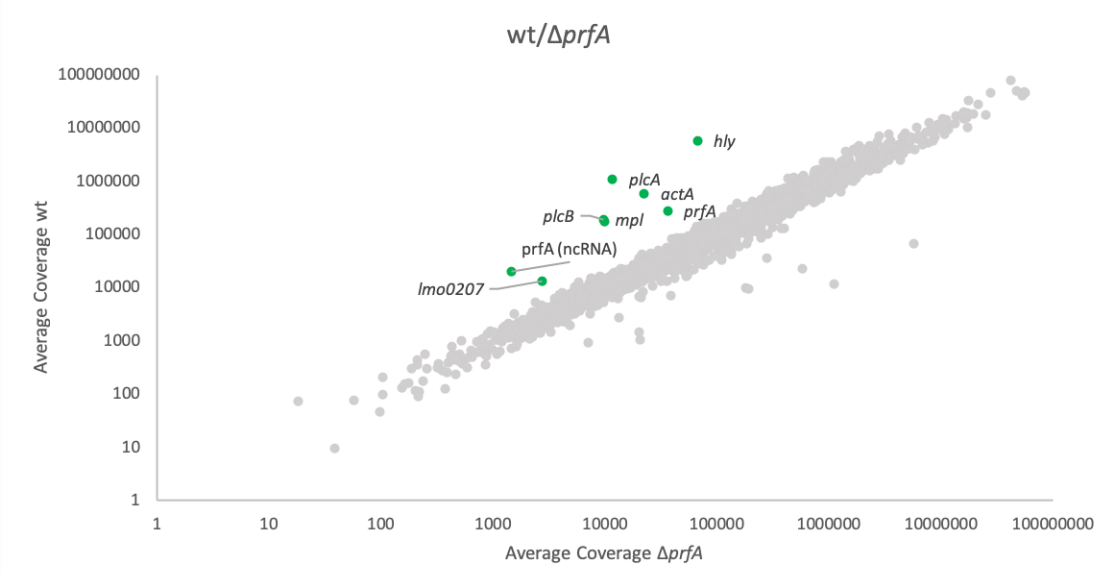


Figure 5.1: Differential expression of *L. monocytogenes* strains wt (10403S) and Δ*prfA* grown under PrfA-inducing conditions. The scatterplot represents the average expression levels of genes. Significantly up-regulated transcripts are depicted in green (FDR ≤ 0.05 and FC ≥ 2.0) and non-differentially expressed transcripts are shown in grey.

Table 5.2: Known and putative *L. monocytogenes* PrfA-dependent genes and ncRNAs and their associated PrfA boxes identified in this study.

LMRG gene	EGDe gene	Gene name or annotation	Function	Fold change[1] wt/Δ*prfA*	*prfA**/wt	*prfA**/Δ*prfA*	PrfA box[2] Canonical: **TTAACA**nn**TGTTAA**
Known PrfA-dependent							
LMRG_01613	*lmo2219*	*prsA*	Protein folding chaperone	*1.99*	*3.71*	7.39	
LMRG_02261	*lmo0838*	*hpt*	Sugar phosphate transporter	*5.54*	85.2	472	a**TAACA**AG**TGTTAA**
LMRG_02622	*lmo0200*	*prfA*	Virulence regulation	7.55	5.46	41.2	c**TAACA**AT**TGTTA**g
LMRG_02623	*lmo0201*	*plcA*	Vacuole escape	93.8	6.06	569	
LMRG_02624	*lmo0202*	*hly*		85.9	*8.10*	696	**TTAACA**AA**TGTTAA**
LMRG_02625	*lmo0203*	*mpl*		18.0	17.7	319	**TTAACA**AA**TGT**a**AA**
LMRG_02626	*lmo0204*	*actA*	Actin assembly (cell-to-cell spread)	25.7	35.3	913	**TTAACA**AA**TGTTA**
LMRG_02627	*lmo0205*	*plcB*	Phospholipase C (vacuole escape)	19.9	41.8	834	
LMRG_02628	*lmo0206*	*orfX*	Unknown	*7.49*	33.1	248	
LMRG_02629	*lmo0207*		Unknown	4.87	34.8	170	
LMRG_02825	*lmo1786*	*inlC*	Cell invasion	2.73	25.5	69.8	
		prfA (ncRNA)	Unknown	13.8	6.22	85.7	c**TAACA**AT**TGTTA**g
		rli57 (ncRNA)	Unknown	*19.3*	*10.3*	199	**TTAACA**AA**TGT**a**AA**
Putative PrfA-dependence							
LMRG_00319	*lmo0636*		Rr2 family transcriptional regulator	*1.22*	3.88	4.75	
LMRG_00320	*lmo0637*		Methyltransferase	*1.16*	3.04	3.51	
LMRG_01109	*lmo1962*		Transcriptional regulator, TetR family	*0.96*	4.31	4.13	
LMRG_01622	*lmo2210*		Unknown	*1.08*	8.36	9.05	
LMRG_02046	*lmo0947*		Cyanate permease	*1.52*	5.03	*7.63*	**TTAACA**AT**TGT**a**AA**

