

**Development Toward a Bovine *Salmonella* Model and a Bacteriophage Treatment Cocktail
to Reduce *Salmonella* Peripheral Lymph Node Carriage in Calves**

by

James Forrest Shirley

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Approved by

Stuart B. Price, Chair, Associate Professor of Pathobiology
Sang-Jin Suh, Associate Professor Biological Sciences
James C. Wright, Professor of Pathobiology

Abstract

Non-typhoidal salmonellosis is the leading bacterial cause of human foodborne disease in the United States and is responsible for numerous hospitalizations and deaths each year. Although several strategies have been successful in controlling other foodborne pathogens such as *Escherichia coli* O157:H7, the incidence of *Salmonella* foodborne disease has been persistently above the national *Healthy People* target for the past decade, emphasizing the importance of the need for increased efforts to control this pathogen.

Cattle are known to harbor *Salmonella* and ground beef has been identified as the source of several foodborne *Salmonella* outbreaks, including a multistate outbreak of *Salmonella* Enteritidis (SE) in ground beef during the summer of 2012. Although cattle hides are considered to be the largest contributor to beef carcass contamination by enteric pathogens, a growing body of evidence suggests that peripheral lymph nodes (PLN) are important sources of *Salmonella* contamination of ground beef. Unlike mesenteric lymph nodes, PLNs are not routinely removed during carcass evisceration and are a potential source of ground beef contamination from infected cattle, underscoring the importance of pre-harvest interventions to reduce pathogens in these sites. Researchers have examined the use of vaccines and direct-fed microbials to reduce *Salmonella* in peripheral lymph nodes with limited success.

Bacteriophage (phage) treatment also has been explored to reduce pathogens and previous studies have demonstrated the efficacy of bacteriophage (phage) treatment to control *E. coli* O157:H7 in pre-harvest cattle. However, to my knowledge, nothing has been published on the use

of orally applied phage as a pre-harvest intervention strategy for, or to reduce peripheral lymph node carriage of, *Salmonella* in cattle.

In response to the 2012 outbreak of SE in ground beef, I hypothesize that SE causes enteric disease in calves and disseminates from the bovine gut to the peripheral lymph nodes, which are not removed at slaughter, and thus contaminate ground beef following carcass processing. The specific aims of this project were to (1) develop an experimental model of SE infection and peripheral lymph node carriage in calves; (2) evaluate the potential for a treatment cocktail of seven lytic phages targeting SE to reduce fecal shedding, disease signs, and peripheral lymph node carriage in the SE calf model, and (3) characterize the seven phages and optimize the treatment by selecting the three phages best suited for pathogen reduction.

To address specific aims one and two, I worked toward developing a model of SE infection and PLN carriage in calves and evaluated the potential for a seven-phage cocktail to reduce disease signs, fecal shedding, and PLN carriage in infected calves. Three pairs of 5-7 week-old calves (four control calves, and two treated calves) were orally challenged with 5.0×10^9 - 1.3×10^{10} CFUs of a bovine SE isolate. Following inoculation, daily fecal samples were enumerated for SE and rectal temperatures were recorded twice daily. Blood, subiliac, and superficial cervical lymph nodes were cultured post-mortem. In treated calves, a cocktail of seven lytic phages targeting SE was orally administered following SE challenge. Oral challenge with SE produced mixed results. Fever spikes were noted for days two or three post inoculation. Although each calf received a high dose of SE, fecal shedding of the organism varied among calves in control (C1, C2, C3, C4) and phage-treated (T1 and T2) groups. Calf T1 shed low amounts of SE ($2-3 \log_{10}\text{CFU/g feces}$); calves C1, C3 and C4 shed moderate amounts of SE ($4-6 \log_{10}\text{CFU/g feces}$); and calves C2 and T2 shed high amounts of SE ($6-8 \log_{10}\text{CFU/g feces}$). Bacteremia was noted for two amongst the three most

severely affected calves and SE was recovered from the PLNs of the same three calves. Following treatment, phages were recovered from PLNs of calf T2. These findings demonstrate that SE causes enteric disease and invades PLNs in calves and that phage treatment may be effective in mitigating *Salmonella* carriage in PLNs. Also, the presence of SE in the PLNs of the three most severely affected calves and its presence in the blood of two of these three suggests that bacteremia may mediate translocation of *Salmonella* from the gut to the PLNs.

Although findings from my calf model of SE suggested that an oral treatment cocktail of seven lytic phages targeting SE may control peripheral lymph node carriage, I sought to reduce the cocktail to the three most suitable phages due to the constraints involved in validating the safety and efficacy of seven phages in a calf model. Characterization experiments were performed for each of the seven phages in the cocktail in order to establish exclusion criteria for cocktail optimization. Electron micrographs were prepared by negatively staining concentrated phage lysates with 2% phosphotungstic acid and viewed with transmission electron microscopy. Qualitative lytic activity was assessed by performing *Salmonella* growth curves in the presence of phage (lysis curves) at varying multiplicities of infection (MOI). Additionally, *Salmonella* host range, efficiency of plating, adsorption rate constants, and ultra-violet (UV) inactivation constants were determined for each phage. Phages were classified into three families based upon morphology: Myoviridae (three phage), Siphoviridae (two phage), and Podoviridae (two phage). Each cocktail phage demonstrated strong lytic activity against SE and was able to lyse or form plaques on multiple *Salmonella* serovars. Except for phage in the Podoviridae family, similarities in the host ranges, efficiencies of plating, lysis curve patterns and adsorption rate constants were found among phages in the same family, suggesting redundancy among cocktail phages in the

Myoviridae and Siphoviridae families. These findings were used to select three of the seven phages for future treatment experiments in my calf model.

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Table of Contents

Abstract	ii
Acknowledgments.....	vi
List of Tables	xiv
List of Figures	xv
List of Abbreviations	xvi
Chapter 1. Literature Review	1
1.1 <i>Salmonella</i> Background	1
1.1.1 Salmonellosis.....	2
1.1.2 <i>Salmonella</i> Virulence Factors	3
1.1.2 Host Specificity	3
1.2 <i>Salmonella</i> Epidemiology and Food Safety.....	5
1.2.1 Serovars in Human Infections	6
1.2.2 Foods Implicated in <i>Salmonella</i> Outbreaks.....	8
1.2.3 Association of Serovars and Food Commodities	9
1.3 <i>Salmonella</i> Enteritidis Background	10

1.3.1	<i>Salmonella</i> Enteritidis Epidemiology.....	10
1.3.2	Foods Associated with <i>Salmonella</i> Enteritidis	11
1.3.3	<i>Salmonella</i> Enteritidis in Poultry and Eggs.....	12
1.3.4	<i>Salmonella</i> Enteritidis Associated with Cattle and Beef	13
1.4	<i>Salmonella</i> in Cattle.....	15
1.4.1	<i>Salmonella</i> Prevalence in Cattle.....	15
1.4.2	<i>Salmonella</i> Serovars Associated with Cattle.....	16
1.4.3	Bovine Salmonellosis	16
1.4.4	Bovine Models of <i>Salmonella</i> Infection in Calves.....	17
1.4.5	Asymptomatic Carrier Status and Fecal Shedding.....	18
1.5	<i>Salmonella</i> Associated with Beef	20
1.5.1	<i>Salmonella</i> Outbreaks Associated with Ground Beef	20
1.5.2	<i>Salmonella</i> Serovars Associated with Ground Beef.....	21
1.5.3	<i>Salmonella</i> Prevalence in Ground Beef.....	21
1.5.4	Common <i>Salmonella</i> Isolates among Humans and Food-Animals	22
1.6	Ground Beef Contamination with <i>Salmonella</i>	23
1.6.1	Bovine Peripheral Lymph Nodes and Ground Beef Contamination	24
1.6.2	<i>Salmonella</i> Prevalence in Bovine Peripheral Lymph Nodes.....	25
1.6.3	<i>Salmonella</i> Concentrations in Bovine Peripheral Lymph Nodes	26
1.6.4	Seasonal Variability in Peripheral Lymph Node Prevalence	27

1.6.5	<i>Salmonella</i> Serotypes in Peripheral Lymph Nodes	28
1.6.6	Mechanism of Peripheral Lymph Node Contamination.....	28
1.7	Bacteriophage Background.....	30
1.7.1	Discovery and Early Research	30
1.7.2	Morphology and Nomenclature.....	31
1.7.3	Infection Cycles.....	32
1.7.4	Bacteriophage Adsorption.....	33
1.8	Pre-harvest Intervention Strategies	34
1.8.1	Sodium Chlorate.....	34
1.8.2	Direct-fed Microbials	35
1.8.3	Vaccines	35
1.8.4	Pre-harvest Interventions in Peripheral Lymph Nodes	36
1.9	Bacteriophage Therapy	37
1.9.1	Advantages of Bacteriophage Therapy	37
1.9.2	Disadvantages of Bacteriophage Therapy	37
1.9.3	Bacteriophage Therapy in Cattle.....	38
1.9.4	Phage Therapy Reduction of Feedlot <i>E. coli</i> O157:H7 Prevalence	39
1.10	Introduction.....	40
1.11	Chapter 1 References	45
Chapter 2.	55

2.1	Abstract	56
2.2	Introduction	58
2.3	Materials and Methods	61
2.3.1	Bacterial Strains and Bacteriophage Treatment Cocktail	61
2.3.2	Animal Care and Housing	61
2.3.3	Calf Experiments	62
2.3.4	Enumeration of <i>Salmonella</i> shed in feces	63
2.3.5	Enumeration of Phage Shed in Feces	63
2.3.6	Post-Mortem Lymph Node Culturing	64
2.3.7	Detection of Phage in Peripheral Lymph Nodes	64
2.4	Results	66
2.4.1	Control Calves C1 and C2	66
2.4.2	Treatment Calves T1 and T2	66
2.4.3	Control Calves C3 and C4	67
2.4.4	Post-Mortem Lymph Node Culturing	68
2.5	Discussion	69
2.5.1	<i>S. Enteritidis</i> Disease in Calves	69
2.5.2	<i>S. Enteritidis</i> Recovery from Peripheral Lymph Nodes	71
2.5.3	Mechanism of <i>Salmonella</i> Dissemination	72
2.5.4	Bacteriophage Treatment	72

2.5.5 Shedding of Bacteriophage in the Feces	73
2.6 Chapter 2 References	75
2.7 Chapter 2 Tables and Figures	79
Chapter 3.....	87
3.1 Abstract.....	88
3.2 Introduction.....	89
3.3 Materials and Methods.....	91
3.3.1 Bacterial Strains and Bacteriophage.....	91
3.3.2 Phage Typing and Pulsed-Field Gel Electrophoresis (PFGE).....	91
3.3.3 Double Agar Overlays.....	92
3.3.4 Screening Veterinary Clinical Samples for <i>Salmonella</i> Bacteriophage	93
3.3.5 Bacteriophage Isolation	93
3.3.6 Bacteriophage Enumeration	94
3.3.7 Preparation of Amplified Bacteriophage Stocks	94
3.3.8 Host Range Determination	95
3.3.9 Efficiency of Plating (EOP)	95
3.3.10 Bacteriophage Concentration and Transmission Electron Microscopy	96
3.3.11 Lysis Curves	96
3.3.12 Adsorption Curves.....	96
3.3.13 Bacteriophage Inactivation by Ultra-Violet Irradiation	97

3.4 Results.....	99
3.4.1 Phage Typing and PFGE of SE Isolates	99
3.4.2 Cocktail Bacteriophages.....	99
3.4.3 Transmission Electron Microscopy of Bacteriophage	99
3.4.4 Bacteriophage Spot Lysis Results	100
3.4.5 Bacteriophage Plaque-formation.....	100
3.4.6 Efficiency of Plating.....	101
3.4.7 Bacteriophage Lysis of SE Broth Cultures.....	102
3.4.8 Adsorption Curves of Cocktail Bacteriophage.....	102
3.4.9 Bacteriophage Inactivation by Ultraviolet Light.....	103
3.5 Discussion.....	104
3.5.1 Host Range of <i>Salmonella</i> Bacteriophages	104
3.5.2 Relative Efficiency of Plating	105
3.5.3 Adsorption Rate Constants	106
3.5.4 Lysis Curves	107
3.5.5 Optimization of SE Bacteriophage Cocktail	107
3.6 Chapter 3 References	109
3.7 Tables and Figures	112
Chapter 4. Discussion	130
4.1 <i>S. Enteritidis</i> Disease in Calves	130

4.2 Recovery of <i>S. Enteritidis</i> from the Peripheral Lymph Nodes	130
4.3 Bacteriophage Treatment	131
4.3.1 Phage Cocktails and Phage Resistance	132
4.3.2 Phage Treatment to Reduce <i>S. Enteritidis</i> Carriage in PLN.....	133
4.3.3 Fecal Shedding of Bacteriophage.....	134
4.4 Bacteriophage Characterization	135
4.5 Chapter 4 References	137
Thesis References	141

List of Tables

Table 2.1: SE in Peripheral Lymph Nodes and Blood	86
Table 3.1: Phage Typing and PFGE of SE Isolates	113
Table 3.2: Transmission Electron Microscopy of <i>Salmonella</i> Bacteriophage.....	114
Table 3.3: Bacteriophage Spot Test on <i>Salmonella</i> Serovars	115
Table 3.4: Bacteriophage Spot Test on SE Isolates	116
Table 3.5: Bacteriophage Lytic Activity and Plaque Formation on <i>Salmonella</i> Serovars	117
Table 3.6: Bacteriophage Lytic Activity and Plaque Formation on SE Isolates	118
Table 3.7: Efficiency of Plating (EOP) on <i>Salmonella</i> Serovars Relative to Bov SE Isolate	119
Table 3.8: Efficiency of Plating (EOP) on SE Isolates Relative to Bov Isolate	120
Table 3.9: Phage Adsorption Rate Constants	128
Table 3.10: Phage UV Inactivation Constants.....	129

List of Figures

Figure 2.1: C1 Fecal Shedding and Body Temperature	79
Figure 2.2: C2 Fecal Shedding and Body Temperature	80
Figure 2.3: T1 Fecal Shedding and Body Temperature	81
Figure 2.4: T2 Fecal Shedding and Body Temperature	82
Figure 2.5: T1 Fecal Shedding of SE and Phage	83
Figure 2.6: C3 Fecal Shedding and Body Temperature	84
Figure 2.7: C4 Fecal Shedding and Body Temperature	85
Figure 3.1: Pulsed-Field Gel Electrophoresis of SE Isolates	111
Figure 3.2: vB_SenM-S7 Lysis of Bovine SE	121
Figure 3.3: vB_SenM-S10 Lysis of Bovine SE	122
Figure 3.4: vB_SenM-S13 Lysis of Bovine SE	123
Figure 3.5: vB_SenS-S56 Lysis of Bovine SE	124
Figure 3.6: vB_SenS-S57 Lysis of Bovine SE	125
Figure 3.7: vB_SenP-S11 Lysis of Bovine SE	126
Figure 3.8: vB_SenP-S25 Lysis of Bovine SE	127

List of Abbreviations

AUCVM	Auburn University College of Veterinary Medicine
Av	Avian
BHI	Brain Heart Infusion
Bov	Bovine
CFU	Colony Forming Unit
DMSO	Dimethyl Sulfoxide
EOP	Efficiency of Plating
EtOH	Ethanol
LB	Lysogeny Broth supplemented with 3% MgSO ₄
LB _c	Lysogeny Broth Broth supplemented with 3% MgSO ₄ and 10mM CaCl ₂
LBM	Lysogeny Broth Agar supplemented with 3% MgSO ₄
LBMt	Lysogeny Broth Top Agar supplemented with 3% MgSO ₄
LC	Left Superficial Cervical Lymph Node
LN	Lymph Node
LS	Left Subiliac Lymph Node
MLN	Mesenteric Lymph Node
MOI	Multiplicity of Infection
Nal	Nalidixic Acid
OD ₆₂₀	Optical Density at 620 Nanometers

OD ₆₅₀	Optical Density at 650 Nanometers
ON	Over Night
PFGE	Pulsed-Field Gel Electrophoresis
PFU	Plaque Forming Unit
PI	Post Inoculation
PLN	Peripheral Lymph Node
PT	Phage Type
RC	Right Superficial Cervical Lymph Node
RS	Right Subiliac Lymph Node
RT	Room Temperature
RV	Rappaport Vassiliadis Broth
<i>Salmonella</i>	<i>Salmonella enterica</i> spp. <i>enterica</i>
SE	<i>Salmonella enterica</i> spp. <i>enterica</i> serovar Enteritidis
SM	Salt Magnesium Buffer
TTh	Tetrathionate Enrichment Broth
UV	Ultra Violet
XLT4	Xylose-Lysine-Tergitol 4 Agar
YS	Chicken Yolk-Sac

Chapter 1. Literature Review

1.1 *Salmonella* Background

Salmonella enterica spp. *enterica* (*Salmonella*) is a genus of gram-negative bacteria comprised of non-spore forming bacilli belonging to the *Enterobacteriaceae* family (Coburn *et al.*, 2007; Dunkley *et al.*, 2009; Agbaje *et al.*, 2011). The genus comprises two species (Reeves *et al.*, 1989; Su and Chiu, 2007), characterized by motility mediated by peritrichous flagella, and facultative anaerobic metabolism (Coburn *et al.*, 2007; Agbaje *et al.*, 2011; Andino and Hanning, 2014). Like other genera in the *Enterobacteriaceae* family, *Salmonella* live within the gastrointestinal tracts of many mammals, birds, and reptiles, but can also persist in the environment (Callaway *et al.*, 2014). The genus is named after an American bacteriologist, D. E. Salmon, after the discovery and isolation of what was then called “bacillus choleraesuis” (now known as *Salmonella* Choleraesuis) from porcine intestines by his research assistant Theobald Smith in 1884 (Agbaje *et al.*, 2011). *Salmonella* nomenclature is considerably complex (Brenner *et al.*, 2000; Dunkley *et al.*, 2009) and many shifts in conventional naming schemes throughout the history of classifying this genus (Salyers 2002; Bopp *et al.*, 1999) have led to confusion among public health officials, scientists, and members of the general public (Brenner *et al.*, 2000). Originally, salmonellae were differentiated into different “serovars” (also called “serotypes”) according to a scheme of serological classification of poly-O and flagellar (H) antigens described by Kaufman and White, who also proposed that each serovar be considered a separate species (Brenner *et al.*, 2000). According to the current Centers for Disease Control and Prevention (CDC) system, the

genus *Salmonella* consists of two species: *Salmonella enterica* (designated as the type species) and *Salmonella bongori* (Su and Chiu, 2007). A third species, *Salmonella subterranean*, was recognized in 2005, but has yet to be adopted by the CDC (Su and Chiu, 2007). Within *Salmonella enterica*, there are six subspecies, (Su and Chiu, 2007), and among these six, the subspecies *enterica* is most relevant to animal disease and is responsible for 99% of Human *Salmonella* infections (Uzzau *et al.*, 2000). *Salmonella enterica* spp. *enterica* (hereafter referred to as *Salmonella*) can be further classified into serovars (Fierer and Guiney, 2001) and more than 2,500 serovars have been described (Hendriksen *et al.*, 2009).

1.1.1 Salmonellosis

The types of disease that are caused by *Salmonella* are classified into two major categories: typhoidal and non-typhoidal salmonellosis (Coburn *et al.*, 2007). Typhoidal disease (or typhoid fever) is a systemic disease generally caused by *S. enterica* subsp. *enterica* serovar Typhi (*S. Typhi*) in humans (Coburn *et al.*, 2007), although other *Salmonella* serovars cause typhoid-like disease in other animals, e.g., Dublin in cattle and Typhimurium in mice (Costa *et al.*, 2012). Paratyphoid fever is another type of salmonellosis that is very similar to typhoid fever, but is attenuated in terms of its severity and duration. Paratyphoid fever is caused by *Salmonella enterica* subsp. *enterica* serovar Paratyphi, a human-specific pathogen. Both typhoid fever and paratyphoid fever have been referred to as “enteric fevers” (Bhan *et al.*, 2005). Clinical manifestations of typhoidal disease in humans include bacteremia, fever, nausea, anorexia, myalgia and headache. Gastrointestinal symptoms, such as diarrhea, can occur occasionally in immunocompromised individuals, but are not primary features of the disease. In contrast, non-typhoidal salmonellosis is primarily an infection of the gastrointestinal tract (enteritis) characterized by fever, diarrhea, malaise, nausea and vomiting (Coburn *et al.*, 2007).

1.1.2 *Salmonella* Virulence Factors

Several virulence factors mediate the ability of *Salmonella* to invade gut epithelia and evade host immune responses, both of which are fundamental features underlying the production of gastrointestinal disease and survival within the host (Mohler *et al.*, 2009). The genes that encode these virulence determinants are found in *Salmonella* pathogenicity islands (SPI) which are located on the chromosome or plasmids (Knodler *et al.*, 2005; Ibarra and Steele-Mortimer, 2009). A total of 23 SPI's have been described in the literature so far. However, the functions of the genes within these islands have yet to be completely elucidated (Sabbagh *et al.*, 2010; Hayward *et al.*, 2013). Of these twenty-three, only five, SPI1-SPI5, are found in all *Salmonella enterica* serovars (Hurly *et al.*, 2014). These genes encode for effector proteins necessary for *Salmonella* invasion and intracellular survival as well as a type III secretion system involved in translocation of these proteins across the plasma membrane directly into the mammalian cells (Coburn *et al.*, 2007; Fierer 2001; Garai *et al.*, 2012). The functions that are mediated by SPI's include gut epithelial invasion, stimulation of cytokine secretion, stimulation of fluid secretion in the ileum, intracellular growth through modification of the intracellular environment, and the full manifestation of enterocolitis, and systemic salmonellosis (Coburn *et al.*, 2007; Fierer 2001; Garai *et al.*, 2012; Hurly *et al.*, 2014).

1.1.2 Host Specificity

Salmonella serovars have evolved various predilections for particular hosts. This preference for particular hosts is thought to involve differences/specificities in the adhesion of the organism to surface molecules on the mucosal surface in the gastrointestinal tract (Bäumler *et al.*, 1998). Some serovars preferentially infect a specific host and are referred to as “host-adapted” (Bäumler *et al.*, 1998). Examples of these serovars and their respective hosts include serovars

Choleraesuis in swine, and Dublin in Cattle (Uzzau *et al.*, 2000; Coburn *et al.*, 2007; Costa *et al.*, 2012; Chen *et al.*, 2013). While infections with host-adapted serovars are typically observed for a particular host, they are able, rarely, to infect other hosts (Uzzau *et al.*, 2000). For example, although Choleraesuis and Dublin cause severe disease in swine and cattle respectively, they also can cause a milder disease in other mammalian species including humans (Smith and Jones, 1967; Wray and Sojka, 1977; Nnalue 1991). Other host-adapted serovars are almost always associated with producing disease in a single organism, such as serovars Typhi/Paratyphi in Humans and Gallinarum/Pullorum in poultry. These serovars are referred to as “host-restricted” (Uzzau *et al.*, 2000). In contrast, other serovars such as Typhimurium, Enteritidis and Newport are capable of infecting a wide range of hosts and are referred to as “host un-adapted” (Uzzau *et al.*, 2000).

1.2 *Salmonella* Epidemiology and Food Safety

Over the past decade, non-typhoidal *Salmonella* has remained the leading bacterial cause of human foodborne disease in the United States (CDC 2009; CDC 2011; Scallan *et al.*, 2011; Gould *et al.*, 2013; Crim *et al.*, 2014; Crim *et al.*, 2015) and abroad (Scallan *et al.*, 2011). These agents are estimated to be responsible for 1.2 million illnesses in the United States each year, with the overwhelming majority of illnesses transmitted through food (Crum-Cianflone 2008; Scallan *et al.*, 2011). Human *Salmonella* infections are also associated with a high degree of morbidity and mortality (Jones *et al.*, 2008; CDC 2011; Scallan *et al.*, 2011; Gould *et al.*, 2013; Crim *et al.*, 2014, Crim *et al.*, 2015), and represent the leading cause of hospitalizations and deaths each year among foodborne illnesses acquired in the United States (CDC 2011; Scallan *et al.*, 2011; Gould *et al.*, 2013; Crim *et al.*, 2014; Crim *et al.*, 2015). Between 1998 and 2008, *Salmonella* was the most implicated etiology in foodborne outbreaks and caused the most illnesses among bacterial foodborne pathogens, accounting for 1,449 (18%) of 7,998 outbreaks and 39,126 (19%) of 273,120 illnesses in the United States. It was also the single greatest cause of morbidity and mortality among foodborne pathogens during this time, causing 44% of hospitalizations and 30% of deaths during this same period (Gould *et al.*, 2013). In recent years, *Salmonella* was responsible for over 7,000 human illnesses in 2013 and 2014, resulting in more than 2,000 hospitalizations and the largest number of deaths among bacterial pathogens commonly transmitted through food in the United States (Crim *et al.*, 2014, Crim *et al.*, 2015).

There has been no decline in the incidence and prevalence of human *Salmonella* infections for several years (CDC 2011; Crim *et al.*, 2015). In fact, *Salmonella* infections in 2010 were greater than from 2006-2008, and were almost three times the 2010 national health objective (CDC 2011). In 2013 and 2014, *Salmonella* infections decreased slightly compared to 2010-2012 but were not

significantly different from 2006-2008 ($p \leq 0.05$) (Crim *et al.*, 2014; Crim *et al.*, 2015). In response to the major role *Salmonella* infections play in public health, the US Department of Health and Human Services called for a 25% decrease in nation-wide incidence of these infections as part of the Healthy People 2020 national goal (CDC 2011). However, despite previous and ongoing food-safety intervention efforts, little progress has been made to decrease incidence of foodborne infections caused by *Salmonella*, and the incidence of *Salmonella* infections has persistently remained well above the National Healthy People target for 2020 (Crim *et al.*, 2014; McEntire *et al.*, 2014; Wheeler *et al.*, 2014; Crim *et al.*, 2015).

1.2.1 Serovars in Human Infections

Despite the inadequate progress of food safety efforts to reduce overall incidence of human *Salmonella* infections, some interventions may have played a role in decreasing the incidence of infection by particular *Salmonella* serovars (Crim *et al.*, 2015). For example, in 2014, the incidence in human infections of *Salmonella* Typhimurium decreased by 27% compared to 2006-2008, even though overall *Salmonella* incidence remained unchanged (Crim *et al.*, 2015). However, there are over 2,500 *Salmonella* serovars (Brenner *et al.*, 2000) and, over the past two decades, there has been substantial variation among *Salmonella* serovars causing human infections (Sarwari *et al.*, 2001; Jones *et al.*, 2008; CDC 2009; CDC 2011; Gould *et al.*, 2013; Jackson *et al.*, 2013; Crim *et al.*, 2014; Crim *et al.*, 2015). Between 1990 and 1995, the top five serovars identified in human cases were Typhimurium, Enteritidis, Heidelberg, Newport, and Hadar (Sarwari *et al.*, 2001). Similarly, the five most frequent *Salmonella* serovars accounting for 61% of all isolates from 1996 to 2006 were Typhimurium, Enteritidis, Newport, Heidelberg, and Javiana (Jones *et al.*, 2008). In more recent summaries of surveillance data from 1998 to 2008, Enteritidis, Typhimurium, Heidelberg, and Newport were identified as the most frequent *Salmonella* serovars implicated in

human foodborne outbreaks (Gould *et al.*, 2013; Jackson *et al.*, 2013). In 2008, the top ten serovars in 6,750 (91%) serotyped *Salmonella* human isolates were: Enteritidis (20.1%), Typhimurium (16.0%), Newport (10.1%), Javiana (6.3%), Saintpaul (6.0%), I 4,[5],12:i:- (4.0%), Muenchen (3.2%), Heidelberg (2.9%), Montevideo (2.9%), and Braenderup (1.6%). When compared to combined data from 1996-1998 and 2005-2007, significant differences ($p \leq 0.05$) among the top serovars in 2008 were observed for Enteritidis (19% increase), Saintpaul (182% increase), and Heidelberg (38% decrease) (CDC 2009). In 2010, the CDC identified Enteritidis, Newport, and Typhimurium as the most common serotypes among 92% of serotyped *Salmonella* isolates (CDC 2011). Compared with data from 1996-1998, significant differences ($p \leq 0.05$) in the incidence among these serotypes were noted for: Enteritidis (76% increase), Newport (47% increase), and Typhimurium (53% decrease) (CDC 2011). In 2013, the top serovars accounting for 6,520 (90%) of serotyped isolates from human infections in the United States were Enteritidis, 1,237 (19%); Typhimurium, 917 (14%); and Newport, 674 (10%). When 2013 data was compared to data from 2010-2012, significant decreases ($p \leq 0.05$) were noted for Enteritidis and Newport infections. However, significant decreases ($p \leq 0.05$) were observed only for Typhimurium and unchanged for Enteritidis and Newport when comparing 2013 data to 2006-2008 (Crim *et al.*, 2014). In 2014, the top five serotypes accounting for 88% of the 7,452 *Salmonella* isolates from human infections were Enteritidis, Typhimurium, Newport, Javiana, and Infantis. Compared with 2006-2008, the incidence of Typhimurium infection was significantly lower ($p \leq 0.05$) in 2014 whereas the incidence of Infantis infections increased compared to 2011-2013 (Crim *et al.*, 2015).

Fluctuations in the incidence of human infection by *Salmonella* serovars is multifactorial and caution must be exercised before drawing conclusions about the success or failure of food safety efforts. First, variation in the frequency of food contamination or changes in food

commodity consumption both have obvious impacts on the incidence and prevalence of foodborne human infections (CDC 2011; Jackson *et al.*, 2013). Second, the data used to estimate the incidence of *Salmonella* infections include both foodborne outbreaks and sporadic cases of human infections. For example, although 80% of *Salmonella* outbreaks between 1998 and 2008 were caused by a single serotype, only 50% of these had an implicated food (Jackson *et al.*, 2013). Thus, despite the close association of *Salmonella* infections with food (Crim *et al.*, 2014), overall incidence may not precisely reflect the top *Salmonella* serovars causing foodborne infections (CDC 2011; Jackson *et al.*, 2013). Third, governmental regulations and changes in food processing practices likely influence incidence of infections by foodborne pathogens (Crim *et al.*, 2015). Fourth, the increasing reliance on culture-independent tests likely affects the number of reported culture-confirmed cases either by increasing the number of cases due to the ease and convenience of these methods, or decreasing the number of cases through lack of diagnosis by traditional culturing methods (Crim *et al.*, 2015). Finally, population characteristics (i.e. the propensity of individuals to seek treatment) likely influence the number of reported human infections (Crim *et al.*, 2015). These factors, among other unidentified variables, limit the generalizability of these and other studies on the incidence of *Salmonella* infections. However, it can be concluded from the epidemiological discussion above that several specific *Salmonella* serovars are consistently implicated human cases of salmonellosis, and that these serovars are valid intervention targets in the food industry.

1.2.2 Foods Implicated in *Salmonella* Outbreaks

Although the identification of *Salmonella* serovars is critical, it is also important consider the association between particular serovars and specific foods in order to design targeted intervention strategies. *Salmonella* are able to contaminate a vast variety of foods (Crum-Cianflone

2008; CDC 2011; Jackson *et al.*, 2013; Crim *et al.*, 2015) and more than 95% of human cases of salmonellosis are linked to consumption of contaminated food items (Crum-Cianflone 2008). The most common foods implicated in human foodborne *Salmonella* infections and outbreaks are animal products such as poultry and eggs, beef, pork, and dairy (Crum-Cianflone 2008), although fruits/nuts, sprouts, and vine-stalked vegetables have also been implicated in human foodborne infections (Gould *et al.*, 2013). Between 1998 and 2008, the most common food item implicated in 1,491 human *Salmonella* outbreaks were eggs (28%), followed by chicken (16%), pork (9%), beef (8%), fruit (8%), and turkey (7%) (Jackson *et al.*, 2013).

1.2.3 Association of Serovars and Food Commodities

Little has been done to systematically establish associations between the common implicated foods and specific *Salmonella* serotypes (Jackson *et al.*, 2013). However, it has been shown recently that some of the predominant serovars causing human illness demonstrate various predilections for certain foods, with some serovars showing associations over a narrow range and others across wide ranges of food commodities (Jackson *et al.*, 2013). Among the top ten serovars causing the most human outbreaks between 1998 and 2008, serovars Enteritidis, Hadar, Heidelberg were found to have close associations with poultry and eggs, and Infantis with pork. In contrast, serovars Newport and Typhimurium were observed to be associated with a wide variety of foods (Jackson *et al.*, 2013).

1.3 *Salmonella* Enteritidis Background

Salmonella enterica spp. *enterica* serovar Enteritidis (SE) was first isolated by August Gaertner in 1888 from a cow and from a man who had died after consuming meat from the same cow. It has since been recognized as an important pathogen in foodborne illness (Branham 1925; Hardy 2005). In the past decade, SE has been identified as the most frequently implicated *Salmonella* serovar among serotyped cases of human salmonellosis acquired in the United States (CDC 2009; CDC 2011; Jackson *et al.*, 2013; Gould *et al.*, 2013; Crim *et al.*, 2014; Crim *et al.*, 2015), and the reduction of illnesses caused by this serovar has been one of the five high-priority goals of the United States Department of Health and Human Services since 2012 (Crim *et al.*, 2014). Like other *Salmonella* serovars, SE causes acute gastroenteritis in humans that can persist for seven days and generally resolves without treatment (Kimura *et al.*, 2004). Clinical features of SE disease include diarrhea, abdominal cramps, pyrexia, and less frequently, vomiting and bloody stool (Kimura *et al.*, 2004; Hennessy *et al.*, 1996).

1.3.1 *Salmonella* Enteritidis Epidemiology

The recognition of SE as a major public health concern and the interest in investigating the epidemiology of SE human disease began in response to a major increase in incidence of illness caused by this serovar in the late 1970s in the northeast United States (Braden 2006; Callaway *et al.*, 2014). As the number of cases of SE continued to rise, the CDC implemented the *Salmonella* Enteritidis Outbreak Reporting System in 1985. This surveillance program was tasked with collecting detailed information pertaining to all reported outbreaks in the U.S., including case numbers, hospitalizations, deaths, associated foods, and related food production facilities (Braden 2006). The surge in the number of human SE infections during the mid to late 1980s in the U.S. was largely the consequence of eradication of *Salmonella* serovars Gallinarum and Pullorum in

poultry flocks (Callaway *et al.*, 2014). Unlike SE, *S. Gallinarum* and *S. Pullorum* are host-adapted and are extremely virulent in poultry. These serovars have contributed to massive economic losses in the poultry industry. These losses led to the implementation of a depopulation strategy to eradicate both serovars in the U.S. However, as the incidence of the *S. Gallinarum* and *S. Pullorum* decreased, the incidence of SE in poultry flocks and human cases of foodborne illness began to rise as a result of the unoccupied niche left behind by these measures (Callaway *et al.*, 2014). Between 1985 and 2003, SE was responsible for a total of 997 of reported outbreaks resulting in 33,687 illnesses, 3281 hospitalizations, and 82 deaths (Braden 2006). During this same period, a peak of 85 outbreaks was reported in 1990 followed by a slow decline to 34 outbreaks in 2003 (Braden 2006) except for a large outbreak of SE in ice cream during 1994 (Hennessy *et al.*, 1996). Later, between 1998 and 2008, SE was responsible for 36% (144 of 403) of *Salmonella* outbreaks that could be traced to a specific food (Gould *et al.*, 2013; Jackson *et al.*, 2013). In recent years, SE has been implicated in 19% and 21% of human cases of salmonellosis occurring in 2013 and 2014, respectively (Crim *et al.*, 2014; Crim *et al.*, 2015), and continues to pose a major public health concern.

1.3.2 Foods Associated with *Salmonella* Enteritidis

A wide variety of food commodities have been implicated in human infections with SE including poultry, eggs, milk, pork, turkey, ground beef, melons, vegetables, and ice cream (Hennessy *et al.*, 1996; Kimura *et al.*, 2004; CDC 2012; Jackson *et al.*, 2013). Historically, eggs have been the most common food vehicle implicated in human SE foodborne outbreaks (Braden 2006; Gould *et al.*, 2013; Jackson *et al.*, 2013). Following the establishment of the *Salmonella* Enteritidis Reporting Outbreak System in 1985, the CDC reported that 77% of SE outbreaks in the Northeast United States in 1986 and 1987 could be traced to egg consumption (Braden 2006).

Moreover, between the years 1985 and 2003, the overwhelming majority (77%) of the SE outbreaks in which a food vehicle could be identified were traced to eggs and products with egg ingredients (Braden 2006). Furthermore, eggs were implicated in 93 of 114 (65%) of SE outbreaks between 1998 and 2008, accounting for 23% of *Salmonella* outbreaks with a single implicated food (Jackson *et al.*, 2013). Poultry was the second most common single food associated with *Salmonella* outbreaks from 1998 to 2008 (Jackson *et al.*, 2013).

1.3.3 *Salmonella* Enteritidis in Poultry and Eggs

In response to the many reports of SE coming from eggs, several studies have endeavored to elucidate the mechanisms underlying egg contamination (Shivaprasad *et al.*, 1990; Gast and Holt, 1998; Keller *et al.*, 1995). Although several experimental inoculations of chicks, laying hens, and broilers have thoroughly documented and characterized SE disease in poultry (Altekruse *et al.*, 1992; Shivaprasad *et al.*, 1990; Gast and Holt, 1990; Barrow 1991, Van Immerseel *et al.*, 2004), the strains of SE that are able to infect are not typically associated with frank disease in flocks (Keller *et al.*, 1995). Initially, it was hypothesized that the exterior of eggs were contaminated with the feces of laying hens actively shedding SE. This led to the wide adoption of egg washing as a preventative measure in the mid-1980s. However, the limited efficacy of this practice to prevent egg-associated SE infections led some to suggest that the interior of the eggs could be a possible source of egg contamination (Louis *et al.*, 1988). In studies of laying hens, SE was found to colonize the yolk and the albumen as early as one day following oral challenge (Shivaprasad *et al.*, 1990; Schoeni *et al.*, 1994). However, it was unclear from these studies whether the interior of the egg was contaminated as a result of ovarian colonization prior to egg formation or whether SE shed in the feces of laying hens was able to penetrate through the shell (Shivaprasad *et al.*, 1990). Later, in a series of three experiments involving oral challenge of laying

hens with 10^8 CFU of SE, investigators showed that SE colonizes forming eggs and that 73% of colonized forming eggs are associated with a colonized oviduct (Keller *et al.*, 1995). However despite the finding that 66% of forming eggs were positive for SE, only 0.8% of freshly laid eggs were positive for the organism, suggesting that antimicrobial properties (e.g. avidin) of fully formed eggs are able to limit colonization (Keller *et al.*, 1995). In addition to the ability of SE to colonize forming and freshly laid eggs, the organism was found to be highly invasive in chickens (Altekruse *et al.*, 1992; Keller *et al.*, 1995; Gast and Holt, 1998). These and other studies established a continuing understanding that SE infection in laying hens and broilers likely is vertically transmitted from carriers in breeder flocks (Gast and Holt, 1998; Van Immerseel *et al.*, 2004; Barrow 1991).

1.3.4 *Salmonella* Enteritidis Associated with Cattle and Beef

It has also been shown that SE readily infects calves and, unlike infection in chickens, causes frank disease (Petrie *et al.*, 1977). The first descriptions of SE disease in cattle were reported in 1977 by Petrie *et al.* who characterized the clinical and epidemiological features of disease in naturally infected young calves (1977). Major clinical disease features included diarrhea, dullness, and fever, as well as less common features like exudative pneumonia and bacteremia (Petrie *et al.*, 1977). Calves that died from SE exhibited necrosis of the intestinal epithelium similar to bovine infections with *S. Dublin* (Petrie *et al.*, 1977; Costa *et al.*, 2012).

Between 1998 and 2008, SE was identified as one of the top three serovars responsible for human *Salmonella* outbreaks traced to beef products (Jackson *et al.*, 2013). SE was also responsible for a recent multistate outbreak during the summer of 2012 that was traced to ground beef from a single production facility which resulted in 46 illnesses and 12 hospitalizations (CDC

2012). Taken together, these findings suggest that cattle are potential entry points for SE in the food supply.

1.4 *Salmonella* in Cattle

1.4.1 *Salmonella* Prevalence in Cattle

It is well known that cattle harbor *Salmonella* and are important reservoirs of the pathogen (Roels *et al.*, 1997; CDC 2006; Kunze *et al.*, 2008; Brichta-Harhay *et al.*, 2011; Hanson *et al.*, 2015). *Salmonella* also reside in cattle environments and can be found widely in dairies, feedlots, farms and processing plants (Callaway *et al.*, 2008). The bacterium is frequently isolated from the hides and feces of various breeds and types of cattle including feedlot cattle, dairy cattle, calves, culled cattle, and fed cattle (Rhoades *et al.*, 2009; Moussa *et al.*, 2010; Koohmaraie *et al.*, 2012; Loneragan *et al.*, 2012; Gragg *et al.*, 2013b; Mohamed *et al.*, 2014; Schmidt *et al.*, 2015). The prevalence of pathogens, including *Salmonella*, in various breeds and types of cattle varies considerably (Dodd *et al.*, 2011). Studies comparing *Salmonella* prevalence in cattle hide and feces have documented major differences among processing plants in geographically distant regions (Rivera-Betancourt *et al.*, 2004; Brichta-Harhay *et al.*, 2008; Brichta-Harhay *et al.*, 2011; Schmidt *et al.*, 2015) and in animals from different feedlots (Green *et al.*, 2010; Schmidt *et al.*, 2015). Similar findings have been reported among dairy cattle with above-average *Salmonella* prevalence observed in some dairies and others with considerably lower prevalence (Loneragan *et al.*, 2012; Hanson 2015b; Schmidt *et al.*, 2015). In addition to the locational differences, many studies have shown seasonal differences in *Salmonella* prevalence in cattle with a greater frequency of *Salmonella*-positive samples collected from cattle hides and feces during Summer and Fall (Barkocy-Gallagher *et al.*, 2003; Edrington *et al.*, 2004a; Gragg *et al.*, 2013b; Hanson 2015b). Aside from methodological differences that likely influence these findings, other factors such as differences in herd management, sanitation practices, genetics, nutrition, and transit stress have

been suggested to play a role in estimations of *Salmonella* prevalence in cattle (Edrington *et al.*, 2004; Hanson 2015b).

1.4.2 *Salmonella* Serovars Associated with Cattle

Cattle are known to harbor many *Salmonella* serotypes (Cummings *et al.*, 2009). Among studies investigating the prevalence of *Salmonella* in cattle (Edrington *et al.*, 2004a, 2004b; Kunze *et al.*, 2008; Dodd *et al.*, 2011; Gragg *et al.*, 2013b), there is considerable overlap between the common *Salmonella* serovars recovered from cattle hides and fecal samples. The most common *Salmonella* serovars isolated from cattle hides at slaughter include Anatum, Kentucky, Muenster, Montevideo, Mbandaka, and Cerro (Kunze *et al.*, 2008; Gragg *et al.*, 2013b). Similarly, the most common *Salmonella* serovars recovered from cattle feces include: Anatum, Kentucky, Montevideo, Mbandaka, Newport and Senftenberg (Edrington *et al.*, 2004a, 2004b; Kunze *et al.*, 2008; Dodd *et al.*, 2011). Although the vast majority do not appear to be associated with disease in these animals (Kunze *et al.*, 2008), *S.* Newport and *S.* Kentucky were among the top five serovars identified in the majority (83%) of clinical cases of disease among dairy cattle in the northeastern United States (Cummings *et al.*, 2009). Other serovars associated with salmonellosis in cattle include *S.* Typhimurium, *S.* Enteritidis, and *S.* Dublin (Cummings *et al.*, 2009; Mohamed *et al.*, 2014).

1.4.3 Bovine Salmonellosis

In general, *Salmonella* causes gastroenteritis in cattle, characterized by pyrexia, diarrhea, dehydration, and anorexia (de Jong and Ekdahl, 1965; Petrie *et al.*, 1977; Mohler *et al.*, 2009; Rhoades *et al.*, 2009; Costa *et al.*, 2012). The fecal-oral route is the primary means of *Salmonella* transmission among cattle, although other routes such as the mucosa of the respiratory tract and the conjunctiva have been described (Mohler *et al.*, 2009). It has also been suggested that

Salmonella is capable of vertical transmission from dam to fetus *in utero*. In a recent study, investigators isolated *Salmonella* from the lymph nodes, liver, spleen, and gastrointestinal tracts of neonatal calves as early as two minutes after birth (Hanson *et al.*, 2015a). Although several *Salmonella* serovars can cause disease in cattle (de Jong and Ekdahl, 1965; Petrie *et al.*, 1977; Moussa *et al.*, 2010, Mohamed *et al.*, 2014), more is known about cattle infections with serovars Dublin and Typhimurium (Santos *et al.*, 2001). Both *S. Typhimurium* and bovine-adapted *S. Dublin* (Santos *et al.*, 2001; Mohler *et al.*, 2009) primarily cause gastroenteritis in these in cattle (de Jong and Ekdahl, 1965; Smith and Jones, 1967; Wray and Sojka, 1981). However, the disease produced by *Salmonella* Dublin generally is more invasive and is characterized by meningoencephalitis, polyarthritis, osteomyelitis, and pneumonia that later manifest in the absence of diarrhea (Rings 1985). Unlike infections with *S. Typhimurium*, cattle can become chronic carriers of *S. Dublin* (Sojka *et al.*, 1974; Gitter *et al.*, 1978) and infections in pregnant heifers or cows can result in spontaneous abortions in the absence of clinical signs of disease (Hall *et al.*, 1979; Rings 1985).