[1]All differentially expressed genes determined using an FDR ≤ 0.05 and a fold change ≥ 2 in at least two strain comparisons, italics denotes FDR > 0.05.
[2]PrfA box found using HMM and confirmed using Virtual Footprint; Nucleotides in bold denote consensus with the canonical sequence. Mismatch nucleotides are in lowercase.

ble 5.2), which suggests that a different mechanism, involving the *prfA** mutation, independent of G1P and activated charcoal is responsible for increased activity of PrfA. Future studies comparing *prfA** strains grown under inducing and non-inducing conditions are needed to verify this hypothesis. The 5 down-regulated genes are: *LMRG_00012* (*lmo0319*), *LMRG_00033* (*lmo0342*), *LMRG_01923* (*lmo2771*), *LMRG_01924* (*lmo2772*), and *LMRG_02900* (*lmo2773*), which are associated with carbon metabolism. Not surprisingly, these genes are down-regulated under PrfA-inducing conditions as they include several PTS permeases (*LMRG_01924*, *LMRG_01924*, and *LMRG_02900*) [224] and an anti-terminator (*LMRG_00012*) in the BglG-like anti-terminator family [225], as well as a transketolase (*LMRG_00033*), which converts C5 sugars into C6 sugars [226]. Our data are consistent with a previous study that used a micro-array to analyze the gene expression of two *L. monocytogenes* EGD strains (*prfA** and Δ*prfA*) grown in minimal media, supplemented with glucose (MM-Glc) [199]. This study showed that genes involved in virulence were up-regulated and genes associated with the PTS pathway were down-regulated [199]. Additionally, when the *prfA** strain was grown in MM-Glc, growth was inhibited compared to a wt strain; however, there was no difference in growth between the wt and Δ*prfA* strain, suggesting that overexpression of PrfA interfered with PTS-mediated sugar uptake [199].

Lastly, for the *prfA** and Δ*prfA* comparison (*prfA**/Δ*prfA*), 44 of the 2964 genes showed differential transcript levels (20 up-regulated and 24 down-regulated) (Figure 5.3). The 20 up-regulated genes consisted of the 10 known, strongly PrfA-dependent genes [227], 1 weakly PrfA-dependent gene (*inlC*) [227], and 2 ncRNAs, as well as 7 putative PrfA-dependent genes identified in this study (Table 5.2). However, two of the putative PrfA-dependent