1.4.4 Bovine Models of *Salmonella* Infection in Calves

Because the disease produced by *S. Typhimurium* in cattle recapitulates clinical features of human infections in many ways, calf models of infection with this serovar have been used extensively to study *Salmonella*-induced enteritis (Santos *et al.*, 2001). Clinical signs of disease manifest between 12 and 48 hours following oral inoculation of calves with *S. Typhimurium* (Tsolis *et al.* 1999), similar to experimental infections in human volunteers with this serovar (Blaser and Newman, 1982). In general, oral inoculation of calves with does between 10^4 and 10^7 CFU produces self-limiting gastroenteritis (pyrexia, anorexia, and diarrhea) whereas higher doses ranging from 10^8 to 10^{11} CFU result in severe systemic disease and mortality (de Jong and Ekdahl,

1965; Rankin and Taylor, 1966; Wray and Sojka, 1978; Smith *et al.*, 1979; Tsolis *et al.*, 1999). Mortality and morbidity of *S. Typhimurium* infection of calves is inversely proportional to age (Smith *et al.*, 1979; Cummings *et al.*, 2009; Mohler *et al.*, 2009) and the majority (~75%) of natural *S. Typhimurium* infections in cattle occur in pre-weaned calves less than two months of age (Sojka and Field, 1970). Bacteremia is also common in calves dying from salmonellosis (Mohler *et al.*, 2009) and *Salmonella* has been isolated from systemic tissues and blood of calves in both experimental (de Jong and Ekdahl, 1965; Pullinger *et al.*, 2007) and natural (Petrie *et al.*, 1977) infections.

1.4.5 Asymptomatic Carrier Status and Fecal Shedding

Cattle also can be asymptomatic carriers of *Salmonella* (CDC 2006; Stevens *et al.*, 2009). Although *Salmonella* can cause frank disease in cattle, some infections can occur in the absence of gross clinical signs (Cummings *et al.*, 2009; Hanson *et al.*, 2015). Moreover, *Salmonella* have been isolated in the feces of healthy (Rhoades *et al.*, 2009; Koohmaraie *et al.*, 2012; Loneragan *et al.*, 2012; Gragg *et al.*, 2013b; Schmidt *et al.*, 2015) and diseased cattle (Petrie *et al.*, 1977; Moussa *et al.*, 2010; Mohamed *et al.*, 2014). The duration of fecal shedding has been shown to vary among cattle infected with different *Salmonella* serovars. However, fecal sheds of *S. Newport* exceeding one year have been reported (Cummings *et al.*, 2009). Previous experimental infections with *S. Typhimurium* have also shown that severely affected calves can shed up to $10 \log_{10}$ CFU/g of feces, and that the largest numbers of organisms tended to be shed during the periods of high fever (de Jong and Ekdahl, 1965). Although little work has been performed to quantify *Salmonella* concentration in the feces of cattle at slaughter, a recent study of *Salmonella* prevalence in dairy cattle found that fecal samples collected from one feedlot and two dairies contained *Salmonella* concentrations ranging from 2.08 to 5.68 \log_{10} CFU/g with an average of 3.27 \log_{10} CFU/g of feces

(Hanson 2015b). Furthermore, diarrhea, in addition to its importance in clinical disease, is also a major source of environmental contamination and subsequent transmission to other cattle in herds (Costa *et al.*, 2012).

1.5 *Salmonella* Associated with Beef

Although poultry and eggs are the foods most often implicated in cases of human foodborne salmonellosis (Gould *et al.*, 2013), beef, along with other meats including turkey and pork, are subject to *Salmonella* contamination in the food production chain (Crum-Cianflone 2008) and represents an important source of *Salmonella* foodborne illnesses (Gould *et al.*, 2013; McEntire *et al.*, 2014; Crowe *et al.*, 2015; Laufer *et al.*, 2015). Among 1,965 *Salmonella* outbreaks reported to the CDC between 1973 and 2011 in which a specific food was identified, 96 (5%) were attributed to beef, accounting for 3,684 illnesses, 318 hospitalizations, and five deaths (Laufer *et al.*, 2015). Moreover, analyses of outbreak data between 1998 and 2008 identified beef in 10% of *Salmonella* outbreaks (Gould *et al.*, 2013) and as the fourth most common cause of salmonellosis (McEntire *et al.*, 2014) in the U.S. Finally, in recent summaries of outbreak data between 2010 and 2014, *Salmonella* was responsible for five outbreaks attributed to beef (Crowe *et al.*, 2015).

1.5.1 *Salmonella* Outbreaks Associated with Ground Beef

Although studies attributing *Salmonella* infections to specific food vehicles often do not distinguish between various types of beef products (Gould *et al.*, 2013; McEntire *et al.*, 2014), ground beef is one of the most substantial sources among *Salmonella*-contaminated beef products implicated in human foodborne disease (Laufer, *et al.*, 2015). Ground beef was recognized as the greatest source of beef-mediated *Salmonella* outbreaks in the 2000s and was implicated in 17 (45%) of 38 outbreaks occurring between 2002 and 2011 (Laufer *et al.*, 2015). Moreover, several multistate outbreaks of *Salmonella* have been attributed to ground beef, emphasizing the important role of this food commodity in *Salmonella* foodborne infections (CDC 2002; Dechet *et al.*, 2006; Schneider *et al.*, 2011; CDC 2012; Jackson *et al.*, 2013; Crowe *et al.*, 2015).

1.5.2 *Salmonella* Serovars Associated with Ground Beef

Several serovars have been implicated in outbreaks of *Salmonella* in ground beef (Laufer *et al.*, 2015). In a recent analysis of *Salmonella* outbreaks attributed to ground beef from 1973 and 2011, the most common serovars responsible for the majority (73%) of outbreaks were Newport (32%), Typhimurium (27%), and Enteritidis (14%) (Laufer *et al.*, 2015). In another analysis of outbreak data from 1998-2008, the most frequently implicated serovars in ground beef outbreaks were: Typhimurium (18%), Newport (18%), and Enteritidis (18%) (Jackson *et al.*, 2013). Finally, among ground beef outbreaks occurring between 1998 and 2014, the most common serotypes were Typhimurium (38%), Enteritidis (24%), and Newport (17%) according to The Foodborne Outbreak Online Database (wwwn.cdc.gov/foodborneoutbreaks).

1.5.3 *Salmonella* Prevalence in Ground Beef

In response to the public health risk that *Salmonella* poses in ground beef, the Food Safety Inspection Service (FSIS) established the Pathogen Reduction/Hazard Analysis and Critical Control Point Systems (PR/HACCP) to ensure that processing facilities were meeting processing control standards for *Salmonella* in ground meats (FSIS 2011). Testing by the USDA-FSIS found that 2.4% of ground beef was contaminated by *Salmonella* between 1998 and 2011 (FSIS 2011). However, there is a variation in the prevalence of *Salmonella* in ground beef with estimations ranging from as low as 0.55% to 7.0% (Scanga *et al.*, 2000; Bosilevac *et al.*, 2009; Rhoades *et al.*, 2009; Koohmaraie *et al.*, 2012; Vipham *et al.*, 2015) and bacterial loads up to 40 CFU/g have been reported (Bosilevac *et al.*, 2009). While variation in these estimates is likely a consequence of methodological differences and sampling biases, it has been shown that the proportion of test-positive ground beef samples follows a seasonal trend with elevations observed between the months of July and October (Williams *et al.*, 2014).

1.5.4 Common *Salmonella* Isolates among Humans and Food-Animals

When associations between *Salmonella* serovars and food commodities are implicated in human foodborne *Salmonella* infections, the question arises regarding the degree of isolates from human *Salmonella* illnesses also being found in food animals. Recently, when Pulsed Field Gel-Electrophoresis (PFGE) patterns of human clinical *Salmonella* isolates (including sporadic infections and outbreaks) were compared to PFGE patterns of isolates from animals before slaughter in Pennsylvania, it was found that 16 (80%) of the 20 most common *Salmonella* PFGE patterns from humans were also found in pre-slaughter food animals (Sandt *et al.*, 2013). Although this study is the first of its kind, it suggests a link between *Salmonella* strains found in food animals and strains isolated from humans, underscoring the importance of expanding pre-harvest interventions to address the continuing and persistently problematic nature of *Salmonella* foodborne disease.

1.6 Ground Beef Contamination with *Salmonella*

The contamination of beef carcasses by cattle hides, termed “hide-to-carcass transfer”, during hide removal has been traditionally considered to be the principal source of carcass and ground beef contamination during slaughter and processing (Barkocy-Gallagher *et al.*, 2003; Koohmaraie *et al.*, 2005; Brichta-Harhay *et al.*, 2008; Koohmaraie *et al.*, 2012; Schmidt *et al.*, 2015). This hypothesis is supported by studies documenting the high correlation between hide bacterial concentrations and carcass contamination (Barkocy-Gallagher *et al.*, 2003; Brichta-Harhay *et al.*, 2008; Schmidt *et al.*, 2015). Recent studies have documented extremely high prevalence of *Salmonella* on cattle hides in processing facilities (Koohmaraie *et al.*, 2012; Schmidt *et al.*, 2015). Among 100 dairy cows in a U.S. commercial beef processing plant, *Salmonella* was isolated from 96% of cattle hides and 47% of carcasses following hide removal (Koohmaraie *et al.*, 2012). Similarly, in a study estimating *Salmonella* contamination in a Nebraska beef processing facility and three associated feedlots, investigators isolated *Salmonella* from 99.5% of cattle hide samples at processing (Schmidt *et al.*, 2015). However, *Salmonella* prevalence on cattle hides among the three feedlots associated with this processing plant were 26.1, 10.9, and 0.0%, suggesting that *Salmonella* hide prevalence differs among feedlots and beef processing plants (Schmidt *et al.*, 2015). Because the presence of *Salmonella* on cattle hides results from contamination by the feces and animals shedding the organism (Barkocy-Gallagher *et al.*, 2003), the difference between hide prevalence among feedlots and processing plants is likely an example of “lairage contamination” which is a consequence of crowding and fecal contamination during transportation of cattle from feedlots to processing plants and passage of cattle through the processing plant “lairage” environment of alley, pins, and chutes that cattle pass through after arrival at the facility (Schmidt *et al.*, 2015). In recognition of the contribution of cattle hides to

beef carcass contamination, several post-harvest interventions have been developed and implemented in the beef industry and have demonstrated efficacy in reducing bacterial loads on cattle hides and carcass surfaces (Wheeler *et al.*, 2014). However, while these measures have improved the safety of beef commodities, outbreaks of *Salmonella* in ground beef continue to occur according to The Foodborne Outbreak Online Database (wwwn.cdc.gov/foodborneoutbreaks).

1.6.1 Bovine Peripheral Lymph Nodes and Ground Beef Contamination

While there is strong evidence that cattle hides are the largest contributor to carcass contamination, few post-intervention carcass samples are *Salmonella* positive after pre-enrichment, and only a small proportion of positive samples contain countable numbers at low concentrations (Barkocy-Gallagher *et al.*, 2003; Brichta-Harhay *et al.*, 2008). Moreover, *Salmonella* prevalence on post-intervention carcasses was estimated to be 0.1% to 1% in two studies (Barkocy-Gallagher *et al.*, 2003; Brichta-Harhay *et al.*, 2008) and a more recent study failed to isolate the organism after post-harvest interventions (Koohmaraie *et al.*, 2012). Furthermore, in a recent meta-analysis of *Salmonella* ground beef contamination and interventions, Li *et al.* argued that carcass surface contamination contributed little to *Salmonella* load in ground beef (2015). Taken together, these findings raise doubts about the contribution of carcass contamination to ground beef contamination.

Although sources other than cattle hides may contaminate ground beef, a recent, accumulating body of evidence suggests that peripheral lymph nodes are important sources of *Salmonella* in ground beef contamination (Arthur *et al.*, 2008; Brichta-Harhay *et al.*, 2012; Koohmaraie *et al.*, 2012; Haneklaus *et al.*, 2012; Gragg *et al.*, 2013a, 2013b; Edrington *et al.*, 2013a, Vipham *et al.*, 2015). It is well known that *Salmonella* can be found in high numbers within

mesenteric lymph nodes (de Jong and Ekdahl, 1965; Samuel *et al.*, 1979; Paulin *et al.*, 2002; Arthur *et al.*, 2008; Crum-Cianflone 2008; Brichta-Harhay *et al.*, 2012) because these are located in close proximity to the gastrointestinal tract. Up until 2008, many studies of *Salmonella* lymph node contamination focused on mesenteric lymph nodes (Arthur *et al.*, 2008). However, mesenteric lymph nodes are routinely removed during carcass evisceration and do not pose a risk for ground beef contamination (Arthur *et al.*, 2008; Ayala 2013). Unlike mesenteric lymph nodes, peripheral lymph nodes (also referred to as deep-tissue lymph nodes) are widely distributed and interspersed within fatty tissues of beef carcasses (Arthur *et al.*, 2008). Moreover, because these lymph nodes are numerous and often small, they cannot be effectively removed at slaughter. Thus, lean and fat trimmings routinely included in ground beef production inevitably contain some peripheral lymph nodes which are also incorporated into ground beef (Arthur *et al.*, 2008; Gragg, *et al.*, 2013b).

1.6.2 *Salmonella* Prevalence in Bovine Peripheral Lymph Nodes

The first study implicating the peripheral lymph nodes as a potential source of ground beef contamination estimated *Salmonella* prevalence to be 1.6% in chuck and flank nodes (lymph nodes likely to be included in ground beef) in fed and culled cattle from four commercial beef processing plants during the winter and spring in 2007 (Arthur *et al.*, 2008). Since this publication, several recent studies have been conducted to estimate the prevalence and bacterial loads of *Salmonella* in bovine peripheral lymph nodes destined for ground beef production (Brichta-Harhay *et al.*, 2012; Haneklaus *et al.*, 2012; Koohmaraie *et al.*, 2012; Gragg *et al.*, 2013a, 2013b; Brown, *et al.*, 2015b; Hanson *et al.*, 2015). Borrowing from previous methodologies (Arthur *et al.*, 2008), the majority of these studies estimated the prevalence of *Salmonella* in superficial cervical lymph nodes (from the chuck region) (Haneklaus *et al.*, 2012; Koohmaraie *et al.*, 2012; Hanson *et al.*, 2015) and subiliac lymph nodes (from the flank region) (Brichta-Harhay *et al.*, 2012; Haneklaus

et al., 2012; Gragg *et al.*, 2013a, 2013b; Brown, *et al.*, 2015b; Hanson *et al.*, 2015) because these were suggested to be the most likely peripheral lymph nodes included in ground beef (Arthur *et al.*, 2008). Although *Salmonella* was recovered from the peripheral lymph nodes in all of these studies, there is a very large degree of variability in the reported prevalence of *Salmonella* in these nodes. The prevalence ranges from 0.61% to 18% in superficial cervical lymph nodes (Arthur *et al.*, 2008; Koohmaraie *et al.*, 2012; Hanson *et al.*, 2015) and 0.8% to 76% in subiliac lymph nodes (Arthur *et al.*, 2008; Brichta-Harhay *et al.*, 2012; Gragg *et al.*, 2013a, 2013b; Brown, *et al.*, 2015b; Hanson *et al.*, 2015) and with a combined prevalence as high as 88.2% (Haneklaus *et al.*, 2012).

1.6.3 *Salmonella* Concentrations in Bovine Peripheral Lymph Nodes

Although the prevalence of *Salmonella* in the peripheral lymph nodes of cattle at slaughter has been sufficiently examined in eight studies since 2008, only two studies investigated *Salmonella* concentrations in these lymph nodes, (Gragg *et al.*, 2013a; Brown *et al.*, 2015b) and two others reported *Salmonella* concentrations in only one lymph node (Arthur *et al.*, 2008; Hanson *et al.*, 2015). Among 618 lymph nodes sampled from feedlot and culled cattle at harvest in the Summer/Fall of 2011, only 114 (18%) contained enumerable concentrations of *Salmonella*. Although the average *Salmonella* concentration in these nodes was 1.75 log₁₀CFU/g, there was a wide range of concentration in individual nodes, with 58.8% containing 1.3-2.9 log₁₀CFU/g and 41.2% containing 3.0-4.8 log₁₀CFU/g (Gragg *et al.*, 2013a). In a more recent study, enumerable concentrations of *Salmonella* in subiliac lymph nodes were found among 280 (62.1%) of 467 lymph nodes collected from Holstein cattle and 276 (59.7%) of 462 beef cattle in US commercial abattoirs between May and October 2014 (Brown *et al.*, 2015b). Although the geometric mean bacterial concentrations in lymph nodes containing of *Salmonella* were 2.3 log₁₀CFU/g and 2.6 log₁₀CFU/g for Holstein and beef cattle respectively, the majority of subiliac nodes for these two

cattle breeds contained *Salmonella* concentrations above the mean with 71.5% of Holstein nodes containing 2.4-4.1 log₁₀CFU/g and 72.6% of beef cattle nodes containing 2.7-4.1 log₁₀CFU/g (Brown *et al.*, 2015b). These studies suggest that peripheral lymph nodes, and specifically subiliac lymph nodes, have the potential to contribute a substantial load of *Salmonella* if incorporated into ground beef.

1.6.4 Seasonal Variability in Peripheral Lymph Node Prevalence

Although the results of recent studies implicate bovine peripheral lymph nodes as an important source of *Salmonella* contamination in ground beef, the estimations of *Salmonella* prevalence in peripheral lymph nodes are inconsistent (Arthur *et al.*, 2008; Brichta-Harhay *et al.*, 2012; Haneklaus *et al.*, 2012; Koohmaraie *et al.*, 2012; Gragg *et al.*, 2013a, 2013b; Brown *et al.*, 2015b; Hanson *et al.*, 2015), and the few studies estimating bacterial loads in these tissues report different proportions of lymph nodes containing enumerable levels of *Salmonella* with varying concentrations (Gragg *et al.*, 2013a; Brown *et al.*, 2015). Several factors may play a role in these observed differences, such as cattle age, dietary effects, animal husbandry, and environments (Gragg *et al.*, 2013a). Animal source (feedlot or cull) has also been suggested to affect *Salmonella* prevalence in the peripheral lymph nodes (Gragg *et al.*, 2013a). However, the observation of higher prevalence in feedlot cattle than in culled cattle (Gragg *et al.*, 2013a) conflicted with earlier reports that had observed higher prevalence in culled cattle than in feedlot cattle (Arthur *et al.*, 2008). Many of these studies also show seasonal dependence of *Salmonella* prevalence in these lymph nodes, with higher *Salmonella* prevalence observed in the Summer/Fall months compared to Winter/Spring (Arthur *et al.*, 2008; Gragg *et al.*, 2013a, 2013b; Haneklaus 2012; Brown *et al.*, 2015). These findings agree with other studies in demonstrating higher *Salmonella* prevalence in cattle feces, hides, and pre-evisceration carcasses during the Summer/Fall months (Barkocy-

Gallagher *et al.*, 2003; Rivera-Betancourt *et al.*, 2004; Callaway *et al.*, 2008; Kunze *et al.*, 2008; Loneragan *et al.*, 2011; Gragg *et al.*, 2013b). The increased prevalence of *Salmonella* in peripheral lymph nodes in warmer months may explain the higher proportion of ground beef samples testing positive for *Salmonella* between July and October (Williams *et al.*, 2014).

1.6.5 *Salmonella* Serotypes in Peripheral Lymph Nodes

Many *Salmonella* serotypes have been recovered from bovine peripheral lymph nodes, with diversities ranging from 6-24 serotypes reported (Arthur *et al.*, 2008; Ayala 2013; Gragg *et al.*, 2013a, 2013b) and the most common serotypes isolated from the peripheral lymph nodes differed in each of these studies. Among 24 serotypes identified in subiliac lymph nodes from feedlot and cull cattle in US commercial abattoirs, the majority were Montevideo (44.0%) or Anatum (24.8%) (Gragg *et al.*, 2013a). These were not only recovered from the peripheral lymph nodes in other reports (Arthur *et al.*, 2008; Ayala 2013; Gragg *et al.*, 2013b), but were the two most commonly isolated serotypes in ground beef (FSIS 2011; Bosilevac *et al.*, 2009). Among the three most frequently identified serovars in *Salmonella* ground beef outbreaks, Typhimurium, Newport, and Enteritidis (Jackson *et al.*, 2013; Laufer *et al.*, 2015), only Typhimurium and Newport were found (in low frequencies) among *Salmonella* peripheral lymph node isolates (Arthur *et al.*, 2008; Gragg *et al.*, 2013a). However, the vast majority of serotypes isolated from the peripheral lymph nodes (Arthur *et al.*, 2008; Ayala *et al.*, 2013; Gragg *et al.*, 2013a, 2013b) have also been isolated from ground beef (Bosilevac *et al.*, 2009).

1.6.6 Mechanism of Peripheral Lymph Node Contamination

The mechanisms mediating translocation of bacteria, including *Salmonella*, from the gut to systemic tissues is the subject of current investigations and remains to be elucidated (Ayala 2013, Brown *et al.*, 2015a). However, several mechanisms have been suggested and *Salmonella*

dissemination may involve multiple routes. The first is that bacteremia mediates *Salmonella* translocation from the gut to peripheral tissues. Septicemia is common in calves dying from salmonellosis (Mohler *et al.*, 2009) and *Salmonella* has been isolated from systemic tissues and blood of calves as early as 2-4 hours after experimental infection (de Jong and Ekdahl, 1965; Pullinger *et al.*, 2007). Moreover, systemic and peripheral lymph node dissemination of *S. Typhimurium* was found to occur despite surgical interventions to prevent dissemination from the gut (de Jong and Ekdahl, 1965). Another hypothesis is that translocation from the gut occurs via the lymphatic system (Uzzau *et al.*, 2000; Paulin *et al.*, 2002; Pullinger *et al.*, 2007; Brown *et al.*, 2015a). In contrast to these hypotheses which are predicated on *Salmonella* colonization and invasion of the gastrointestinal tract, several recent studies have suggested that *Salmonella* dissemination to peripheral lymph nodes may occur transdermally through biting flies or hide abrasions (Edrington *et al.*, 2013; Gragg *et al.*, 2013a; Gragg *et al.*, 2013b; Brown *et al.*, 2015).

1.7 Bacteriophage Background

Bacteriophages (phages) are small viruses that infect and replicate within bacteria. They are ubiquitous in nature and represent the most abundant biologically active entities on the planet. Phages can be found in a wide variety of ecological niches and it has been estimated that a single drop of seawater can harbor as many as 10^6 virus particles, or “virions”, and one gram of soil can contain approximately 10^8 particles (Guttman *et al.*, 2004; Deresinski 2009). Owing to their abundance, phages play an important role in the regulation of microbial balance in practically every ecosystem where this has been studied (Guttman *et al.*, 2004; Suttle 2005). Thus phages have an important collective influence on the biosphere. It has been estimated that up to 50% of global bacterial populations are killed by phages every 48 hours (Suttle 2005; Deresinski 2009).

1.7.1 Discovery and Early Research

Bacteriophages were identified in the early 1900s although there is some controversy surrounding their discovery (Duckworth 1976). It is generally accepted that phages were discovered through independent observations of two microbiologists, F. W. Twort in 1915 and Félix d’Herelle in 1917 (Duckworth 1976, Summers 2001; Abedon 2009). F. W. Twort, an English microbiologist, is known for his “Glassy Transformation” observation of translucent *Micrococcus* colonies which he later concluded was caused by a filterable, infectious agent that is also able to replicate (Duckworth 1976). The term “bacteriophage”, a combination of “bacteria” and the Greek word “phagein” meaning “to eat”, was coined by the French-Canadian microbiologist Félix d’Herelle in 1917 who isolated a filterable substance that produced small clearings in confluent bacterial growth on agar plates. He later called these clearings as “plaques” (Duckworth 1976; Deresinski 2009), a term still used today to refer to areas of bacterial lysis resulting from phage replication (Guttman *et al.*, 2004). Building on laboratory procedures allowing him to manipulate

these phage, d'Herelle developed the Double-Agar Overlay Plaque Assay which is today considered the gold standard for enumeration of phage particles (Summers 2001). These and other discoveries immediately provoked interest in the therapeutic potential of phages in bacterial infections. Shortly thereafter, D'Herelle began exploring the use of phages as a prophylaxis to control avian typhosis in poultry flocks caused by *S. Gallinarum*, marking the early work in the study of phage therapy. By the early 1920s, the existence of phage and their infectious cycles were accepted and understood (Duckworth 1976; Summers 2001; Guttman *et al.*, 2004).

1.7.2 Morphology and Nomenclature

Studies of the chemical composition of phage in the mid to late 1930s indicated, initially, the proteinaceous character of bacteriophage. However, it wasn't long before the presence of phosphorous suggested another component which was demonstrated to be nucleic acid by the German biochemist Max Schlesinger in 1936 (Ceyssens 2009). Like all viruses, phages are obligate parasites. Although phage genomes contain all of the genes necessary for replication and assembly, phages do not possess the basic metabolic components necessary for energy production and protein synthesis. Thus, the completion of the phage life cycle is fundamentally dependent on the infection of a suitable host (Guttman *et al.*, 2004). Phage particles (virions) are composed of a nucleic acid genome surrounded by a protein coat termed a capsid. Of the phages that have been characterized in the literature, over 95% belong to the Caudovirales order and are characterized by the presence of tails, isometric capsids, and genomes composed of linear double-stranded DNA (Ackermann *et al.*, 2003; Ceyssens 2009; Salmond and Fineran, 2015). Tailed phages are the oldest of all phages and are thought to have risen close to the origin of life due their high degree of diversification (Ackermann 1998). Most (85%) families of tailed phages have isometric capsids with helical symmetry (Lwoff *et al.*, 1962; Ackermann *et al.*, 2003). Tails may consist of helical

structures or stacked discs along with terminal proteins structures such as base plate, fibers, or spikes (Ackermann *et al.*, 2003). The three most prominent families within the Caudovirales are Siphoviridae, Myoviridae, and Podoviridae, distinguished according to tail morphology (Ackermann 2001; Ceyssens 2009; Salmond and Fineran, 2015). Of the phage that have been characterized, 60% belong to the Siphoviridae which have long flexible tails, 25% are Myoviridae which have a double-layered contractile tail, and 15% are members of the Podoviridae characterized by short tails (Ackermann 2001; Guttman *et al.*, 2004). The remaining 3-4% of characterized phages consist of polyhedral, filamentous, and pleomorphic phages belonging to ten families (Ceyssens 2009). The content and composition of phage genomes vary considerably and generally reflect the genetic material of their respective hosts (Ackermann 2003). Siphoviridae phages are typically associated with Gram-positive bacteria including streptococci, and lactococci. Myoviridae and Podoviridae are frequently associated with γ -proteobacteria and other bacilli (Ackermann 2001). Considering that all the phages characterized in this work are Caudovirales, discussion of the isolation, characterization, and life cycles of the other phages will be not be included.

1.7.3 Infection Cycles

Bacteriophages can undergo two different life cycles upon successful infection of a suitable host. In a lytic infection cycle, phage rapidly multiply within the host bacterium, eventually lysing the cells and releasing new phage progeny (Skurnik and Strauch, 2006; Patel *et al.*, 2015; Salmond and Fineran, 2015). In the lysogenic infection cycle, the injected phage genetic material becomes incorporated into the host genetic material and remains in a dormant state as a “prophage” and is replicated along with its host (Skurnik and Strauch, 2006; Patel *et al.*, 2015; Salmond and Fineran, 2015). Because the work described herein pertains exclusively to lytic phages, a detailed

discussion of phage lysogeny will be not be included. The lytic infection cycle can be divided into several phases: 1) phage adsorption to the surface of a host, 2) injection of phage genome into the cytosol of the host bacterium, 3) expression of phage genes necessary for phage replication and utilization of the host's intracellular machinery, 4) replication and assembly of phage progeny, and 5) lysis of the host cell and release of phage progeny to infect other cells (Skurnik and Strauch, 2006; Shao and Wang, 2008; Patel *et al.*, 2015; Salmond and Fineran, 2015).

1.7.4 Bacteriophage Adsorption

The fundamental step in the lytic infection cycle is successful attachment, or adsorption, of phage to the host bacterium (Skurnik and Strauch, 2006). Phage adsorption involves recognition of surface molecules, or phage receptors, by tail fibers and other phage appendages (Katsura 1983; Goldberg *et al.*, 1994). Phages are known to use a range of bacterial surface molecules as receptors including lipopolysaccharide, outer membrane proteins, capsule, fimbriae, and flagella (Levin and Bull, 2004; Skurnik and Strauch, 2006; Patel *et al.*, 2015). It has also been shown that the maximum yield of phage progeny per bacterial cell is dependent on the rate of phage adsorption (Delbrück 1940).

1.8 Pre-harvest Intervention Strategies

In an effort to decrease the prevalence of pathogens in food animals, governmental officials, investigators, and food-animal producers sought to put in effect a so called “farm to table” food safety strategy involving mutual cooperation among all members within the food production chain. However, much of work aimed at improving food safety has focused on post-harvest interventions to be implemented by food producers and processors, and little energy has been allocated to develop pre-harvest intervention strategies to be applied by food animal producers (Dahl *et al.*, 2004). Moreover, the difficulty of describing complex host-microbe interactions, gaining regulatory approval, and ensuring economic feasibility makes the design and execution of pre-harvest interventions challenging, especially considering the problem of lairage environmental contamination once cattle arrive in processing plants (Wheeler *et al.*, 2014). Nevertheless, several investigators have explored pre-harvest interventions and argued for the efficacy of the approaches in reducing pathogens in food animals. Some of the most promising of these approaches include sodium chlorate addition to cattle feed and water, vaccines against animal-associated pathogens, pro-biotics/direct-fed microbials, and cocktails of lytic phages targeting pathogens of interest (Callaway *et al.*, 2002; Dahl *et al.*, 2004; Wheeler *et al.*, 2014).

1.8.1 Sodium Chlorate

Sodium Chlorate has shown promise to reduce pathogens in food animals (Callaway *et al.*, 2002; Dahl *et al.*, 2004; Wheeler *et al.*, 2014). Its efficacy stems from reduction to chlorite by nitrate reductase in the cytoplasm of bacteria resulting in accumulation of chlorite and death of the cell (Stewart 1988). The addition of sodium chlorate to feed and water has been shown to reduce fecal shedding of *E. coli* O157:H7 and *S. Typhimurium* DT104 in cattle rumen contents (Anderson *et al.*, 2000; Anderson *et al.*, 2002). However, large amounts of sodium chlorate cause toxicity in

cattle and the U.S. Food and Drug Administration has yet to approve using the additive in cattle for human consumption (Dahl *et al.*, 2004; Wheeler *et al.*, 2014).

1.8.2 Direct-fed Microbials

The administration of direct-fed microbials, or pro-biotics, is another pre-harvest intervention strategy which is aimed at introducing populations of bacteria to out-compete and/or antagonize pathogens within the gastrointestinal tract (Callaway *et al.*, 2002; Dahl *et al.*, 2004). It is well known that lactic acid producing bacteria have an inhibitory effect on *Salmonella* and *E. coli*, both within living animals and in refrigerated food animal products, and direct-fed microbials have also shown some success in reducing the shedding of *E. coli* O157:H7 and other pathogens in cattle and in poultry to reduce *Salmonella* (Dahl *et al.*, 2004). Although recent accumulating evidence for potential food safety applications have contributed to a growing interest in using direct-fed microbials (Wheeler *et al.*, 2014), this approach has not been widely adopted often due to incompatibility with existing practices (i.e. inclusion antibiotics in feed) (Callaway *et al.*, 2002).

1.8.3 Vaccines

Vaccination of cattle is another pre-harvest approach that aims at preventing the colonization of pathogens by inducing protective immunity (Loneragan and Brashears, 2005). Vaccines against *Salmonella* (Wheeler *et al.*, 2014; Heithoff *et al.*, 2015) and *E. coli* O157:H7 (Wheeler *et al.*, 2014) have been developed for commercial use and are frequently used in the dairy industry (House *et al.*, 2001; Wheeler *et al.*, 2014; Heithoff *et al.*, 2015). However, the adoption of vaccination as a pre-harvest intervention in the beef industry has been minimal (Wheeler *et al.*, 2014), and it has been estimated that less than 1% of beef cattle producers utilized this approach (USDA-APHIS, 2010) and fewer than 6% of fed cattle are vaccinated against *Salmonella* (USDA-APHIS, 2013).

1.8.4 Pre-harvest Interventions in Peripheral Lymph Nodes

In light of recent evidence documenting the prevalence of *Salmonella* in bovine peripheral lymph nodes, recent research efforts have evaluated the potential for some of these pre-harvest interventions to reduce *Salmonella* in peripheral lymph nodes (Edrington *et al.*, 2013a; Vipham *et al.* 2015). In a recent study developing a model of *Salmonella* peripheral lymph node carriage in calves, the application of a commercially available *Salmonella* vaccine to reduce peripheral lymph node carriage produced mixed results, although modest treatment effects were observed in one group (Edrington *et al.*, 2013a). Administration of a direct-fed microbial containing *Lactobacillus animalis* and *Propionibacterium freudenreichii* to feedlot cattle has been shown to modestly reduce *Salmonella* concentrations in peripheral lymph nodes (Vipham *et al.*, 2015). It is important to consider that effective pathogen control in pre-harvest animals is unlikely to result from a single intervention. Because bacteria are versatile organisms with varying susceptibilities to a single treatment, the use of pre-harvest interventions in combination may increase the efficacy of single interventions, and combination strategies are likely to be more effective than a single strategy alone (Wheeler *et al.*, 2014). Thus, future intervention strategies to reduce *Salmonella* in the peripheral lymph nodes may possess increased efficacy when used in combination with those discussed above.

1.9 Bacteriophage Therapy

1.9.1 Advantages of Bacteriophage Therapy

The use of phages to reduce pathogens in food animals possesses several advantages. Unlike antibiotics, phages are natural, nontoxic, and have been successfully used to treat human bacterial infections in (Dahl *et al.*, 2004). Because phage treatments can also be designed to target specific pathogens, there is also less risk of creating imbalances in intestinal microflora, which is essential to the digestive capacity of ruminants (Dahl *et al.*, 2004, Carvalaho *et al.*, 2012). phage treatment also has the added benefit of replicating in the process of killing its host (Smith and Huggins, 1982; Callaway *et al.*, 2002), which may increase the duration that the treatment may persist unlike the degradation of antibiotics over time (Callaway *et al.*, 2002). In the case of treating intestinal pathogens in cattle, phage shed in the feces can be passed to other food animals, reducing the need to administer treatment to each animal within a feedlot (Dahl *et al.*, 2004; Rozema *et al.*, 2009) and controlling environmental deposition of pathogens into feedlots (Stanford *et al.*, 2010).

1.9.2 Disadvantages of Bacteriophage Therapy

Despite these advantages, phage therapy is not currently approved for use in humans, food, or food animals (Dahl *et al.*, 2004). Although many studies have documented the safety and efficacy of phage treatment, more work will be needed in order to gain regulatory approval (Dahl *et al.*, 2004). Unlike antibiotics, there is a paucity of data pertaining to the pharmacokinetics and pharmacodynamics of phage therapy (Skurnik and Strauch, 2006). Several studies have detailed phage growth dynamics *in vitro* (Ellis and Delbrück, 1939; Delbrück 1940a, 1940b; Shao and Wang, 2008; Marco *et al.*, 2010). However, these data don't necessarily extrapolate into phage growth dynamics *in vivo* (Payne and Jansen, 2003). Phage may also carry harmful genes that can be passed on to their hosts (Skurnik and Strauch, 2006) and phage-mediated transmission of toxin

genes has been demonstrated for *Streptococcus pyogenes* (Broudy and Fischetti, 2003), *Vibrio cholerae* (Davis *et al.*, 2000), *Clostridium botulinum* (Brüssow *et al.*, 2004) and others.

Another important challenge in the development of phage therapy is the problem of phage resistance. Successful infection of a suitable host bacterium requires the adsorption of phage to bacterial surface molecule or phage receptors (Tanji *et al.*, 2004; Skurnik and Strauch, 2006). Alteration of these receptors resulting from spontaneous mutations confers resistance of the host bacterium to phage adsorption and subsequent infection (Levin and Bull, 2004; Tanji *et al.*, 2004; Callaway 2008). Moreover, phages may utilize common receptors of the host bacterium. Thus, if two different phages bind to the same receptor, alteration of this surface molecule would confer resistance to both phages (Tanji *et al.*, 2004). However, phages are known to attach to a range of bacterial surface molecules such as lipopolysaccharide, outer membrane proteins, capsule and flagella (Levin and Bull, 2004). Thus the use of cocktails containing phages that recognize different receptors may mitigate or perhaps prevent, the emergence of phage resistant bacteria (Carvalaho *et al.*, 2012).

1.9.3 Bacteriophage Therapy in Cattle

Although nothing has been published on the use of phage to reduce *Salmonella* in cattle, several studies have explored phage treatment as a pre-harvest intervention strategy to reduce *E. coli* in cattle (Smith *et al.*, 1987a; Rozema *et al.*, 2009; Stanford *et al.*, 2010) and the efficacy of phage to treat *E. coli* O157:H7 infections has been documented in a mouse model (Tanji *et al.*, 2005). Previous studies have shown that phage treatment in calves experimentally infected with *E. coli* O157:H7 reduces fecal shedding (Smith *et al.*, 1987a; Rozema *et al.*, 2009; Stanford *et al.*, 2010). Treatment cocktails of several phages have demonstrated superior efficacy to control *E.*

coli O157:H7 in cattle than single phage treatments (Tanji *et al.*, 2005; Rozema *et al.*, 2009; Tanji *et al.*, 2004).

1.9.4 Phage Therapy Reduction of Feedlot *E. coli* O157:H7 Prevalence

In addition to reducing pathogen levels in cattle, phages have also been shown to affect the prevalence of *E. coli* O157:H7 in feedlot environments (Oot *et al.*, 2007; Niu *et al.*, 2009). It has been established that phage treatment of *E. coli*-infected calves results in fecal shedding of phage in their feces (Smith and Huggins, 1983; Smith *et al.*, 1987a; Rozema *et al.*, 2009; Stanford *et al.*, 2010). Also, several studies have reported high concentrations of bacteriophage, ranging from 10^6 to 10^{10} PFU/g, can be shed in feces of *E. coli* O157:H7-infected cattle following oral treatment (Smith and Huggins, 1983; Smith *et al.*, 1987a; Rozema *et al.*, 2009) which may limit environmental deposition and transmission of pathogens among feedlot cattle (Rozema *et al.*, 2009). In previous work examining the prevalence of both *E. coli* O157:H7 and phage in feedlots, it was found that *E. coli* O157:H7 prevalence was inversely proportional to prevalence of its infecting phage (Oot *et al.*, 2007; Niu *et al.*, 2009). It has also been suggested that phage can be transmitted among feedlot cattle. In a study designed to recapitulate feedlot conditions, Rozema *et al.* (2009) detected *E. coli* O157:H7-infecting phage in the feces of control calves two days after phage administration to the treatment group. Similarly, phage were isolated from control cattle environments in a study examining microencapsulated phage to reduce *E. coli* O157:H7, supporting the notion that phage can easily be transferred among feedlot cattle (Stanford *et al.*, 2010).

1.10 Introduction

Non-typhoidal salmonellosis, is the leading bacterial cause of human foodborne disease in the U.S. and represents the leading cause of hospitalizations and death among foodborne illnesses acquired in the country (Scallan *et al.*, 2011; Crim *et al.*, 2014, Crim *et al.*, 2015). In 2014, cases of foodborne disease caused by *Salmonella* totaled 7,452 per 100,000 population, resulting in 2,141 hospitalizations and 30 deaths (Crim *et al.*, 2015). Among 88% serotyped *Salmonella* cases of foodborne disease in 2014, the top serotype, identified in 21% of cases, was *Salmonella enterica* serovar Enteritidis (SE) (Crim *et al.*, 2015). Although several strategies have been successful in controlling other foodborne pathogens such as *E. coli* O157:H7 (Wheeler *et al.*, 2014), the incidence of *Salmonella* foodborne disease has persistently been above the national *Healthy People* target in the past decade (Crim *et al.*, 2014) underscoring the need for increased effort to control this pathogen.

Cattle are known to harbor *Salmonella* and the pathogen is frequently isolated from the feces and hides of healthy animals at slaughter (Rhoades *et al.*, 2009; Moussa *et al.*, 2010; Koohmaraie *et al.*, 2012; Gragg *et al.*, 2013b; Mohamed *et al.*, 2014, Loneragan *et al.*, 2012). Moreover, ground beef has been identified as the source of several foodborne *Salmonella* outbreaks (Dechet *et al.*, 2006; CDC 2002; Schneider *et al.*, 2011; Jackson *et al.*, 2013) including an outbreak caused by SE during the summer of 2012 (CDC 2012). It is known that cattle hides represent a primary source of ground beef contamination by enteric pathogens (Koohmaraie *et al.*, 2005). However, a recent, accumulating body of evidence suggests that peripheral lymph nodes are important sources of *Salmonella* in ground beef (Arthur *et al.*, 2008; Brichta-Harhay *et al.*, 2012; Koohmaraie *et al.*, 2012; Haneklaus *et al.*, 2012; Gragg *et al.*, 2013a, 2013b; Edrington *et al.*, 2013a, Vipham *et al.*, 2015). Although mesenteric lymph nodes are routinely removed during

the evisceration process, peripheral lymph nodes are far too numerous and interspersed to be effectively removed and inevitably become incorporated into ground beef. National *Salmonella* prevalence in ground beef has been estimated to be anywhere from 2.2% (FSIS 2011) to 4.2% and bacterial loads up to 40 CFU/g have been observed (Bosilevac *et al.*, 2009). However, estimations of *Salmonella* prevalence in peripheral lymph nodes are inconsistent, likely arising from methodological differences. (Arthur *et al.*, 2008; Haneklaus *et al.*, 2012; Koohmaraie *et al.*, 2012; Gragg *et al.*, 2013a, 2013b). Nevertheless, the repeated isolation of *Salmonella* from peripheral lymph nodes warrants pre-harvest efforts to reduce *Salmonella* contamination in these sites.

In an effort to decrease the prevalence of pathogens in food animals, governmental officials, investigators, and food-animal producers sought to put in effect a so called “farm to table” food safety strategy involving mutual cooperation among all members within the food production chain (Dahl *et al.*, 2004). Some of the more promising pre-harvest intervention strategies to reduce enteric pathogens in cattle include sodium chlorate, vaccines, pro-biotics/direct-fed microbials, and phage cocktails (Callaway *et al.*, 2002; Dahl *et al.*, 2004; Wheeler *et al.*, 2014), and recent research efforts have examined some of these approaches to reduce *Salmonella* in peripheral lymph nodes, albeit with limited success (Edrington *et al.*, 2013a; Vipham *et al.*, 2015). In a recent study developing a model of *Salmonella* peripheral lymph node carriage in calves, the application of a commercially available *Salmonella* vaccine to reduce peripheral lymph node carriage produced mixed results, although modest treatment effects were observed in one group (Edrington *et al.*, 2013a). Administration of a direct-fed microbial containing *Lactobacillus animalis* and *Propionibacterium freudenreichii* to feedlot cattle has been shown to modestly reduce *Salmonella* concentrations in peripheral lymph nodes (Vipham *et al.*, 2015). These strategies were found to be only moderately effective. However, it is important to note that bacteria are versatile organisms

with varying susceptibilities to a single treatment and that a combination of pre-harvest interventions are likely to be more effective than a single strategy alone (Wheeler *et al.*, 2014). Thus, future intervention strategies to reduce *Salmonella* in the peripheral lymph nodes may possess increased efficacy when used in combination with those discussed above.

Bacteriophage treatment is another intervention strategy that has been explored to reduce pathogens in pre-harvest cattle. Bacteriophages (phages) are small viruses that infect and replicate within bacteria (Guttman *et al.*, 2004; Deresinski 2009). Like all viruses, phages are obligate parasites. Although phage genomes contain all of the necessary genes for replication and assembly, phages do not possess the basic metabolic components necessary for energy production and protein synthesis. Thus, the completion of the phage life cycle is fundamentally dependent on the infection of a suitable host (Guttman *et al.*, 2004).

Since the discovery, the bactericidal properties of phages have generated interest in the therapeutic potential of phages to control bacterial pathogens. Previous studies have shown that phage treatment possesses remarkable efficacy to control *E. coli* infection and/or fecal shedding in cattle (Smith *et al.*, 1987a; Smith and Huggins, 1983; Rozema *et al.*, 2009; Greer 2005), and commercially available cocktails of lytic phages against *E. coli* O157:H7 and *Salmonella* have also been developed and approved for hide washing (Wheeler *et al.*, 2014; Kropinski *et al.*, 2012). However, to my knowledge, nothing has been published on the use of oral application of phages as a pre-harvest intervention strategy to reduce peripheral lymph node carriage of *Salmonella* in cattle.