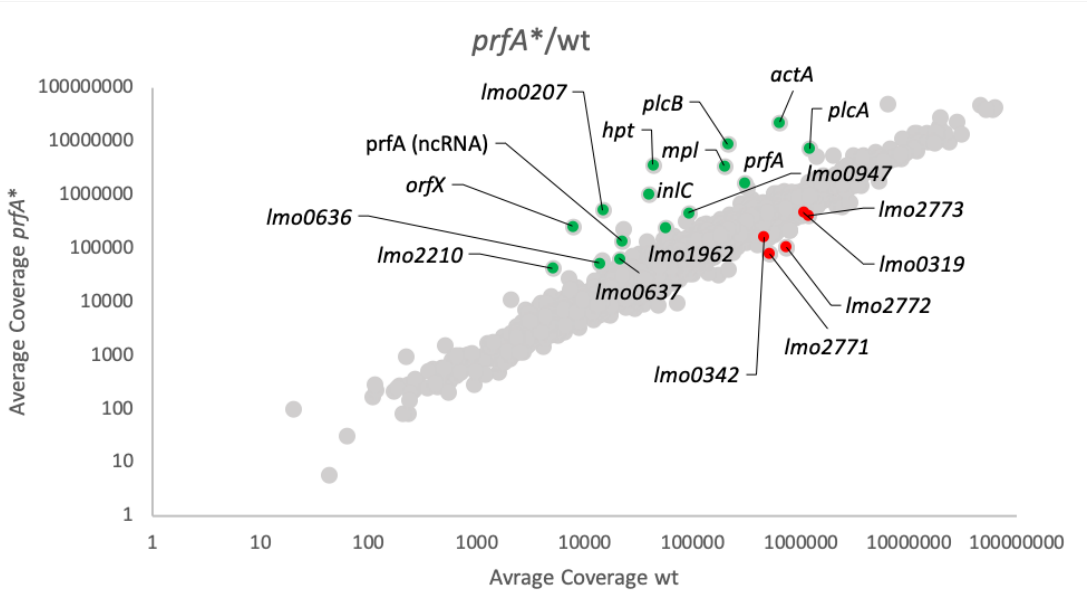


Figure 5.2: Differential expression of *L. monocytogenes* strains *prfA** and wt (10403S) grown under PrfA-inducing conditions. The scatterplot represents the average expression levels of transcripts. Significantly up-regulated transcripts are depicted in green (FDR $\leq$ 0.05 and FC $\geq$ 2.0), signifcantly down-regulated transcripts are in red (FDR $\leq$ 0.05 and FC $\leq$ 0.5), and non-differentially expressed transcripts are shown in grey.

genes (*LMRG_01829/lmo2419* and *LMRG_02751/lmo0186*) were excluded from the study as they had low coverage and were not significant in at least two of the strain comparisons. Of the 24 down-regulated genes, the majority are genes associated with carbon and nitrogen metabolism, sugar ABC transporters, and glycine cleavage [198, 228, 196, 229]. It has been suggested that down-regulation of these genes could be a result of different regulation of PrfA-dependent genes given the growth condition [198]. For example, slower growth of *L. monocytogenes* has been observed intracellularly compared to growth in a rich laboratory medium, in which glucose or other PTS sugars are not limited [196]. Overall, our data show that PrfA induction up-regulated genes associated with intracellular survival and virulence and repressed genes associated with carbon utilization, specifically non-phosphorylated sugars.

5.4.2 RNA-seq confirms 13 previously identified PrfA regulon members as PrfA-dependent

Our RNA-seq analysis of *L. monocytogenes* strains (wt, *prfA**, and Δ*prfA*) under PrfA-inducing conditions identified 16 genes and 2 ncRNAs (Table 5.2; Figure 5.4), 11 of which that have previously been reported as PrfA-dependent or involved in PrfA-mediated control of virulence in *L. monocytogenes* using transcriptomic and/or mechanistic studies [228, 230, 231]. Previous transcriptomic studies have used DNA macro-array data to identify PrfA-dependent genes under PrfA-inducing and -repressing conditions such as a rich medium, supplemented with activated charcoal or glucose-6-phosphate for induction or glucose or cellobiose for repression [199, 198]. In our analysis, we used a rich medium