In response to the 2012 outbreak of SE in ground beef, I hypothesize that SE causes enteric disease in calves and disseminates from the bovine gut to the peripheral lymph nodes to contaminate ground beef following carcass processing. Thus, in the work described in this thesis,

I sought to assess the potential for oral phage treatment to *Salmonella* peripheral lymph node carriage in a calf infection model. The specific aims of this project were as follows: 1) develop a model of SE infection and peripheral lymph node carriage in calves, 2) evaluate the potential for a treatment cocktail of seven lytic phages targeting SE to reduce fecal shedding, disease signs, and peripheral lymph node carriage in the SE calf model and 3) characterize and optimize the seven phage treatment cocktail to three phages.

In chapter 2, I addressed the first specific aim by working toward developing a model of SE infection in 5-7 week-old calves using a bovine isolate of SE. I found that SE causes enteric disease in 5-7 week-old calves and that the bacterium disseminates from the gut to peripheral lymph nodes following oral inoculation. I also addressed specific aim two by treating a pair of experimentally infected calves with a cocktail of seven SE-targeted bacteriophages. Findings in phage-treated calves suggest that phage treatment has the potential to reduce disease signs and fecal shedding in SE-infected calves and to control *Salmonella* carriage in peripheral lymph nodes by disseminating from the gut along with its host.

In chapter 3, I addressed the third specific aim to characterize and optimize the phage cocktail to three phages. Findings from my calf model of SE suggested that an oral treatment cocktail of seven lytic phages targeting SE reduces *Salmonella* disease signs, fecal shedding, and peripheral lymph node contamination in experimentally infected calves. Although it has been demonstrated that cocktails of several phages are more efficacious than single phage treatments against *E. coli* O157:H7 in cattle (Tanji *et al.*, 2005; Rozema *et al.*, 2009; Tanji *et al.*, 2004), both experiments involving single phage treatments and cocktail treatments are necessary in order to evaluate the safety and efficacy of multi-phage cocktails. Because calf animal models are considerably more laborious and time-consuming than smaller animal models (e.g. mice), the

completion of sufficient numbers of experimental repetitions required to evaluate the safety and efficacy of a seven phage cocktail is impractical. In light of these limitations, I determined the host range, efficiency of plating, adsorption kinetics, and lytic properties of the all seven SE-targeted phages and used these parameters to reduce the cocktail to three phages.

1.11 Chapter 1 References

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Chapter 2.

Development Toward a Model to Evaluate Bacteriophage Treatment to Control *Salmonella* Peripheral Lymph Node Carriage in Calves

2.1 Abstract

Salmonella is the leading bacterial cause of human foodborne disease in the United States and is responsible for numerous illnesses, hospitalizations, and deaths each year. Cattle are known to harbor *Salmonella* and ground beef has been identified as the source of several foodborne *Salmonella* outbreaks. Among cases of foodborne disease in 2014, the top serotype was *Salmonella enterica* serovar Enteritidis (SE) which was responsible for a multistate outbreak linked to *Salmonella* in ground beef during the summer of 2012. Although cattle hides are considered to be the largest contributor to beef carcass contamination by enteric pathogens, a growing body of evidence indicates that peripheral lymph nodes (PLN) are important sources of *Salmonella* contamination of ground beef. Unlike mesenteric lymph nodes, PLNs are not routinely removed during carcass evisceration and are a potential source of ground beef contamination from infected cattle, underscoring the importance of pre-harvest interventions to reduce pathogens in these sites. Among current pre-harvest strategies in cattle, previous work has demonstrated the efficacy of bacteriophage (phage) treatment to control *Escherichia coli* O157:H7 in cattle. In light of these findings, I worked toward developing a model of SE infection and PLN carriage in calves and evaluated the potential for oral phage treatment to reduce disease signs, fecal shedding, and PLN carriage in infected calves.

Three pairs of 5-7 week-old calves (four control calves, and two phage-treated calves) were challenged orally with between 5.0×10^9 and 1.3×10^{10} CFUs of a bovine SE isolate. Following inoculation, daily fecal samples were enumerated for SE and rectal temperatures were recorded

twice daily. Blood, subiliac, and superficial lymph nodes were cultured post-mortem. In treated calves, a cocktail of seven lytic phages which targeted SE was orally administered following SE challenge. Oral challenge with SE produced mixed results. Fever spikes were noted for days two or three post inoculation. Although each calf received a high dose of SE, fecal shedding of the organism varied among calves in control (C1, C2, C3, C4) and phage-treated (T1 and T2) groups. Calf T1 shed low amounts of SE (2–3 log₁₀CFU/g feces); calves C1, C3 and C4 shed moderate amounts of SE (4–6 log₁₀CFU/g feces); and calves C2 and T2 shed high amounts of SE (6–8 log₁₀CFU/g feces). Bacteremia was noted for two of the three most severely affected calves and SE was recovered from the PLNs of the same three calves. Following treatment, phages were recovered from PLNs of calf T2. These findings demonstrate that SE causes enteric disease and invades PLNs in calves and that phage treatment may be effective in mitigating *Salmonella* carriage in PLNs. Also, the presence of SE in the PLNs of the three most severely affected calves and its presence in the blood of two of these three suggests that bacteremia may mediate translocation of *Salmonella* from the gut to PLNs.

2.2 Introduction

Non-typhoidal salmonellosis, is the leading bacterial cause of human foodborne disease in the United States and represents the leading cause of hospitalizations and death among foodborne illnesses in the country (Scallan *et al.*, 2011; Crim *et al.*, 2014, Crim *et al.*, 2015). In 2014, cases of foodborne disease caused by *Salmonella enterica* (hereafter referred to as *Salmonella*) totaled 7,452 per 100,000 in population, resulting in 2,141 hospitalizations and 30 deaths (Crim *et al.*, 2015). Among 88% Serotyped *Salmonella* cases of foodborne disease in 2014, the top serotype, identified in 21% of cases, was *Salmonella enterica* serovar Enteritidis (SE) (Crim *et al.*, 2015). Although several strategies have been successful in controlling other foodborne pathogens such as *Escherichia coli* O157:H7 (Wheeler *et al.*, 2014), the incidence of *Salmonella* foodborne disease has persistently been above the national *Healthy People* target in the past decade (Crim *et al.*, 2014) underscoring the need for increased effort to control this pathogen.

Cattle are known to harbor *Salmonella* and the pathogen is frequently isolated from the feces and hides of healthy animals at slaughter (Rhoades *et al.*, 2009; Moussa *et al.*, 2010; Koohmaraie *et al.*, 2012; Loneragan *et al.*, 2012; Gragg *et al.*, 2013b; Mohamed *et al.*, 2014). Moreover, ground beef has been identified as the source of several foodborne *Salmonella* outbreaks (CDC 2002; Dechet *et al.*, 2006; Schneider *et al.*, 2011; Jackson *et al.*, 2013) including an outbreak caused by SE during the summer of 2012 (CDC 2012). Cattle hides represent the greatest source of ground beef contamination by enteric pathogens (Koohmaraie *et al.*, 2005). However, a recent, accumulating body of evidence suggests that peripheral lymph nodes are

important sources of *Salmonella* in ground beef (Arthur *et al.*, 2008; Brichta-Harhay *et al.*, 2012; Koohmaraie *et al.*, 2012; Haneklaus *et al.*, 2012; Edrington *et al.*, 2013a; Gragg *et al.*, 2013a, 2013b; Vipham *et al.*, 2015). Although mesenteric lymph nodes are routinely removed during the evisceration process, peripheral lymph nodes are far too numerous and interspersed to be effectively removed and inevitably become incorporated into ground beef. National *Salmonella* prevalence in ground beef has been estimated to be anywhere from 2.2% (FSIS 2011) to 4.2% and bacterial loads up to 40 CFU/g have been observed (Bosilevac *et al.*, 2009). However, estimations of *Salmonella* prevalence in peripheral lymph nodes are inconsistent, likely arising from methodological differences. (Arthur *et al.*, 2008; Haneklaus *et al.*, 2012; Koohmaraie *et al.*, 2012; Gragg *et al.*, 2013a, 2013b). Nevertheless, the repeated isolation of *Salmonella* from peripheral lymph nodes warrants pre-harvest efforts to reduce bacterial contamination in these sites.

Bacteria are versatile organisms with varying susceptibilities to a single treatment and, it has been mentioned, that a combination of pre-harvest interventions should be considered to effectively control enteric pathogens in cattle (Wheeler *et al.*, 2014). Currently, the use of sodium chlorate, direct-fed microbials, vaccines and phages are some pre-harvest approaches to reduce enteric pathogens in cattle (Wheeler *et al.*, 2014), and recent research efforts have examined some of these approaches to reduce *Salmonella* in peripheral lymph nodes, albeit with limited success (Edrington *et al.*, 2013a, Vipham *et al.* 2015). In a recent study developing a model of *Salmonella* peripheral lymph node carriage in calves, the application of a commercially available *Salmonella* vaccine to reduce peripheral lymph node carriage produced mixed results, although modest treatment effects were observed in one group (Edrington *et al.*, 2013a). Administration of a direct-fed microbial containing *Lactobacillus animalis* and *Propionibacterium freudenreichii* to feedlot cattle has been shown to modestly reduce *Salmonella* concentrations in peripheral lymph nodes

(Vipham *et al.*, 2015) and may increase the efficacy of other interventions when used in combination. Several studies have shown, however, that phage possesses remarkable efficacy to control *E. coli* infection and/or fecal shedding in cattle (Smith and Huggins, 1983; Smith *et al.*, 1987a; Greer 2005; Rozema *et al.*, 2009). Commercially available cocktails of lytic phages against *E. coli* O157:H7 and *Salmonella* have also been developed and approved for hide washing (Kropinski *et al.*, 2012; Wheeler *et al.*, 2014). However, to my knowledge, nothing has been published on the use of oral application of phages in pre-harvest control of *Salmonella* or as a reduction strategy for peripheral lymph node carriage in cattle. Here, I show preliminary findings from experiments developing a model of SE peripheral lymph node carriage in 5-7 week old calves and show results suggesting the potential for oral phage treatment to control *Salmonella* in peripheral lymph nodes.

2.3 Materials and Methods

2.3.1 Bacterial Strains and Bacteriophage Treatment Cocktail

An isolate of *S. Enteritidis* (SE) from a Bovine fecal culture, was received from the Auburn University College of Veterinary Medicine (AUCVM) Diagnostic Bacteriology and Mycology Laboratory and was nalidixic acid resistant mutant (Nal^R) was isolated for selection convenience in fecal cultures. Nal^R SE -80°C freezer stocks were prepared by suspending over-night (ON) LBM slant growth in Dulbecco's phosphate buffered saline without calcium and magnesium (PD) (137 mM NaCl, 2.7 mM KCl, 10mM NaHPO₄, 1.8mM KH₂PO₄) and transferring one milliliter of suspension to four milliliters of glycerol. Phage -80°C stocks were prepared by adding dimethyl sulfoxide (DMSO) (7% v/v) to high titer phage lysates. Phage treatment cocktail was developed by selecting seven virulent phages exhibiting strong lytic-activity against SE from a collection of phages isolated from *Salmonella*-positive clinical veterinary fecal cultures received from the AUCVM Diagnostic Bacteriology and Mycology Laboratory. Treatment cocktail was prepared by suspending a combination of all seven phages in salts-magnesium (SM) buffer at a concentration of 10¹⁰ plaque forming units (PFU) each.

2.3.2 Animal Care and Housing

The animal protocols performed herein were approved by the Auburn University Institutional Animal Care and Use Committee. All calves in these experiments received colostrum. Prior to weaning, calves were treated with Amprolium (Corid[®] 9.6% oral solution, Merial Ltd., Duluth, GA) at 10 mg/kg body weight for 5 days and a single treatment of Fenbendazole (Panacur[®]

Suspension (Drench) 100 mg/mL, Intervet/Merck Animal Health, Millsboro, DE) at 10 mg/kg body weight. Pairs of just weaned calves between five-to-seven weeks were co-housed in a Biosafety Level 2 isolation unit and allowed to acclimate for one week. During acclimation, fecal samples were cultured to confirm that calves were *Salmonella* negative and submitted to AUCVM's parasitology lab to ensure the absence of intestinal parasites. Calves were fed a diet of antibiotic-free growth-starter calf feed and hay.

2.3.3 Calf Experiments

Three pairs (two control, one treated) of five-to-seven week-old calves were inoculated with $0.56 - 1.7 \times 10^{10}$ CFUs of stationary-phase SE. Inocula were prepared from (ON) cultures of SE grown in Brain-heart Infusion (BHI) broth (Bacto™ Brain Heart Infusion, Becton, Dickson and Company, Sparks, MD). Cultures were centrifuged ($12,000 \times g$, 15 min, 4°C), and pellets were washed twice with 0.85% NaCl solution. Five to ten milliliters of washed cells, diluted to $\text{OD}_{620} = 1.0$, were drawn into a 30mL dosing syringe used for oral inoculations and placed on ice. Viable cell counts of inocula were determined by serially diluting washed cell ($\text{OD}_{620} = 1.0$) in 1XPD and spread-plating in triplicate onto lysogeny broth agar plates (Difco™ LB Agar, Miller, Becton, Dickinson and Company, Sparks, MD) supplemented with 5mM MgSO_4 (LBM) followed by ON incubation at 37°C . The administered dose was calculated by multiplying the concentration of Bov SE determined from plate counts by the volume used in the inocula. Following inoculation, fecal samples were collected once daily and observed for stool consistency. Rectal temperatures were recorded twice daily. Electrolytes were administered to calves exhibiting dehydration resulting from severe disease. Following the onset of fever, one of the three pairs of SE-challenged calves was administered the phage treatment cocktail each day for five days.

2.3.4 Enumeration of *Salmonella* shed in feces

Daily fecal *Salmonella* shedding was determined by spread plating. One gram of feces was added to nine milliliters of 1XPD and mixed thoroughly. Fecal suspensions were serially diluted in 1XPD and 100µL of the appropriate dilutions were spread-plated in duplicate onto Xylose-Lysine-Tergitol 4 (Difco™ XLT4 Agar Base, Becton, Dickinson and Company, Sparks, MD) agar (XLT4) containing nalidixic acid (35 µg/mL). Plates were incubated ON at 37°C. Viable cell counts per gram of feces were calculated by multiplying the average colony counts by the inverse of the dilution $\times 10$.

2.3.5 Enumeration of Phage Shed in Feces

Following phage treatment, one gram of the fecal sample was added to nine milliliters 1XPD, mixed thoroughly and centrifuged (12,000 x g, 15 min). Supernatants were serially diluted in SM buffer and enumerated for phage by the double-agar overlay plaque assay using SE as the indicator strain. Bacteriophage were enumerated by the double agar overlay plaque assay described by Kropinski *et al.* (2009b) with modifications. Briefly, ten-fold serial dilutions of supernatants were prepared in Salt Magnesium (SM) buffer (100mM NaCl, 8mM MgSO₄, 50mM Tris-HCl, 0.01% w/v Gelatin) and 100 µL of phage dilutions was added to 200µL of exponential-phase cultures of SE in LB. Phage-cell mixtures were subsequently incubated for 10 minutes at 37°C to allow for phage adsorption. Afterwards, phage-cell mixtures were combined with 3mL of LBM top agar (LB + 0.7% agarose) containing 1% tetrazolium dye (LBMt) and poured over pre-warmed (37°C) LBM agar plates in duplicate or triplicate. After solidifying uncovered for 30 min under a biosafety cabinet, plates were incubated ON at 37°C. Following incubation, isolated plaques were counted and concentrations were calculated from countable plates containing 30-300 plaques.

2.3.6 Post-Mortem Lymph Node Culturing

Left and right superficial cervical (LC & RC), left and right subiliac (LS & RS), and one mesenteric lymph node (MLN) were excised after calves were euthanized. Adipose trim of the excised lymph nodes was removed and the exterior was sterilized three times by submersion in 95% ethanol (EtOH) for ten seconds. Excess EtOH was removed by passage through an open flame. Lymph nodes were then cut into ~1 cm³ pieces with sterile scissors, transferred to 25 mL of buffered peptone water (BPW) (Difco™ Buffered Peptone Water, Becton, Dickinson and Company, Sparks, MD), and incubated ON at 37°C. The next day, 0.1 mL and 0.5 mL aliquots were subcultured to Rappaport-Vassiliadis broth (RV) and tetrathionate (Difco™ Tetrathionate Broth Base, Becton, Dickinson and Company, Sparks, MD) broth (TTh) respectively, and were incubated at 37°C ON. The following day, cells grown in RV and TTh broths were streaked for isolation onto XLT4 agar plates and incubated at 37°C ON. Putative *Salmonella* colonies growing on XLT4 agar plates were picked and streaked on XLT4 + Nal to select for my marked SE isolate. SE colonies from XLT4 + Nal agar plates were confirmed by slide agglutination with *Salmonella* O Group D1 antiserum (Difco™, *Salmonella* O Antiserum Group D1 Factors 1, 9, 12, Becton, Dickinson and Company; Sparks MD).

2.3.7 Detection of Phage in Peripheral Lymph Nodes

Bacteriophage in lymph nodes were detected by spot test (Kutter 2009). One milliliter of ON lymph nodes cultures was centrifuged (12,000 x g, 5 min) and supernatants were filtered through a 0.2µm syringe filter. Filtered supernatants were subsequently spotted on a LBM double agar lawn containing SE. Spots were allowed to dry uncovered in a biosafety cabinet for one hour. Afterwards, plates were inverted and incubated ON at 37°C. The following day, cores from clearing were transferred to SM buffer saturated with chloroform and allowed to diffuse at room

temperature for 1 hour. Diffused core solutions were serially diluted in SM buffer and 100μL of each dilution was plated using the Double Agar Overlay Plaque Assay (Kropinski *et al.*, 2009a) and incubated ON at 37°C. Plates were observed the following day for plaque formation indicative of phage presence.

2.4 Results

2.4.1 Control Calves C1 and C2

The first pair of control calves (C1 and C2) were Holstein bull calves and were orally inoculated with 1.3×10^{10} CFU of SE. Inoculation produced an acute gastroenteritis characterized by fever, diarrhea, and shedding (Figures 2.1 and 2.2). Fever was noted for Calf C1 (Figure 2.1) from days two through five post-inoculation (PI), and days one through five PI for Calf C2 (Figure 2.2). Diarrhea was noted for calf C1 from days three through day fifteen PI, and for calf C2 from days one to fourteen PI (data not shown). Calf C1 shed moderate numbers of SE in the feces from days one through twelve PI and SE was detected intermittently in the feces only after enrichment in TTh broth from days thirteen through thirty PI. (Figure 2.1, data not shown). Calf C2, however, shed increasingly higher numbers of SE in the feces for days one through fourteen PI. On days ten through fourteen PI, when Calf C2's fecal shedding of SE was the highest, hypothermia was noted with brief return to normal temperature on mornings of days twelve and fourteen (Figure 2.2). On day fourteen, calf C2 was found to be depressed, recumbent, severely anorexic, and was euthanized in accordance with my protocol.

2.4.2 Treatment Calves T1 and T2

A pair of free-martin Holstein calves in the treatment group was orally inoculated with 1.7×10^{10} CFU of SE following acclimation in the isolation unit. Oral inoculation of Calf T1 produced a mild disease, with fever noted from days two through five PI. On days three through day seven PI, Calf T1 was treated daily with the phage cocktail. Following treatment, temperature in Calf T1

slowly returned to normal by day fourteen PI, with slight elevations during this time period. This calf shed low numbers of SE in the feces from days one through eleven and day thirteen PI, with quantifiable numbers only on days one through three and seven (Figure 2.3).

In contrast, Calf T2 exhibited an acute, severe gastroenteritis with a high fever on day two PI followed by acute hypothermia and accompanying recumbence on day three. Calf T2 shed increasingly high numbers of SE in the feces for day one through three post inoculation and was treated with phage cocktail in the afternoon of Day 3 (Figure 2.4). However, on the morning of day 4 PI, calf T2 was found dead in its isolation unit stall. Excision of the left subiliac and right superficial cervical LN was performed shortly after discovery of calf T2. *Rigor mortis* prevented successful, aseptic excision of right subiliac, left superficial cervical, as well as mesenteric LN.

Due to the unexpected death of Calf T2, enumeration of phages shed in the feces could only be performed for Calf T1. Phages were detected in the feces of Calf T1 for days four, five, and six PI, at concentrations of $3.97 - 4.18 \log_{10}$ (PFU/g). After day six PI, phages were intermittently detected in TTh enrichment cultures of Calf T1 feces (Figure 2.5).

2.4.3 Control Calves C3 and C4

A pair of Holstein-Jersey crossed calves in the second control group were orally inoculated with 5.6×10^9 CFU of SE. For calf C3, oral inoculation resulted in severe disease characterized by high fever from days two through five (Figure 2.6) and diarrhea on days one and five PI (data not shown). Calf C3 shed moderate numbers of SE in the feces which increased from days two through five. On day five, the animal was found to be recumbent and bloated, and was euthanized (Figure 2.6). In contrast, disease signs for calf C4 were moderate with fever noted from days two through five PI, followed by return to normal body temperature. Calf C4 shed moderate to high numbers of SE in the feces up to thirteen days PI (Figure 2.7) with diarrhea noted for days one, two and

eight through eleven PI (data not shown). Poor appetite, lethargy and bloody diarrhea were observed for this calf on days eight through ten, when fecal shedding was highest.

2.4.4 Post-Mortem Lymph Node Culturing

Post-Mortem culturing of peripheral lymph nodes was performed in order to determine the potential for both SE and orally administered phage to reach peripheral lymphatics. SE was recovered from PLN enrichment broths of the three most severely affected calves: Calves C2 and C3 in the control group, and calf T2 in the phage-treated group (Table 2.1). Cocktail phage were recovered from SE-positive PLN pre-enrichment broths of Calf T2. SE was also recovered from ante-mortem blood cultures of Calves T2 and C3, and mesenteric lymph nodes of Calves C2, C3, and C4 (Table 2.1).

2.5 Discussion

2.5.1 *S. Enteritidis* Disease in Calves

Although SE is not commonly found in cattle in the United States (Kunze *et al.*, 2008; Brichta-Harhay *et al.*, 2011; Dodd *et al.*, 2011; Loneragan *et al.*, 2012), natural infections with this serovar have been reported abroad (Petrie *et al.*, 1977; Moussa *et al.*, 2010; Mohamed *et al.*, 2014) and SE isolation has been reported in cattle from slaughtering facilities in Pennsylvania (Sandt *et al.*, 2013). Moreover, SE is one of the three top serotypes responsible for human *Salmonella* outbreaks traced to beef products (Jackson *et al.*, 2013) and was implicated in a human outbreak linked to consumption of ground beef (CDC, 2012). In light of these findings, I chose serovar Enteritidis for this study.

In this study, fecal shedding and disease outcomes were notably different among the calves in this study. Signs of self-limiting enteric disease (pyrexia, anorexia, and diarrhea) were observed for control calves C1 and C4, and treatment calf T1 and was accompanied by low to moderate fecal shedding (Figures 2.1, 2.7, and 2.3). However, severe, disseminated disease was observed for control calves C2 and C3 and treatment Calf T2, and was characterized by high fever, progressively increasing fecal shedding, and mortality (Figures 2.2, 2.6, and 2.4). These disease presentations, both self-limiting gastroenteritis and lethal disseminated salmonellosis, mirror previous clinical observations in calves naturally infected with SE (Petrie *et al.*, 1977). Although it is tempting to infer that lethal disseminated disease is a feature of SE infection in calves, mortality has also been observed in clinical calf infections with several other *Salmonella* serovars

which predominantly cause gastroenteritis in calves, such as *S. Typhimurium* and *S. Newport*, and was not associated with a particular serovar (Cummings *et al.*, 2009). Moreover, the ability to invade and replicate intracellularly, avoid host immune defenses, and cause disseminated disease are key features of *Salmonella* pathogenicity, irrespective of the serovar (Mohler *et al.*, 2009). Host-associated factors such as species, immune factors, age, and infectious dose, also play important roles in outcomes of *Salmonella* infections (Santos *et al.*, 2001). In calves, factors such as age, infectious dose, weaning stress and colostrum quality are known to play a role in *Salmonella* disease severity (Mohler *et al.*, 2009; de Jong and Ekdahl, 1965). Although all of the calves in my study received colostrum, I were unable to assess colostrum quality and the degree to which colostrum quality affected disease outcomes is uncertain. Lethal disease in calves infected with *Salmonella* is also associated with age (Smith *et al.*, 1979; Cummings *et al.*, 2009; Mohler *et al.*, 2009), and I observed disseminated disease in three calves in this study (C2, T2, and C3). The infectious dose also plays a role in disease severity. Previous experiments examining the effect of *S. Typhimurium* infectious doses on disease outcomes in two – four day-old calves found that oral challenge with $\sim 10^{11}$ CFU was associated with severe disseminated disease with high mortality, whereas doses of $\sim 10^8$ CFU resulted in self-limiting gastroenteritis (pyrexia, anorexia, and diarrhea) (de Jong and Ekdahl, 1965). In my experiments, SE doses of 0.5 and 1.3×10^{10} CFU produced enteric and disseminated disease in untreated calves and, although more work is needed, it is possible that these doses represent, or approximate, a median lethal dose. Furthermore, it is my opinion that observed disease outcomes in my calves is likely a combination of age and dose-dependent effects in calf-infections with broad host-range serovars, rather than a unique and specific SE pathogenicity in calves.

2.5.2 *S. Enteritidis* Recovery from Peripheral Lymph Nodes

In previous and recent studies of experimental *Salmonella* infection in calves, it has been shown that substantial challenge doses ($\sim 10^{10}$ CFU) are required to successfully recover the pathogen from peripheral lymph nodes (de Jong and Ekdahl 1965; Edrington *et al.*, 2013a; Brown *et al.*, 2015a). After oral challenge of calves with $\sim 10^{10}$ CFU, I recovered SE from the peripheral lymph nodes of calves with severe disseminated disease and the highest fecal *Salmonella* shedding but not from calves with gastroenteritis and low to moderate fecal shedding (Table 2.1). An obvious explanation for this finding is that severe disease facilitates peripheral lymph node dissemination of this pathogen. Similar to my findings, *S. Typhimurium* was found to be present in peripheral lymph nodes of experimentally infected calves that had died but not from surviving calves (de Jong and Ekdahl, 1965). However, *Salmonella* has been recovered from peripheral lymph nodes of apparently healthy animals at slaughter (Arthur *et al.*, 2008; Brichta-Harhay *et al.*, 2012; Haneklaus *et al.*, 2012; Gragg *et al.*, 2013a, 2013b). Moreover, Edrington *et al.* (2013a) state that recovery from the peripheral lymph nodes is a function of incidence or the rate at which lymph nodes become infected with and clear *Salmonella*. Thus, while large doses are needed to efficiently model peripheral lymph node dissemination, the condition of these animals does not necessarily reflect animals at slaughter nor is severe disease apparently required for *Salmonella* peripheral lymph node dissemination. Instead, repeated exposure to lower doses of *Salmonella* are likely what results in peripheral lymph node carriage by cattle, as has been suggested by Brown *et al.* (2015a). In my experiments, SE was recovered from post-mortem culturing of four peripheral lymph nodes only after extensive pre-enrichment cultures (Table 2.1) and could not be isolated by direct plating (data not shown). Thus, it is possible that peripheral lymph nodes other than those selected in these experiments could have harbored *Salmonella* in surviving calves.

2.5.3 Mechanism of *Salmonella* Dissemination

The mechanisms mediating translocation of bacteria, including *Salmonella*, from the gut to systemic tissues remain to be elucidated. In my experiments, SE was isolated from blood cultures of two of the three severely affected calves (Table 2.1) with positive peripheral lymph nodes. Similar associations between lethal disease, peripheral lymph node dissemination, and positive blood cultures have been observed in natural calf infections with SE (Petrie *et al.*, 1977). Bacteremia is common in calves dying from salmonellosis (Mohler *et al.*, 2009) and *Salmonella* has been isolated from systemic tissues and blood of calves as early as 2-4 hours after experimental infection (de Jong and Ekdahl, 1965; Pullinger *et al.*, 2007). Moreover, systemic and peripheral lymph node dissemination of *S. Typhimurium* was found to occur despite surgical interventions to prevent dissemination from the gut (de Jong and Ekdahl, 1965). Taken together, these results suggest that hematogenous dissemination is a mechanism mediating *Salmonella* translocation to peripheral lymph nodes. In contrast, several studies argue that translocation from the gut occurs via the lymphatic system (Paulin *et al.*, 2002; Pullinger *et al.*, 2007; Brown *et al.*, 2015a). Alternatively, necrosis of the intestinal epithelium similar to *S. Dublin* infection was observed in calves naturally infected with SE (Petrie *et al.*, 1977). Thus, bacteremia maybe a consequence of compromised gut integrity. More work is needed to ascertain precisely what role bacteremia plays in disseminated salmonellosis and contamination of peripheral lymph nodes.

2.5.4 Bacteriophage Treatment

Many studies have explored phage treatment as a pre-harvest intervention strategy to reduce *E. coli* in cattle (Rozema *et al.*, 2009; Stanford *et al.*, 2010). However, to my knowledge, this work is the first to explore the potential of phage treatment to reduce *Salmonella* in cattle. In previous studies using phage to control *E. coli* O157:H7, it has been shown that a cocktail of

phages is more effective at reducing target bacteria than treatment with a single phage (Tanji *et al.*, 2004; Tanji *et al.*, 2005; Rozema *et al.* 2009). Thus, I chose to treat calves with a cocktail of seven bacteriophages. Of the two calves that received phage treatment, phages were found in two PLN of Calf T2 (Table 2.1), and fecal *Salmonella* numbers were much decreased in Calf T1 (Figure 2.3). The recovery of phage from the peripheral lymph nodes of an experimentally infected calf suggests that phage treatment can penetrate the PLN and may reduce *Salmonella* past carcass processing by becoming incorporated in ground beef along with their hosts. Although neither *Salmonella* nor phage were isolated from the lymph nodes of Calf T1, fecal *Salmonella* shedding for this calf was the lowest among all of the calves in this study despite receiving the highest challenge dose. Future experiments in SE infected calves are needed to confirm peripheral lymph node penetration and fecal *Salmonella* shedding reduction by my phage cocktail.

2.5.5 Shedding of Bacteriophage in the Feces

It has long been established that *E. coli*-infected calves treated with phage shed phage in their feces (Smith and Huggins, 1983; Smith *et al.*, 1987a; Rozema *et al.*, 2009; Stanford *et al.*, 2010). In my study, Calf T1 shed $\sim 10^4$ PFU/g of phage in the feces for days 2-4 post-treatment (Figure 2.5) which is considerably lower than other work in *E. coli*-infected calves reporting phage sheds in the range of $10^6 - 10^{10}$ PFU/g after oral treatment (Smith and Huggins, 1983; Smith *et al.*, 1987a; Rozema *et al.*, 2009). Nevertheless, shedding of phage may have advantages extending beyond pathogen control in cattle at slaughter. In previous work examining the prevalence of both *E. coli* O157:H7 and phage in feedlots, it was found *E. coli* O157:H7 prevalence was inversely proportional to prevalence of its infecting phage (Oot *et al.*, 2007; Niu *et al.*, 2009). Also, in a study designed to recapitulate feedlot conditions, Rozema *et al.* (2009) suggested that *E. coli* O157:H7-infecting phage can be transmitted among feedlot cattle after detecting phage in the feces

of control calves two days after phage administration to the treatment group. Similarly, isolation of phage from control cattle environments were reported in a study examining microencapsulated phage to reduce *E. coli* O157:H7 (Stanford *et al.*, 2010). In my study, Calf T1 shed phage concomitantly with SE in the feces for nine days after initial treatment (Figure 2.5). Taken together, these findings warrant future work investigating the potential for phage treatment to limit environmental deposition and transmission of *Salmonella* among feedlot cattle.

Although only one calf (T1) treated with phage survived experimental infection with *Salmonella*, the fecal shedding and disease of calf T2 was particularly severe and likely beyond treatment, similar to observations in phage treatment of calves experimentally inoculated with *E. coli* (Smith and Huggins, 1983). Nevertheless, because calf T1 exhibited the mildest disease and lowest fecal *Salmonella* shedding amongst the calves in these experiments, and orally administered phage were recovered from the peripheral lymph nodes of calf T2, both warrant future investigation. Ongoing treated and untreated calf experiments are being conducted to confirm the viability of pre-harvest phage treatment to control and prevent *Salmonella* contamination of peripheral lymph nodes.

2.6 Chapter 2 References

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2.7 Chapter 2 Tables and Figures

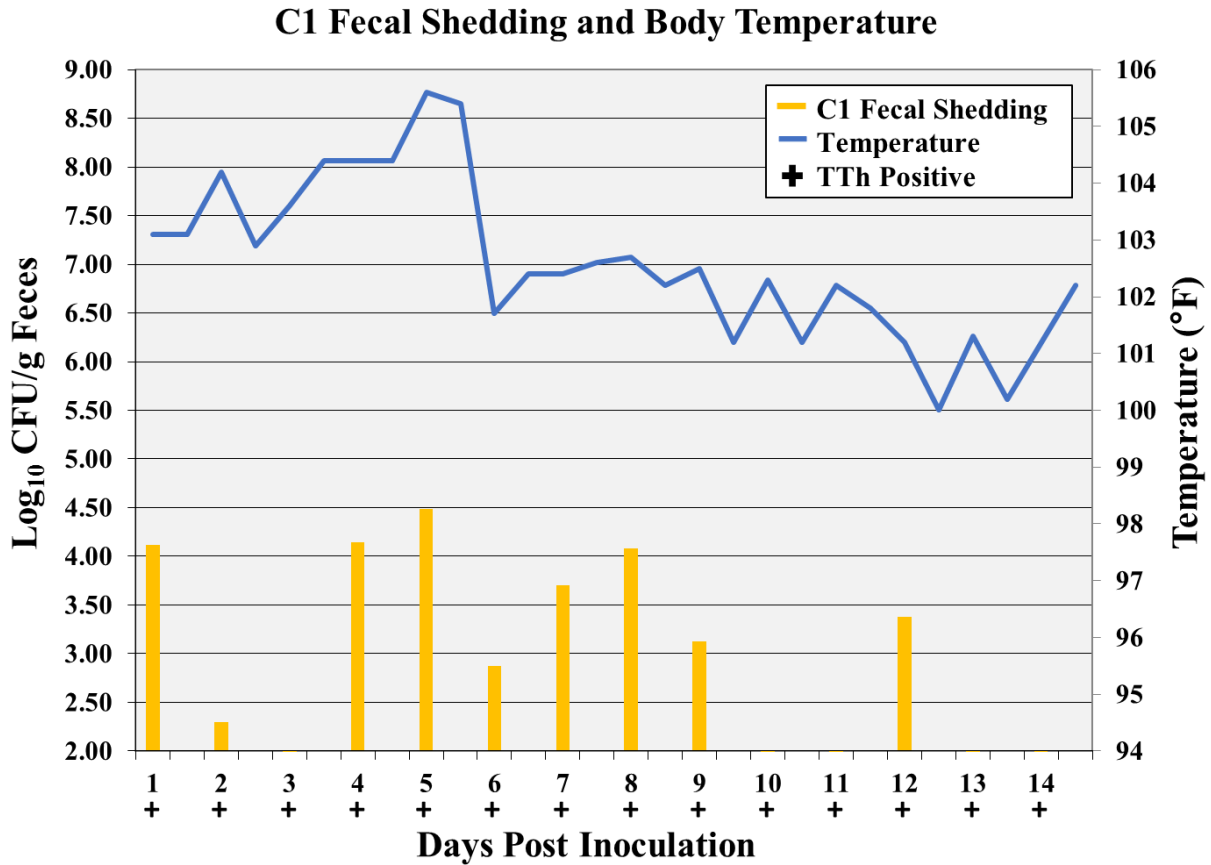


Figure 2.1. Calf C1 fecal shedding (yellow bars) and rectal temperature (blue line) vs. time following inoculation with 1.3×10^{10} CFU SE on day 0. (+) denote SE positive tetrathionate (TTh) enrichment fecal cultures.

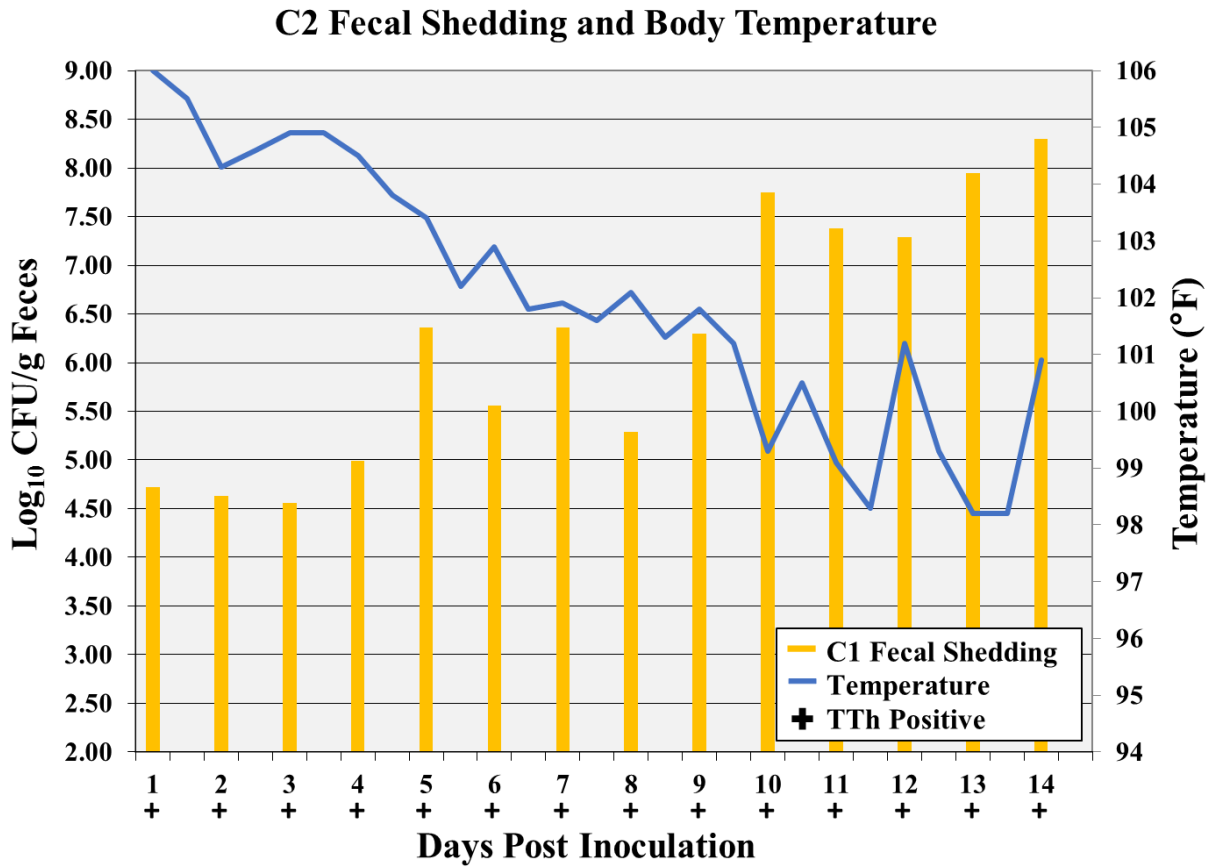


Figure 2.2. Calf #14 fecal shedding (yellow bars) and rectal temperature (blue line) vs. time following inoculation with 1.3×10^{10} CFU SE on day 0. (+) denote SE positive tetrathionate (TTh) enrichment fecal cultures.

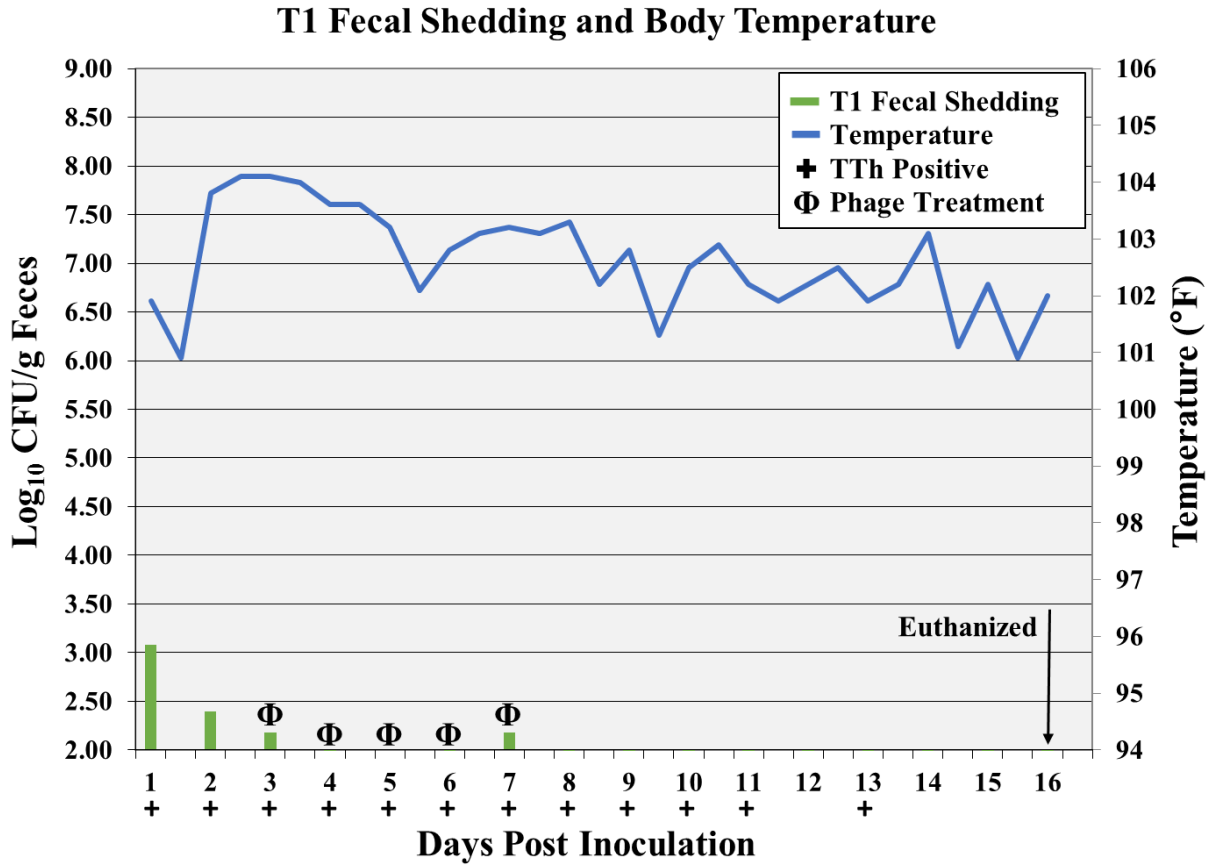


Figure 2.3. Calf #24 fecal shedding (green bars) and rectal temperature (blue line) vs. time following inoculation with 1.7×10^{10} CFU SE on day 0. (+) denote SE positive tetrathionate (TTh) enrichment fecal cultures and (Φ) marks days phage treatment was administered

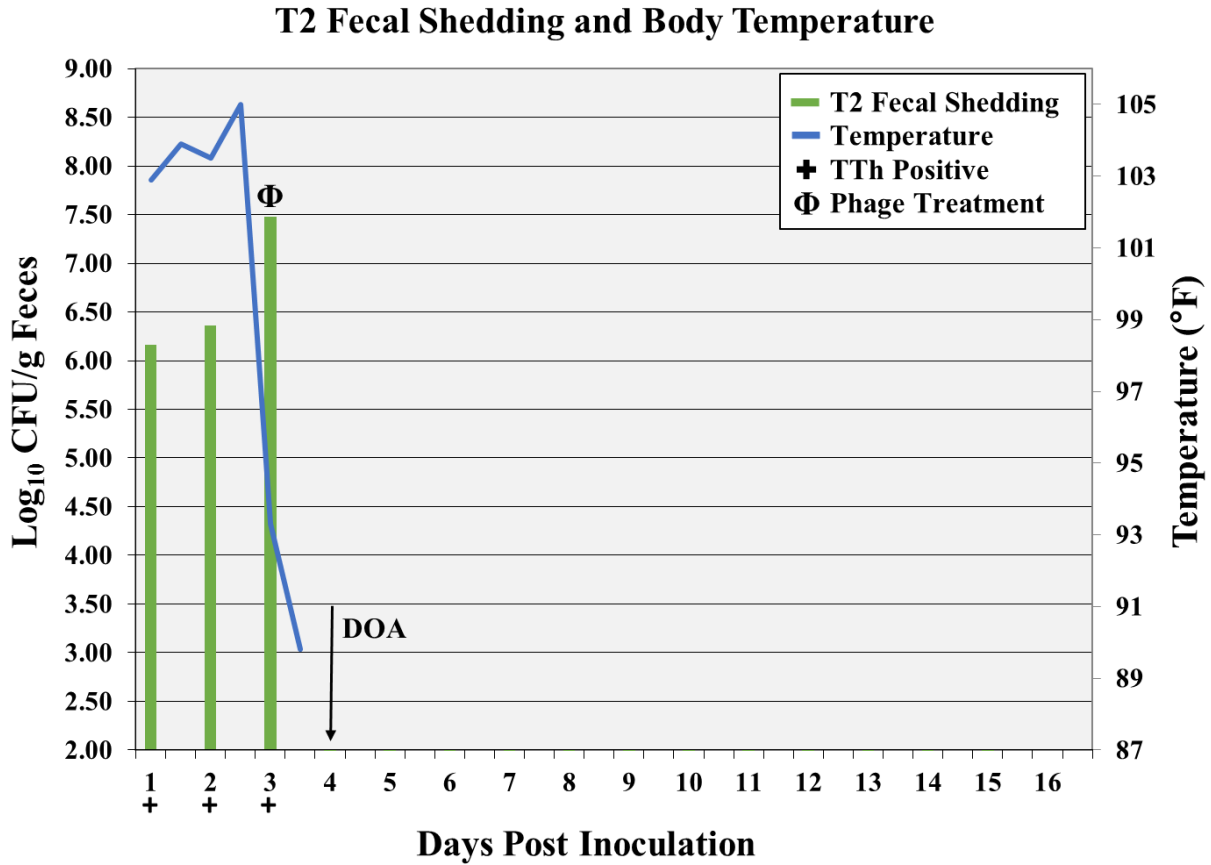


Figure 2.4. Calf #25 fecal shedding (green bars) and rectal temperature (blue line) vs. time following inoculation with 1.7×10^{10} CFU SE on day 0. (+) denote SE positive tetrathionate (TTh) enrichment fecal cultures and (Φ) marks days phage treatment was administered