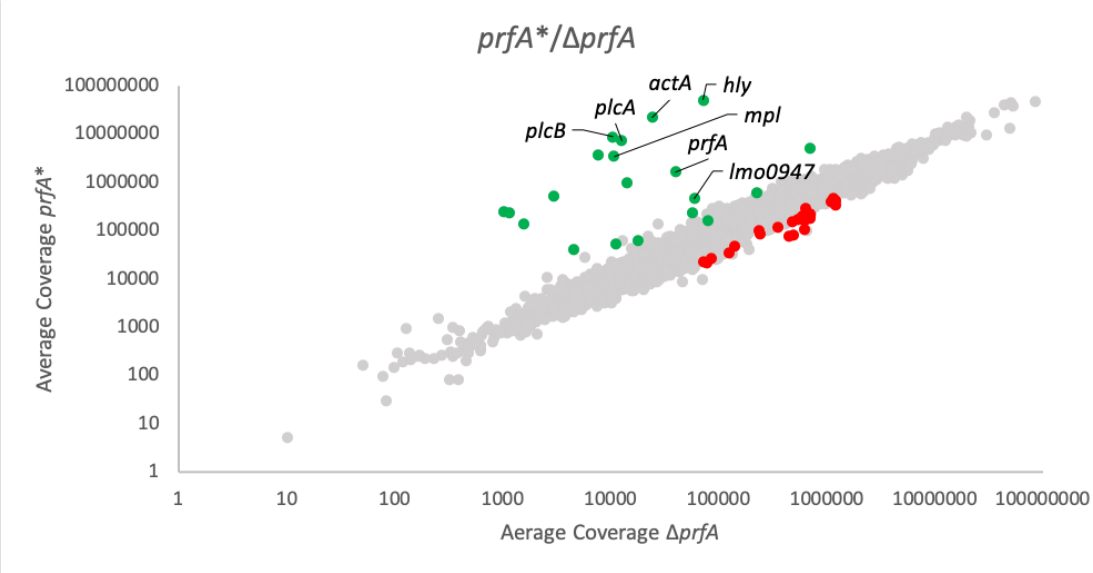


Figure 5.3: Differential expression of *L. monocytogenes* strains *prfA** and Δ*prfA* grown under PrfA-inducing conditions. The scatterplot represents the average expression levels of transcripts. Significantly up-regulated transcripts are depicted in green (FDR ≤ 0.05 and FC ≥ 2.0), signifcantly down-regulated transcripts are in red (FDR ≤ 0.05 and FC ≤ 0.5), and non-differentially expressed transcripts are shown in grey. Due to the large number of significant transcripts in this comparison, only transcripts belonging to the core PrfA regulon and the novel, putative PrfA-dependent gene are labeled.

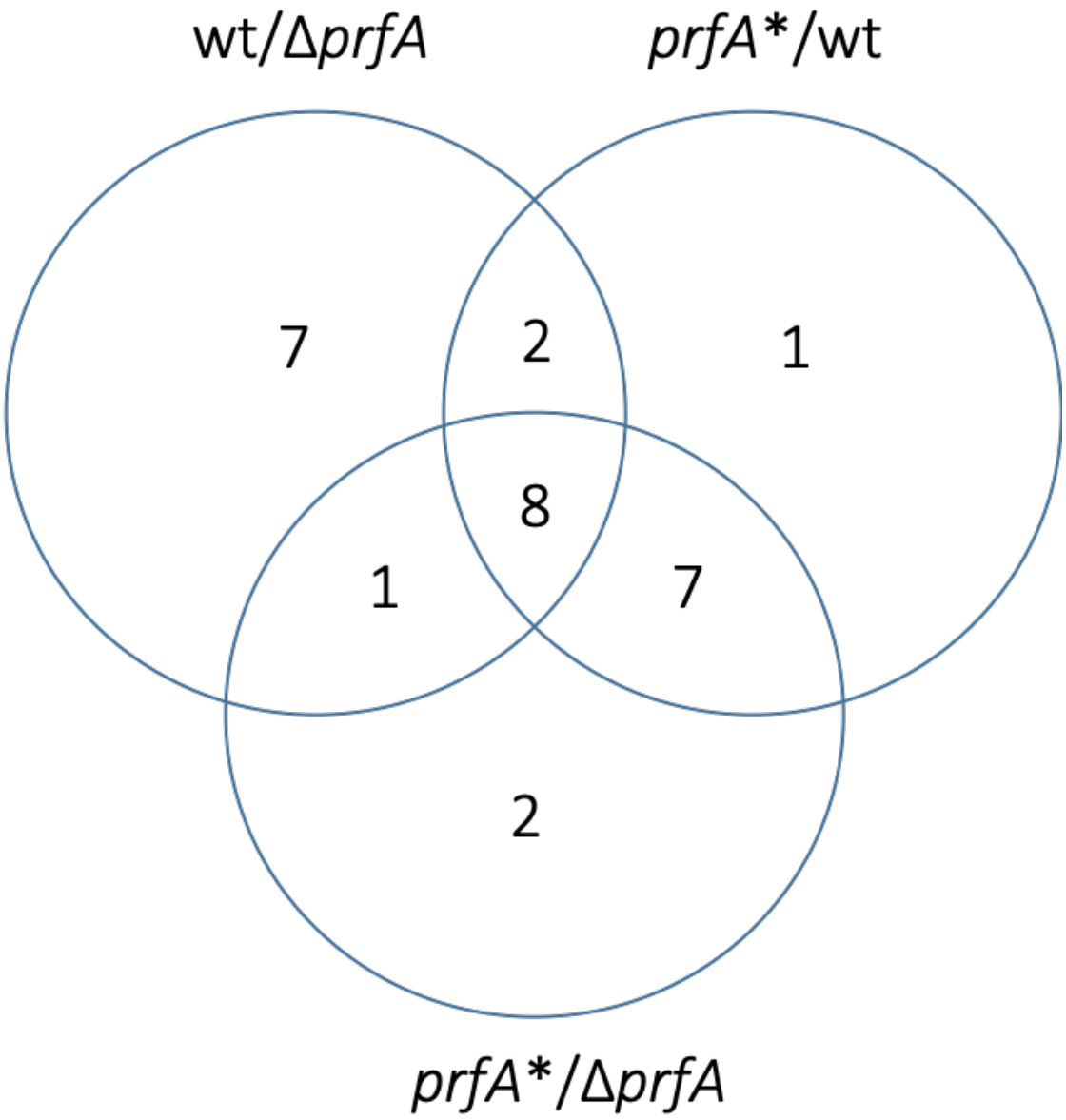


Figure 5.4: Distribution of significantly up-regulated (FDR ≤ 0.05, FC ≥ 2) transcripts in *L. monocytogenes* for each strain comparison. The numbers shown here represent the transcripts listed in 5.2.

pre-treated with activated charcoal and supplemented with G1P.

Of the 18 transcripts identified as significantly up-regulated, 8 were shared across all comparisons (Figure 5.4), most of which belong to the core PrfA regulon (Table 5.2); however, identification of weakly or indirectly regulated genes varied among our study and previous studies, as *inlAB* were not significant in our study compared to other transcriptomic studies [198, 227]. Most notably, our analysis was able to identify genes belonging to the LIPI-1 (*prfA*, *plcA*, *hly*, *mpl*, *actA*, and *plcB*). These genes had a high FC in relation to other PrfA-regulated genes in each comparison, with the highest FC in the *prfA**/Δ*prfA* comparison (40- to 900-fold). This is not surprising since in the *prfA** mutant PrfA is constitutively active. Furthermore, these genes make up the core PrfA regulon and are essential for *L. monocytogenes* survival as an intracellular pathogen [228, 190, 230].

5.4.3 RNA-seq identifies 5 new putative PrfA regulon members, including one with a putative upstream PrfA box located within an ORF

RNA-seq analysis of *L. monocytogenes* strains (wt, *prfA**, and Δ*prfA*) under PrfA-inducing conditions identified 5 putative PrfA-dependent protein-coding genes that have not been previously classified, including *LMRG_00319* (*lmo0636*), *LMRG_00320* (*lmo0637*), *LMRG_01109* (*lmo1962*), *LMRG_01622* (*lmo2210*), and *LMRG_02046* (*lmo0947*) (Table 5.2). Further analysis of these genes identified a PrfA box upstream of one of these genes, *LMRG_02046*

(*lmo0947*).

PrfA regulates gene expression by binding to a specific DNA motif known as the PrfA box, which is located upstream of the promoters -35 region. Traditionally, searching for different regulator binding sites was restricted to intergenic regions. However, transcription start sites within ORFs have been increasingly detected in several bacteria [232]. DNA sequences of known PrfA boxes were used to construct an HMM and a position weight matrix that were used to search for previously unidentified PrfA binding sites across the *L. monocytogenes* genome. All reported putative PrfA boxes were detected using our search criteria. Interestingly, a putative PrfA box was predicted within the 3 region of *LMRG_02047* (*lmo0948*) ORF and upstream of a predicted σ^{A}-dependent -35 and -10 promoter elements (Figure 5.6). Transcription from the predicted promoter is expected to increase the expression of the downstream gene, *LMRG_02046* (*lmo0947*) in PrfA-dependent manner but not *LMRG_02047* (*lmo0948*). Indeed, in the *prfA** mutant, expression of *LMRG_02046* (*lmo0947*) was increased by 5- and 7-fold compared to wt or Δ*prfA*, respectively (Figure 5.6). On the other hand, expression of *LMRG_02047* (*lmo0948*) was not significantly changed among the tested conditions, showing an increase of expression from 1- to 2-fold (Figure 5.6). *LMRG_02047* (*lmo0948*) encodes a GntR-type DNA-binding transcription factor and *LMRG_02046* (*lmo0947*) encodes a putative transmembrane transport protein from the major facilitator superfamily (MFS). MFS transport systems allow the uptake of essential nutrients, excretion of deleterious substances, and communication between cells and the environment [233]. PrfA-regulation of genes related to MFS transport could be essential in quickly sensing the intracellular environment to allow for uptake of nutrients that are essential for survival and excrete potentially harmful products. It