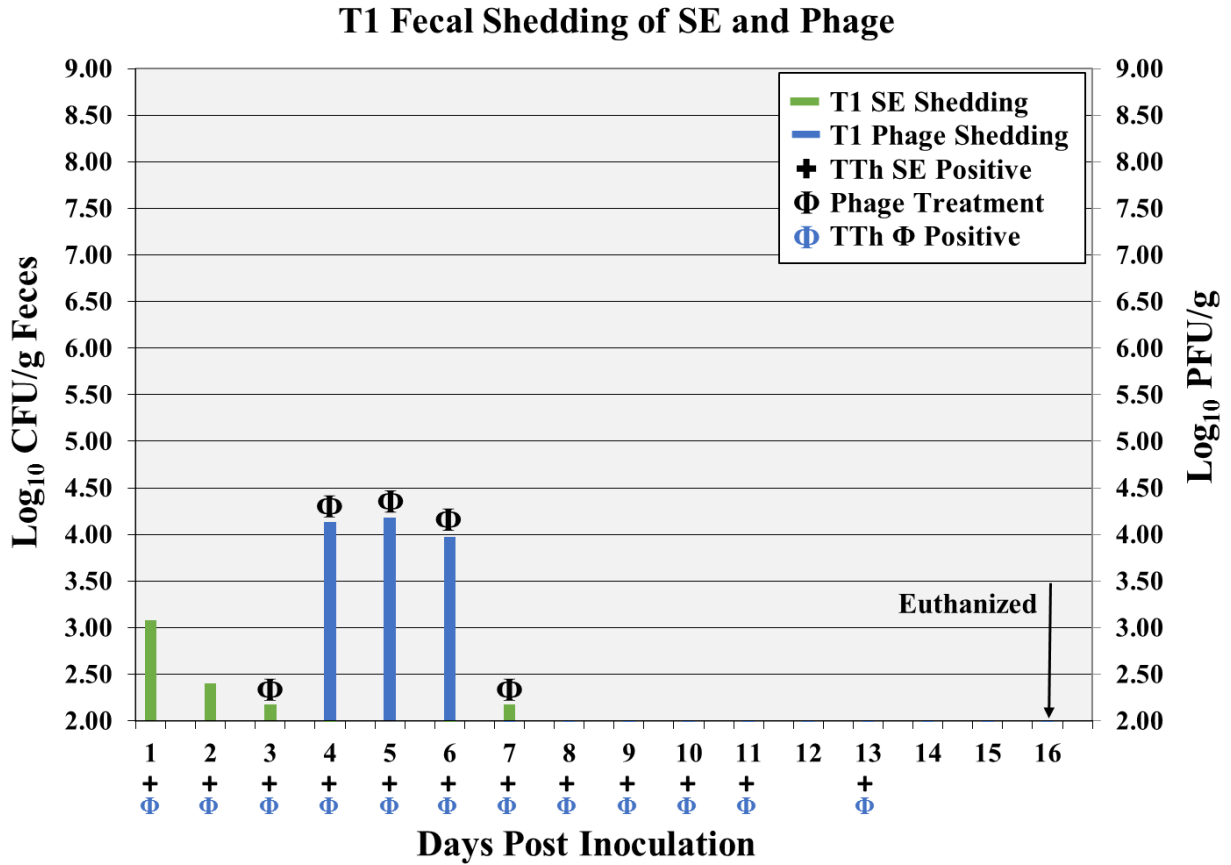


Figure 2.5. Calf #24 shedding of SE (green bars) and bacteriophage (blue bars) in the feces vs. time following inoculation with 1.7×10^{10} CFU SE. (Φ) marks days phage treatment was administered. (+) represents SE positive and (Φ) bacteriophage positive tetrathionate (TTh) enrichment fecal cultures.

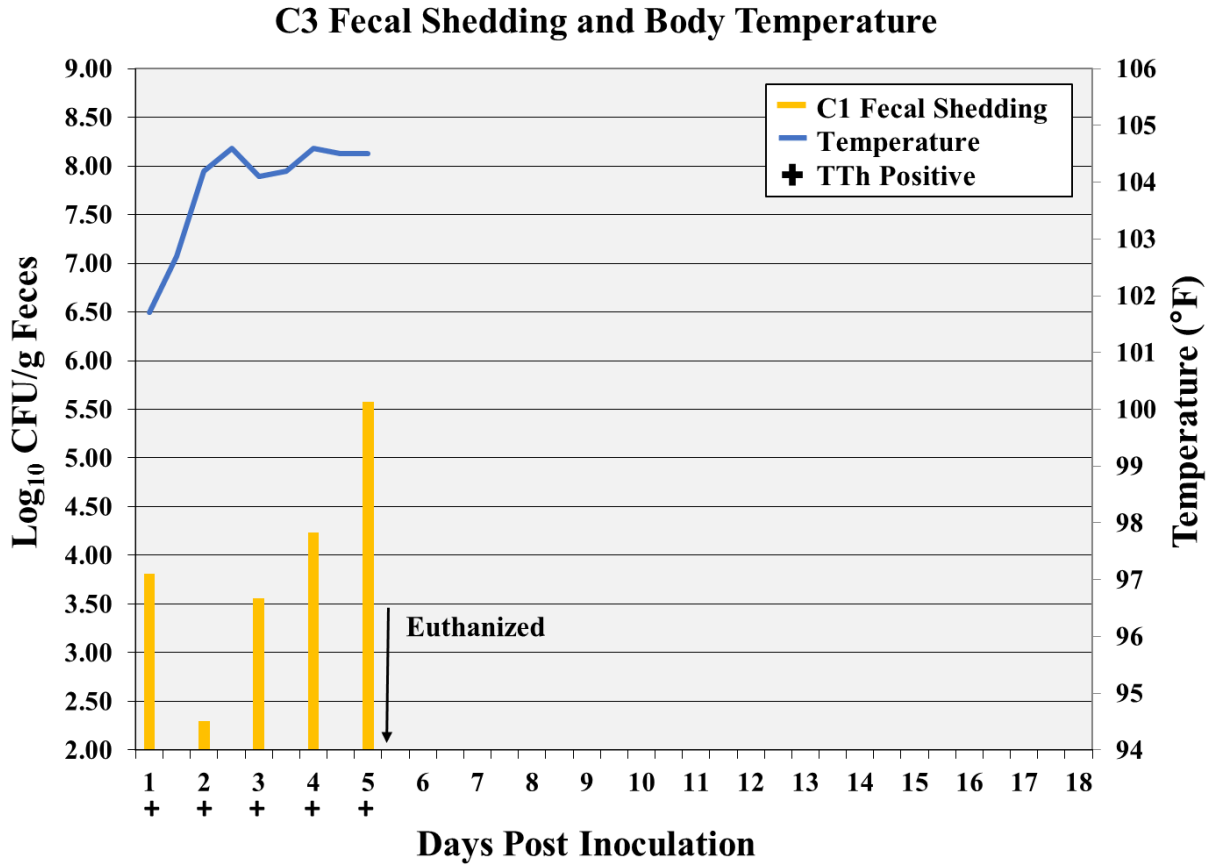


Figure 2.6. Calf #D1 fecal shedding (yellow bars) and rectal temperature (blue line) vs. time following inoculation with 0.56×10^{10} CFU SE on day 0. (+) denote SE positive tetrathionate (TTh) enrichment fecal cultures.

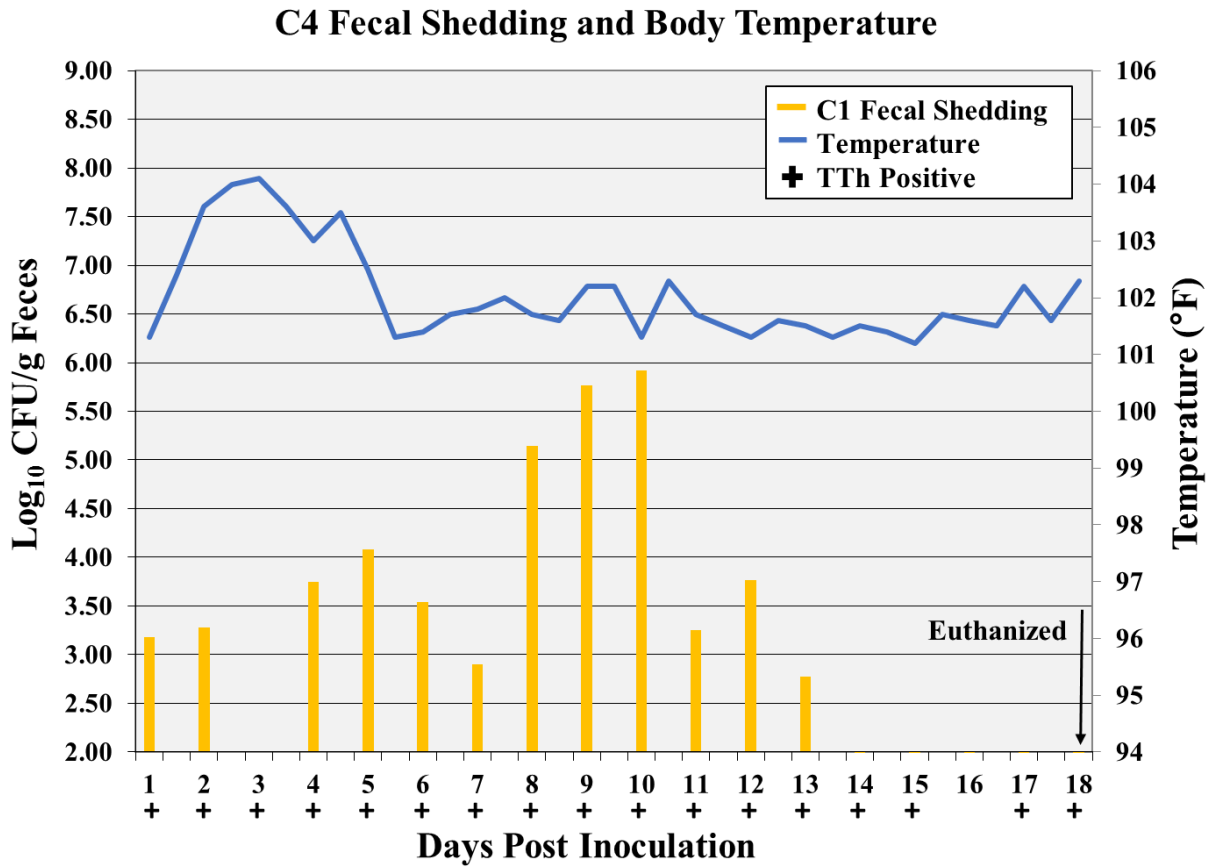


Figure 2.7. Calf #D2 fecal shedding (yellow bars) and rectal temperature (blue line) vs. time following inoculation with 0.56×10^{10} CFU SE on day 0. (+) denote SE positive tetrathionate (TTh) enrichment fecal cultures.

Table 2.1: SE in Peripheral Lymph Nodes and Blood

Calf	Blood	Lymph Node				
		RC	LC	RS	LS	MLN
C1	- ^a	-	-	-	-	-
C2	-	+ ^b	+	+	-	+
C3	+	N/D ^c	N/D	+	N/D	+
C4	-	-	N/D	-	N/D	+
T1	-	-	-	-	-	-
T2	+	+ / Φ ^d	N/D	N/D	+ / Φ	N/D

^a Culture negative for SE.

^b Culture positive for SE.

^c Not done.

^d Phage recovered from lymph node.

Chapter 3.

Characterization and Optimization of a Bacteriophage Cocktail to Reduce *Salmonella* Peripheral Lymph Node Carriage in Calves

3.1 Abstract

Preliminary findings in experiments developing a model of *Salmonella* Enteritidis (SE) peripheral lymph node contamination in five to seven week-old calves suggested that seven SE-targeting phages (phages) in a treatment cocktail were able to penetrate peripheral lymph nodes and were shed in appreciable numbers in the feces following oral administration. However, due to constraints involved in implementing a seven phage cocktail, characterization experiments were performed for each of the seven phages in the cocktail in order to establish exclusion criteria for cocktail optimization. Electron micrographs were prepared by negatively staining concentrated phage lysates with 2% phosphotungstic acid and viewed with transmission electron microscopy. Qualitative lytic activity was assessed by performing *Salmonella* growth curves in the presence of phage (lysis curves) at varying multiplicities of infection (MOI). Additionally, *Salmonella* host range, efficiency of plating, adsorption rate constants, and ultra-violet (UV) inactivation constants were determined for each cocktail phage. Phages were classified into three families based upon morphology: Myoviridae (three phages), Siphoviridae (two phages), and Podoviridae (two phages). Each cocktail phage demonstrated a strong lytic activity against SE and was able to lyse or form plaques on several *Salmonella* serovars. Except for phage in the Podoviridae family, similarities in the host ranges, efficiencies of plating, lysis curve patterns and adsorption rate constants were found among phages in the same family, suggesting redundancy among cocktail phages in the Myoviridae and Siphoviridae families. These findings were used to optimize the treatment cocktail to three phages.

3.2 Introduction

Bacteriophages (phages) are small viruses that infect and replicate within bacteria. They are ubiquitous in nature, and represent the most abundant biologically active entities on the planet (Guttman *et al.*, 2004; Deresinski 2009). Like all viruses, phages are obligate parasites. Although phage genomes contain all of the necessary genes for replication and assembly, phages do not possess the basic metabolic components necessary for energy production and protein synthesis. Thus, the completion of the phage life cycle is fundamentally dependent on the infection of a suitable host (Guttman *et al.*, 2004).

The tailed phages are organized in the order Caudovirales. The three most prominent families within the Caudovirales order are Siphoviridae, Myoviridae, and Podoviridae, which are distinguished according to tail morphology (Ceyssens 2009). Of the Caudovirales phages that have been characterized, 60% belong to the Siphoviridae which have long flexible tails, 25% are Myoviridae phages with double-layered contractile tails, and 15% are members of the Podoviridae family, characterized by short tails (Guttman *et al.*, 2004). Shortly after their discovery in the early 1900s, many investigators sought ways to exploit the bactericidal properties of phages in order to treat or control bacterial infections (Duckworth 1976; Summers 2001; Guttman *et al.*, 2004).

In previous work, I performed experiments toward developing a model of *S. enterica* subsp. *enterica* serovar Enteritidis (SE) infection in calves to evaluate the ability of a seven phage treatment cocktail to reduce *Salmonella* in cattle. To my knowledge, nothing has been published on the use of phage to reduce *Salmonella* in cattle. However, several studies have successfully

used phage treatment to control *E. coli* infections in cattle (Smith and Huggins, 1987a; Rozema *et al.*, 2009; Stanford *et al.*, 2010) and successful treatment of *E. coli* O157:H7 infections in a mouse model has been reported (Tanji *et al.*, 2005). Preliminary findings in experiments developing a calf model of SE suggest that an oral treatment cocktail of seven lytic phages targeting SE reduces *Salmonella* disease signs, fecal shedding, and peripheral lymph node contamination in experimentally infected calves. Although it has been demonstrated that cocktails of several phages are more efficacious than single phage treatments (Tanji *et al.*, 2004; Tanji *et al.*, 2005; Rozema *et al.* 2009), it is necessary to conduct experiments involving single phage treatments in addition to cocktail treatments in order to evaluate the safety and efficacy of phage cocktails. Also, because calf animal models are considerably more laborious and time-consuming than models in smaller animals (e.g. mice), the completion of a sufficient number of experimental repetitions required to evaluate the safety and efficacy of a seven phage cocktail is impractical.

In light of these limitations, I sought to reduce my treatment cocktail to three phages by characterizing the host range, efficiency of plating, adsorption kinetics, and lytic properties of the all seven SE-targeting phages. The three phages chosen will be tested in my calf experimental infection model to determine efficacy in reducing disease signs, fecal shedding, and peripheral lymph node colonization.

3.3 Materials and Methods

3.3.1 Bacterial Strains and Bacteriophage

Sixteen *Salmonella* serovars, including two *S. Typhimurium* serovars, and seven SE isolates were received from the Auburn University College of Veterinary Medicine (AUCVM) Diagnostic Bacteriology and Mycology Laboratory collection of *Salmonella* isolates from clinical veterinary samples. Two additional SE isolates were received from the Alabama State Diagnostic Lab and an isolate of *E. coli* O157:H7 was obtained from Dr. John (Skip) Foster of University of South Alabama (Tables 3.1 and 3.3).

Seven virulent phages exhibiting strong lytic-activity against SE were selected from a collection of phages isolated from *Salmonella*-positive clinical veterinary fecal cultures and necropsy samples received from the AUCVM Diagnostic Bacteriology and Mycology Laboratory and included in a phage treatment cocktail targeting SE (Table 3.2).

3.3.2 Phage Typing and Pulsed-Field Gel Electrophoresis (PFGE)

Nine SE isolates were submitted to the National Veterinary Services Laboratory (Ames, IA) for *Salmonella enterica* serovar Enteritidis phage typing (Table 3.1).

Pulsed-Field Gel Electrophoresis of SE isolates was performed as described by Palmer (2014) with modifications. Briefly, cells from overnight (ON) growth on Lysogeny Broth agar plates (Difco™ LB Agar, Miller, Becton, Dickinson and Company, Sparks, MD) containing 3% MgSO₄ (LBM) were suspended in Cell Suspension Buffer (100mM Tris Base, 100mM EDTA, pH 8.0) and mixed thoroughly by vortex. Cell concentrations were adjusted to an OD₆₀₀ between 1.3

and 1.4 followed by the addition of proteinase K to a final concentration of 1mg/mL. Plugs were prepared by casting a combination of melted 1% SeaKem® Gold agarose (Lonza, Allendale, NJ) containing 1% sodium dodecyl sulfate (SDS) and an equal volume of the cell suspension in wells of 50-well disposable plug molds (Bio-Rad, Hercules, CA). After casting, plugs were allowed to solidify at room temperature (RT) for 20 min. Plugs were subsequently transferred to 1.5mL of lysis buffer (50mM Tris base, 50mM EDTA pH 8.0 + 1% Sarcosyl) containing 0.33mg/mL proteinase K and were incubated for one hour in a 55°C shaking water bath at 175 rpm. Following lysis, plugs were washed twice with MilliQ water, four times with TE buffer (10mM Tris base, 1mM EDTA, pH 8), and stored ON at 4°C. The next day, plug slices were digested with 30 U of *Xba*I for 3 hours in a 37°C water bath. Digested plug slices were extensively dried using Chemiwipes (Kimberly-Clark, Roswell, GA) and loaded onto a comb. Plugs of *S. Branderup* H9812 digested with *Xba*I were included as a molecular weight (MW) marker. Digested plugs were resolved in a 1.2% SeaKem® Gold Agarose gel using a CHEF mapper® Pulsed Field Electrophoresis System (Bio-Rad, Hercules, CA). Running conditions were as follows: 16 hour run-time at 14°C with a 120°Angle, 30 kb-low MW, 600 kb-high MW. Initial switch time was 2.16s and final switch time was 63.8s. Gels were stained with 50µL GelRed™ (Biotium, Hayward, CA) in 500mL of MilliQ water for 20 min. Gels were de-stained twice with MilliQ water for 20 minutes each. Stained gels were visualized and gel photos were captured with a GelDoc-It® Imager (UVP, Upland, CA).

3.3.3 Double Agar Overlays

Double agar overlays used to determine lytic phage activity or titer on *Salmonella* subsp. *enterica* (*Salmonella*) serovars were prepared as described by Kropinski *et al.* (2009b) with modifications. Briefly, 200µL of exponential-phase *Salmonella* cultures in Lysogeny Broth

containing 3% MgSO₄ (LB) (Difco™ LB Agar, Miller, Becton, Dickinson and Company, Sparks, MD) were combined with 3mL of LBM top agar (LB + 0.7% agarose) containing 1% tetrazolium dye (LBMt), and poured over LBM plates. Plates were allowed to solidify uncovered under a biosafety cabinet for 30 min.

3.3.4 Screening Veterinary Clinical Samples for *Salmonella* Bacteriophage

Salmonella enterica-positive tetrathionate (TTh) enrichment broth (Difco™ Tetrathionate Broth Base, Becton, Dickinson and Company, Sparks, MD) grown cultures of clinical veterinary samples obtained from the AUCVM Bacteriology and Mycology Laboratory were screened for *Salmonella*-infecting phages against a panel of sixteen *Salmonella* serovars. TTh broths (~1mL) were pelleted by centrifugation for 2 min at 12,000 x g in an Eppendorf 5415 C centrifuge to remove debris. Supernatants were subsequently sterilized by filtration through 0.45µm syringe filters. Afterwards, 10 µL of filtered, TTh supernatants were spotted onto double agar overlay lawns of sixteen *Salmonella* serovars. After allowing drops to dry uncovered under a biosafety cabinet for 30 min, plates were incubated ON at 37°C. Following incubation, plates were observed for clearings in the bacterial lawns indicative of phage plaques. *Salmonella* serovars on which the filtered, culture supernatants produced the strongest clearings were selected to isolate, amplify, and enumerate phages in the following procedures.