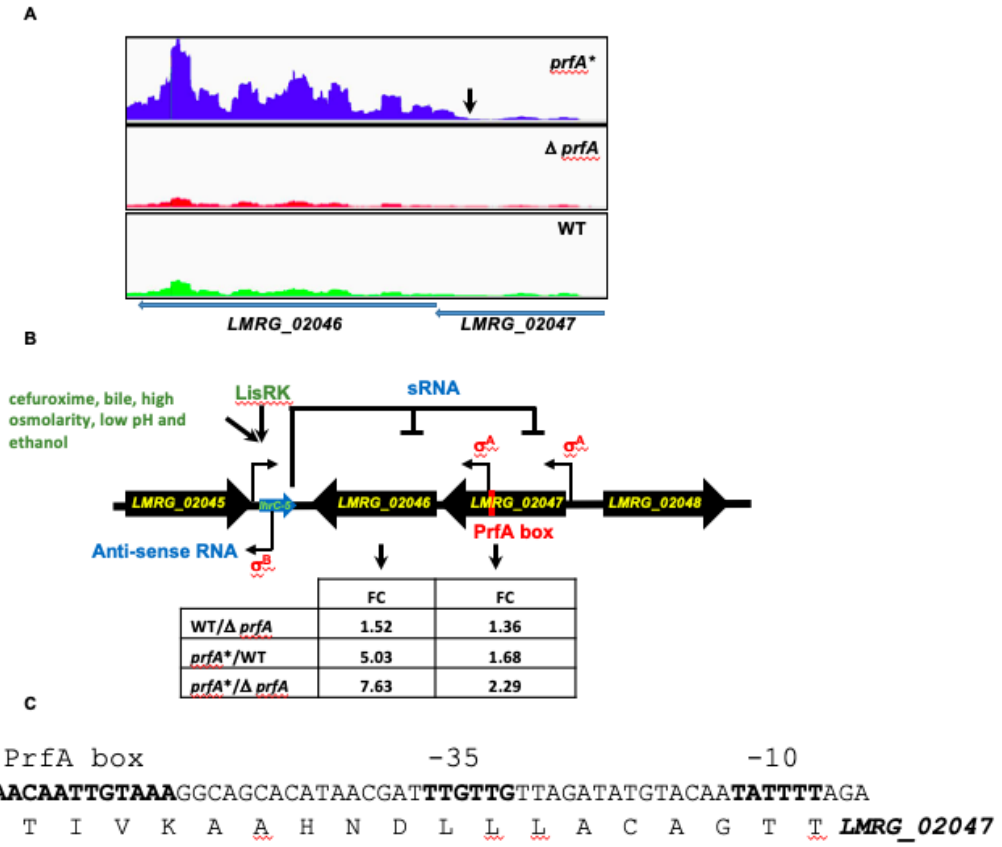
has been suggested that *LMRG_02046* (*lmo0947*) and *LMRG_02047* (*lmo0948*) are regulated post-transcriptionally by a ncRNA expressed from the downstream *lhrC-5* locus (Figure 5.6), which is in turn regulated by the σB-dependent antisense RNA, anti-*lhrC-5* [234, 235]. LhrC is a multicopy sRNA that acts as a repressor of the cell envelope-associated adhesin, LapB in response to cell envelope stress [234]. It is also known to be induced during infection by bacterial pathogens [234]. Given that surface proteins are recognized by the host immune system, down-regulation of genes by LhrC might be an attempt by *L. monocytogenes* to evade detection by immune cells [234]. Similarly, both *LMRG_02046* (*lmo0947*) and *LMRG_02047* (*lmo0948*) might be down-regulated by LhrC during the initial stages of infection by *L. monocytogenes*, followed by PrfA-dependent up-regulation of *LMRG_02046* (*lmo0947*) when virulence genes are necessary for intracellular survival.

Although, we did not identify PrfA binding sites for the other 4 putative PrfA-dependent genes, PrfA could still play an indirect role in expression of these genes. Previous reports have indicated low PrfA-associated differential expression for a number of genes [199, 198]. Functions for genes indirectly regulated by PrfA include, but are not limited to transporters, transcriptional regulators (*LMRG_00319/lmo0636* and *LMRG_01109/lmo1962*), metabolic enzymes (*LMRG_00320/lmo0637*), and proteins of unknown function (*LMRG_01622/lmo2210*), which are putative functions of the genes identified in this study (Table 5.2). Furthermore, it is possible that PrfA plays an indirect role in regulation of these genes. Previous studies have shown that σB regulates many genes that are also regulated by PrfA [236, 237], creating a co-regulatory network between the general stress response and virulence; therefore, future studies are needed to determine the mechanism of PrfA regulation for these

genes.

5.4.4 Conservation of PrfA box, including associated σA-dependent -35 and -10 promoter regions

The PrfA box is a conserved 14-bp sequence located approximately -40 bp upstream of the transcription start site of PrfA-regulated genes [190, 187, 238]. PrfA-dependent genes in *L. monocytogenes* are associated with a consensus PrfA box TTAACAnnTGTTAA [239, 227], which is conserved in *Listeria ivanovii* (an animal pathogen) and *Listeria seeligeri* (a non-pathogenic species) [240]. A previous study performed a comparative analysis of PrfA boxes, which revealed a pattern in the consensus sequence that is essential for the interaction of PrfA with the PrfA DNA-binding motif and proposed a canonical PrfA box defined by a consensus sequence TTAACANNTGTTAA, with seven invariant nucleotides (underlined) and tolerance for one or two mismatched non-obligatory nucleotides [227]. Our *in silico* analysis showed that *LMRG_02046* (*lmo0947*) contains the seven critical nucleotides and one mismatch nucleotide TTAACAATTTGTAAA, providing further evidence that this gene might be PrfA-dependent. Moreover, the newly identified PrfA box shares sequence similarity to PrfA boxes of known PrfA-dependent genes and aligns with genes included in the core PrfA regulon (Figure 5.5). Additionally, the associated -35 and -10 elements of the newly identified promoter region is similar to that of previously described σ^{A}-dependent promoters [190, 241] (Figure 5.6). Taken together, these data support that we potentially identified a novel PrfA-dependent gene; however, future studies are needed to confirm PrfA binding to the puta-



	FC	FC
WT/Δ *prfA*	1.52	1.36
*prfA**/WT	5.03	1.68
*prfA**/Δ *prfA*	7.63	2.29

Figure 5.5: Identification of a new putative PrfA-regulated genes. (A) expression of the *LMRG_02046-02047* locus in wt, *prfA* and *prfA**. (B) Organization and regulation of of the *LMRG_02046-02047* locus with fold change of gene expression in RNA-seq data. (C) DNA sequence of the putative PrfA box and promoter of *LMRG_02046*.

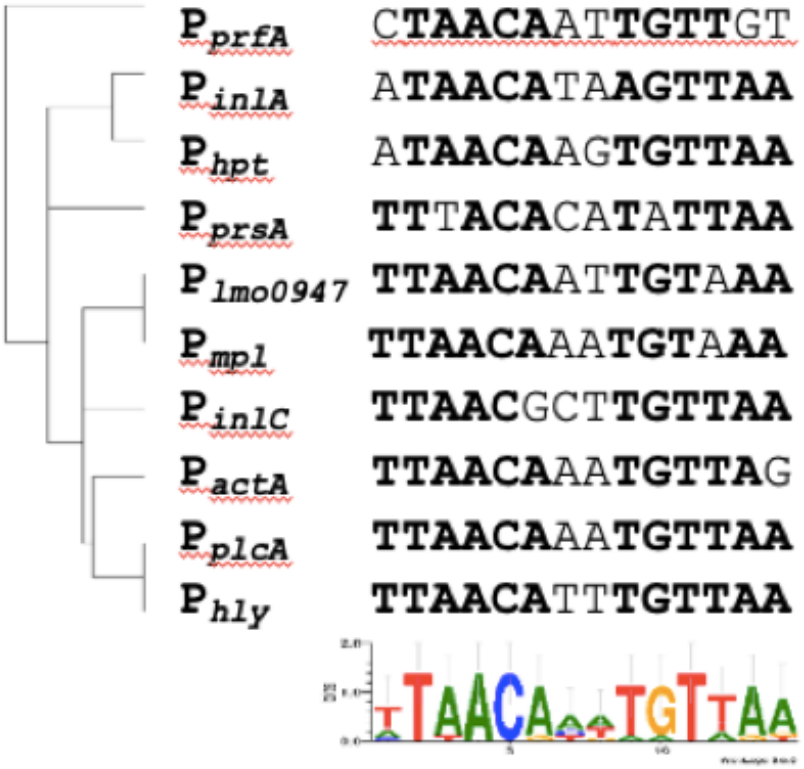


Figure 5.6: Variation of PrfA box sequences among different PrfA-regulated genes, compared to the putative PrfA-dependent gene *LMRG_02046* (*lmo09047*) described here. Sequence logo of the consensus sequence of the PrfA binding site.

tive PrfA box.

5.5 CONCLUSIONS

Combined with many studies that have investigated the PrfA regulon and virulence in *L. monocytogenes*, we have shown tha PrfA directly regulates a small subset of genes; however, using RNA-seq and bioinformatics we were able to identify a novel PrfA-dependent gene and its putative PrfA box. This finding highlights the importance of using advanced approaches to analyze RNA-seq data, which can potentially reveal previously unidentified genes regulated under specific conditions.

CHAPTER 6

CONCLUSIONS AND FUTURE WORK

6.1 Conclusions

In this thesis we show the critical role of temperature, pH, and *L. monocytogenes* serotype in the effectiveness of antimicrobials intended to control foodborne pathogens. Our results provide additional insights that go beyond the traditional approach of studying bacterial sensitivity to antimicrobials using broth studies. We show the complex interaction among the environment, the food matrix, the bacterial pathogen, and the control strategy. This is important as our data suggest that nisin- and phage-based control strategies could have limited impact on reducing public health risks if the physiochemical characteristics of the food product, as well as storage temperature are not considered.

We also identified a novel PrfA-dependent gene in *L. monocytogenes* using advanced bioinformatics approaches to analyze the RNA-seq data. Previous work done by micro-array is limited in its approach because it relies upon existing knowledge of genome sequences. However, RNA-seq experiments allow for investigation of known transcripts and exploring new ones. Therefore, our findings not only confirmed our existing knowledge of PrfA regulation in *L. monocytogenes*, but also shed light on putative novel genes regulated by PrfA.

6.2 Future Work

This research provides the framework for future investigations regarding *L. monocytogenes* resistance to nisn and phage, as well as contributions to virulence. Given that under certain environmental conditions, *L. monocytogenes* is less sensitive to nisin and phage, there is a risk that some strains might acquire resistance upon repeated exposure to these interventions. Thus, without novel antimicrobial treatments *L. monocytogenes* can continue to contaminate foods and subsequently cause disease in humans. Future investigations aimed at expanding our understanding of the potential for nisin and phage resistance should include (i) determining whether surviving cells after exposure to nisin or phage are truly resistant and (ii) whether that resistance is transient or heritable. It is likely that *L. monocytogenes* found in food and food-processing environments can become resistant to phage as phage-resistance has been observed in *L. monocytogenes* isolated from natural environments [163].

Further characterization of the novel PrfA-dependent gene (*LMRG_02046*) identified here is needed to determine (i) if PrfA binds to its DNA-binding site (PrfA box) and (ii) its putative function/role. A gel shift assay can be used to verify protein-DNA binding; however, defining the function of *LMRG_02046* is challenging given that it could be co-regulated by other transcriptional regulators.

Overall, efforts to control *L. monocytogenes* and understand its regulation are still being explored. Each new discovery leads to an exciting contriubiton to the story of this important foodborne pathogen.

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