3.3.5 Bacteriophage Isolation

Bacteriophages were isolated by single-plaque propagation described by Serwer *et al.* (2009) with modifications. Briefly, LBM agar plates were stabbed using 1 µL loops dipped in the filtered, TTh culture supernatants that generated clearings on the double-agar overlays of *Salmonella* serovars. Afterwards, melted LMBt (~3mL) combined with log-phase cultures (200µL) of *Salmonella* serovars were poured over stabbed plates and swirled. Plates were allowed

to solidify uncovered under a biosafety cabinet for 30 min and incubated ON at 37°C. For filtered TTh culture supernatants failing to propagate single plaques, phages were isolated using the double agar overlay plaque assay (see below). The next day, isolated plaques from either single plaque propagation or double agar overlay plaque assay were cored using a Pasteur pipette and transferred into Salt Magnesium (SM) buffer (100mM NaCl, 8mM MgSO₄, 50mM Tris-HCl, 0.01% w/v Gelatin) containing 2% chloroform as described by Serwer *et al.* (2009). Cores were incubated at room temperature for ≥ 4 hours to allow phage to diffuse into the SM buffer.

3.3.6 Bacteriophage Enumeration

Bacteriophage were enumerated by the double agar overlay plaque assay described by Kropinski *et al.* (2009b) with modifications. Briefly, ten-fold serial dilutions of phage samples were prepared in SM buffer and 100 μ L of phage dilutions was added to 200 μ L of exponential-phase cultures of *Salmonella* serovars in LB. Phage-cell mixtures were subsequently incubated for 10 minutes at 37°C to allow for phage adsorption. Afterwards, phage-cell mixtures were combined with 3mL of LMBt and poured over pre-warmed (37°C) LBM agar plates in duplicate or triplicate. After solidifying uncovered for 30 min under a biosafety cabinet, plates were incubated ON at 37°C. Following incubation, isolated plaques were counted and titers were calculated from countable plates containing 30-300 plaques.

3.3.7 Preparation of Amplified Bacteriophage Stocks

Bacteriophages were amplified as described by Fortier and Moineau (2009) with modifications. Briefly, 10 μ L of diffused phage core supernatant were added to 12.5 mL of exponential-phase *Salmonella* cultures in LB and incubated ON at 37°C with shaking. Following incubation, phage-*Salmonella* cultures (lysates) were treated with 30 μ L chloroform and pelleted by centrifugation for 15 minutes at 12,000 x *g*. Supernatants were subsequently sterilized by

filtration through 0.2 µm syringe filters. Amplified phage working stocks were stored at 4°C. Stocks for long-term storage in -20°C and -80°C freezers were prepared by addition of DMSO 7% (v/v) to 1 ml of high titer lysates in 2.0 mL cryogenic vials (Nalgene® cryogenic vials, Thermo Fisher Scientific, MA) (Sambrook and Russel, 2001).

3.3.8 Host Range Determination

The lytic activity of SE-targeted phages against *Salmonella* serovars was determined by the spot test described by Kutter (2009). Briefly, ten-fold serial dilutions of amplified phage stocks were prepared in SM buffer and 10 µL of each dilution were spotted onto double agar overlays of sixteen *Salmonella* serovars and the 1993 Jack-in-the-Box outbreak strain of *E. coli* O157:H7. After allowing spots to dry uncovered under a biosafety cabinet for 30min, plates were incubated ON at 37°C. Following incubation, the amount of clearing of bacterial growth within spots was evaluated based on a scoring scheme described by Turner *et al.* (2012). Single-plaque propagation was used to determine phage plaque production on serovars which produced spot clearings.

3.3.9 Efficiency of Plating (EOP)

The efficiency of SE-targeted phages to produce plaques on *Salmonella* serovars relative to the bovine SE isolate was conducted as described by Kutter (2009). Briefly, amplified phage working stocks were diluted in SM buffer to concentrations between 1.0 and 3.0×10^3 PFU/mL and 100µL of dilutions were plated in triplicate on the sixteen *Salmonella* serovars and one *E. coli* O157:H7 strain using the double agar overlay plaque assay. Following incubation, the number of plaques was counted on each plate and EOP values were calculated by dividing the number of plaques produced on a particular bacterial host by the number of plaques produced on the bovine SE isolate. A mixed statistical model for analysis of variance and Scheffe's test for multiple

comparisons were used to detect differences in EOP values. Statistical analysis was performed using SAS version 9.4 (SAS Institute, Cary, NC).

3.3.10 Bacteriophage Concentration and Transmission Electron Microscopy

Bacteriophage lysates were concentrated by polyethylene glycol (PEG) precipitation as described by Carlson (2005) and visualized with transmission electron microscopy as described by Ackermann (2009). Briefly, concentrated phage samples were negatively stained with 2% (w/v) phosphotungstic acid and adjusted to pH 7.0 with KOH. High magnification (45,000X or 71,000X) images were captured using a Philips EM 301 Transmission Electron Microscope (TEM) and edited with ImageJ software.

3.3.11 Lysis Curves

Growth curves of bovine SE in the presence of cocktail phage were performed to compare bacterial lysis at varying multiplicities of infection (MOI). Overnight cultures of bovine SE in LB were diluted in LB to an OD₆₂₀ of 0.5 and added to six 25 mL Erlenmeyer flasks. At time=0 hour, phage stocks were added to flasks in ten-fold multiplicities of infection (MOI) (10^{-4} – 1 phage to cell) along with a control flask to which no phage was added. Flasks were incubated at 37°C with shaking for 6-7 hours and OD readings of each flask were taken hourly.

3.3.12 Adsorption Curves

Adsorption curves were conducted as described by Hyman and Abedon (2009) with modifications. Briefly, $\sim 10^5$ PFU of phage were added to a 125 mL Erlenmeyer flask containing mid-log SE in LB supplemented with 10 mM CaCl₂ (LB_C) diluted to OD₆₅₀ 0.1-0.3 and to a control flask containing LB_C. Both flasks were incubated in a 37°C water bath for ten minutes. Samples were taken from the experimental flask at 1 min intervals, combined with LB_C saturated with chloroform, and thoroughly mixed by vortex followed by centrifugation for 15min at $12,000 \times g$.

Phage in sample supernatants were subsequently enumerated by the double-agar overlay plaque assay (single plate per sample). Cell densities of diluted SE broth cultures in each flask were determined by spread plating on LBM agar plates in triplicate. All plates were incubated ON at 37°C. Following incubation, plaques and colonies on plates were counted. Afterwards, the natural logarithm of plaque counts from each plate were plotted as a function of time and a best-fit line was drawn through the data points. The adsorption rate constant was calculated by dividing the slope of the best-fit line by the bacterial density (CFU/mL) and multiplied by -1 (to remove the negative sign). Correlation coefficients and R^2 values were used to assess curve quality and data linearity, respectively. Adsorption rates of each phage were determined from the results of at least three experimental runs. A general linear model for analysis of variance and Scheffe's test for multiple comparisons were used to test for differences in adsorption rate constants. Statistical analysis was performed using SAS version 9.4 (SAS Institute, Cary, NC).

3.3.13 Bacteriophage Inactivation by Ultra-Violet Irradiation

Inactivation of phage by ultra-violet (UV) radiation was performed using a bench-top scale collimated beam apparatus which was constructed according to recommendations by Bolton and Linden (2003) with a UV disinfection wand as a UV source. UV irradiance was measured using a radiometer. Amplified phage stocks were diluted in SM buffer to a concentration of $\sim 10^9$ PFU/mL and 75 mL of phage dilutions were transferred to a glass Petrie dish. Phage dilutions were exposed to UV radiation at 30 s intervals for 5 min under continuous stirring. 100 μ L samples were collected every 30 s and transferred to 900 μ L SM buffer. After UV exposure, samples were serially diluted in SM buffer and plated using the double agar overlay plaque assay. Plates were incubated ON at 37°C. Inactivation curves were constructed using a model assuming first order inactivation kinetics proposed by Havelaar *et al.* (1990):

$$N / N_0 = e^{kEt}$$

where,

N = phage concentration in samples collected at each time point (PFU/mL)

N_0 = the initial phage concentration prior to UV exposure (PFU/mL)

E = the effective UV irradiance (W/m²)

t = time (s)

k = rate constant (m²/J).

Following incubation, plaques were counted and UV inactivation curves were generated by plotting the natural logarithm of the ratio of plaque counts to initial phage concentration ($\ln N/N_0$) as a function of time (s). UV inactivation rate constants (k) were calculated by dividing the slope of the best of fit line (slope = kEt) by the measured irradiance (E) and total exposure time ($t = 300$ s).

3.4 Results

3.4.1 Phage Typing and PFGE of SE Isolates

SE isolates from various hosts and sources were submitted to the National Veterinary Services Laboratory (Ames, IA) for *Salmonella enterica* Serovar Enteritidis phage typing. Phage types were determined for only five of nine SE isolates and with four isolates failing to conform to established patterns (Table 3.1).

Because four of the nine SE isolates could not be phage typed, pulsed-field gel electrophoresis was performed to differentiate SE isolates based on banding patterns produced from *Xba*I digests of total genomic DNA (Figure 3.1). PFGE analysis revealed three banding patterns among the SE isolates, allowing classification of the isolates into three groups (Table 3.1).

3.4.2 Cocktail Bacteriophages

Bacteriophages vB_SenM-S7 (S7), vB_SenM-S10 (S10), vB_SenM-S13 (S13), vB_SenP-S11 (S11), vB_SenP-S25 (S25), vB_SenS-S56 (S56) and vB_SenS-S57 (S57) were selected from a collection of *Salmonella* phages isolated from *Salmonella*-positive TTh enrichment cultures from clinical veterinary samples. Phages were selected based on their strong lytic activity against wild-type, bovine (Bov) SE isolate, and named according to recommendations by Kropinski *et al.* (2009).

3.4.3 Transmission Electron Microscopy of Bacteriophage

The morphologies of all seven phages were determined by TEM. Examination of electron photomicrographs revealed that phages belonged to three families in the Caudovirales order: three

from Myoviridae (S7, S10 and S13), two from Podoviridae (S11 and S25), and two from Siphoviridae (S56 and S57) (Table 3.2). S7, S10, and S13 phage particles were shown to have non-contractile tails consistent with Myoviridae morphology, and tail fibers resembling the *Salmonella* phage Vi I. (Ackermann 2007). Both S11 and S25 particles were shown to have short, non-contractile tails consistent with Podoviridae morphology with S11 tail fibers similar to *Salmonella* phage P22, and S25 fibers resembling T7 (Ackermann 2007). S56 and S57 phage particles contained long, flexible, non-contractile tails consistent with Siphoviridae tail morphologies, and tail fibers resembling *Salmonella* phage Vi II (Ackermann 2007). The genomes of phages in all three families consists of double-stranded DNA (Ackermann 2007) and all cocktail phages were found to be resistant to chloroform (Table 3.2).

3.4.4 Bacteriophage Spot Lysis Results

Host range determination of cocktail phages by spot lysis test revealed that all seven cocktail phage commonly lysed several of the 16 serovars in my panel. All phages exhibited lytic activities against serovars Agona, Choleraesuis, Dublin, Enteritidis, Heidelberg, Javiana, and Typhimurium. Three phages were found to have additional lytic activities, with S11 producing clearings on double agar overlays of serovars Bardo and Newport, and both S7 and S10 exhibiting lytic activity against the 1993 Jack In The Box® outbreak strain of *E. coli* O157:H7 in hamburger (Table 3.3). Spot lysis tests of cocktail phage on nine clinical veterinary SE isolates revealed that each cocktail phage produces clearings on SE isolates from a variety of hosts (Table 3.4).

3.4.5 Bacteriophage Plaque-formation

The plaque-forming ability of S7, S10, S13, S11, S25, S56, and S57 on *Salmonella* serovars in my panel was determined by single-plaque propagation. All phages produced plaques on *Salmonella* serovars Enteritidis and Javiana and subsets of three or four phages produced plaques

on serovars Choleraesuis, Dublin, Heidelberg, and Typhimurium. Plaque production on serovars Agona, Bardo, and Newport was demonstrated for S11 only. Both phages exhibiting lytic activity against *E. coli* O157:H7 (S7 and S10) in spot lysis tests also produced plaques on this strain. Cocktail phage tended to produce spot lysis and plaques on *Salmonella* serovars belonging to the same serogroup. For instance, each of the seven phages lysed and/or formed plaques on all of the *Salmonella* serovars in groups B (Agona, Heidelberg, and Typhimurium) and C3 (Dublin and Enteritidis) as well as serovar Choleraesuis in group C₁ (Table 3.5). Cocktail phages produced plaques on most of the SE isolates, however no plaques were produced by S11, S56, and S57 on YS and 775 isolates (Table 3.6).

3.4.6 Efficiency of Plating

The efficiency of plating was performed to determine the ratio of plaques produced by phages on *Salmonella* serovars relative to the number produced on the bovine SE isolate. Phages S7, S10, S13, S11 and S25 generated plaques on *Salmonella* serovars in numbers less than, equal to, or greater than the plaque numbers on the bovine SE isolate (Table 3.7). All Myoviridae phage (S7, S10, and S13) generated significantly fewer plaques on serovar Heidelberg than on the bovine SE isolate ($p < 0.0001$). Significantly fewer plaques of both S10 and S13 were produced on serovar Javiana, S10 on Choleraesuis, and S13 on serovar Typhimurium ($p \leq 0.05$). Except for S11 EOP on serovar Javiana, which was not significantly different from bovine SE ($p > 0.05$), Podoviridae phage (S11 and S25) generated fewer plaques on all *Salmonella* serovars than on the bovine SE ($p < 0.0001$). In contrast, Siphoviridae phages (S56 and S57), which only produced plaques on serovars Javiana and Enteritidis, generated approximately 50% more plaques on Javiana than on the bovine SE ($p < 0.0001$) (Table 3.7).

Plating efficiencies for phages were also determined for eight SE isolates relative to the bovine isolate. Most of the phages produced plaque numbers on SE isolates similar to numbers produced on the bovine isolate. However, S13 phage generated significantly fewer plaques on 420, 775, and YS isolates and S7 on isolate 775 ($p \leq 0.05$). In contrast, S25, S56, and S57 produced significantly more plaques on SE isolates 420 and 1660, along with higher plaque numbers of S25 and S56 on isolates 2480 and Av respectively ($p \leq 0.05$) (Table 3.8).

3.4.7 Bacteriophage Lysis of SE Broth Cultures

Growth curves of SE in the presence of phage were conducted to assess the effect of various multiplicities of infection on bacterial lysis in broth and to compare lysis patterns among the phages examined. Myoviridae phages (S7, S10 and S13) exhibited similar lysis patterns to one another with the largest reductions in optical density at 620 nm (OD_{620}) observed for phage-SE MOIs of 10^{-3} and 10^{-4} (Figures 3.2–3.4). Siphoviridae phages (S56 and S57) also produced visually similar lysis patterns with MOIs of 10^{-2} and 10^{-3} reducing OD_{620} to below the starting point after 6.5 hours of incubation (Figures 3.5 and 3.6). However, Podoviridae phages (S11 and S25) produced lysis curve patterns distinct from one another. S11 produced pronounced lysis of bovine SE, with even the lowest MOI (10^{-6}) reducing OD_{620} to below the starting point after only three hours of incubation (Figure 3.7). In contrast, S25 exhibited moderate lysis in broth, with the largest reduction in OD_{620} observed for 10^{-4} MOI at seven hours of incubation. However, the optical density for this MOI was well above the starting OD_{620} (Figure 3.8).

3.4.8 Adsorption Curves of Cocktail Bacteriophage

Adsorption rate constants for S7, S10, S13, S11, S25, S56 and S57 were determined to compare the rates that phage adsorb to the bovine SE isolate and are summarized in Table 3.9. Although no significant differences in adsorption rate constants were found among phage within

the same family, significant differences were observed among families ($p \leq 0.05$). Adsorption rate constants of Myoviridae phage were found to be significantly different from both the Podoviridae and Siphoviridae families ($p \leq 0.05$); however, Siphoviridae and Podoviridae constants were not significantly different.

3.4.9 Bacteriophage Inactivation by Ultraviolet Light

UV inactivation constants for phage are summarized in Table 3.10.

3.5 Discussion

3.5.1 Host Range of *Salmonella* Bacteriophages

The host range of phages is defined as the genera, species, subspecies or strain that a bacterial virus can infect and is a defining characteristic identifying a particular phage (Kutter 2009). Phages are generally thought to infect a narrow range of hosts, often a specific species or strain within a single bacterial genus (Rakhuba *et al.*, 2010). However, phages capable of infecting a wide range of *Salmonella* serovars and multiple genera have been isolated and characterized (Bielke *et al.*, 2007). Similarly, phages in my experiments were shown to collectively produce spot lysis clearings or plaques on nine of the sixteen serovars in my panel and two phages (S7 and S10) demonstrated activity against *E. coli* O157:H7 (Table 3.3). The finding that phages lysed and/ or formed plaques on *Salmonella* belonging to the same serogroup (Table 3.5) is consistent with the well-known association between O antigens and phage susceptibility (Lindberg 1973). It is also worth noting that collectively, the seven phages examined in this study lyse and/or infect five of the most frequently isolated *Salmonella* serovars implicated in human illnesses in the U.S. over the past two decades (Sarwari *et al.*, 2001; Jones *et al.*, 2008; CDC 2009; CDC 2011; Gould *et al.*, 2013; Jackson *et al.*, 2013; Crim *et al.*, 2014; Crim *et al.*, 2015). This finding indicates that serovar-targeted mixtures of phages used to treat food animals have the potential to reduce the number of human salmonellosis cases.

Although the phages in my experiments exhibited lytic activity against most of the *Salmonella* serovars in my panel, they were only able to produce plaques on a smaller subset of

these same serovars (Table 3.5). Myoviridae phages S7, S10, and S13, as well as Podoviridae phage S11 produced plaques on most of the serovars identified in spot tests. However, Siphoviridae phages S56 and S57 generated plaques on only two of the eight clearing-positive serovars in spot lysis tests. These observations likely are a consequence of abortive infection, in which the host bacterial cells undergo programmed cell death upon binding of lytic phages to the cellular surface (Kutter 2009), or “lysis from without” in which binding of large numbers (e.g. MOI = 200) of phages to the bacterial cell induces swelling, resulting in lysis (Delbrück 1940a; Visconti 1953). In both of these circumstances, the death of the host bacterium occurs before phage successfully replicates and releases the progeny required for plaque formation (Abedon and Yin 2009). The challenge presented by the abortive infection and lysis from without phenomena is a practical one – serovars on which a phage does not form plaques cannot be used to titer that phage, a necessary pre-requisite for employing that phage in an animal treatment protocol. Thus, phages in these experiments exhibit considerable antimicrobial activity despite the inability to complete successful lytic cycles in particular serovars.

3.5.2 Relative Efficiency of Plating

Efficiencies of plating were determined for each of the seven phages against my *Salmonella* panel in order to assess a phage’s predilection for particular serovars. The efficiency of plating is determined by calculating the titer a phage produces on a particular bacterial strain and dividing it by the maximum titer produced on a phage’s preferred host or indicator strain, and thus is always relative to the bacterial strain producing the maximum titer for the phage in question (Kutter 2009). In these experiments, however, I calculated EOPs for all phages relative to the bovine SE isolate used in my calf infection and treatment models so that meaningful comparisons could be made, although a single preferred host could not be identified for each of the seven phages (Tables 3.7

and 3.8). Among *Salmonella* serovars in my panel, S25 produced the largest plaque numbers on the bovine SE isolate and S56 and S57 produced the most plaques on *S. Javiana* (Table 3.7). However, Myoviridae phages (S7, S10, and S13) did not exhibit a preference for the bovine SE isolate, both Typhimurium strains, and serovar Dublin, except for S13, which produced significantly fewer plaques ($p < 0.05$) on wild type *S. Typhimurium* (Table 3.7). Among SE isolates, S56 and S57 demonstrated a clear preference for PFGE pattern B isolates and PT 9a (Table 3.8). However, other meaningful associations between EOPs of different phage types or PFGE patterns were not observed.

3.5.3 Adsorption Rate Constants

Bacteriophage growth in broth cultures of a suitable bacterial host can be divided into three phases: 1) phage adsorption to the surface of a suitable host and injection of viral genetic material, 2) utilization of host cellular machinery to produce phage progeny, and 3) lysis of the host cell and release of phage progeny to infect other cells (Shao and Wang, 2008). The adsorption of phage to its host involves the recognition of surface molecules or phage receptors by tail fibers and other phage appendages (Katsura 1983; Goldberg *et al.*, 1994). Considering that the maximum yield of phage progeny per bacterial cell has been shown to be dependent on the rate that phage adsorb to cells (Delbrück 1940a), adsorption curves for S7, S10, S13, S11, S25, S56, and S57 on the bovine SE isolate were conducted to compare phages within the same family and between phage families and used as an exclusion criterion for cocktail optimization. No significant differences were found among the adsorption rate constants of phages within the same family ($p > 0.05$), and Myoviridae adsorption rates were significantly lower than either Podoviridae or Siphoviridae phages ($p < 0.05$) (Table 3.9). Siphoviridae phage had the largest adsorption rate constants, although these were not significantly different from Podoviridae constants ($p > 0.05$).

3.5.4 Lysis Curves

Lysis curves were conducted for each phage in this study by comparing OD₆₂₀ of SE broth cultures with phage to control growth of the bovine SE isolate without phage addition. These curves were used to qualitatively examine the contribution of MOI to a phage's antimicrobial activity against bovine SE in broth, and to compare lysis curve patterns among phages in the same family and between phage families. The largest differences between the OD₆₂₀ of negative control cultures and phage-containing SE cultures after 6-7 hours of incubation were found for MOIs in between the lowest and highest phage-to-cell ratios, suggesting that an ideal MOI is required for maximum lysis in bovine SE broth cultures.

Visual similarities were observed for lysis curve patterns among morphologically similar phage within the same families. I observed similar lysis curve patterns among all phage in the Myoviridae family (S7, S10, and S13) which bear close similarity to the *Salmonella* phage Vi I (Figures 3.2–3.4), and both Siphoviridae phages (S56 and S57; Figures 5 and 6) which resemble Vi II (Ackermann 2007). In contrast, the two Podoviridae phages (S11 and S25), which differ in tail fiber morphology (Table 3.2), produced distinct lysis curve patterns (Figures 3.7 and 3.8). These results suggest that morphologically similar phages within the same family can exhibit similar antimicrobial activities against the bovine SE isolate and this may allow us to exclude one of my Myoviridae phage and one of my Siphoviridae in the optimized phage cocktail.

3.5.5 Optimization of SE Bacteriophage Cocktail

The phage characterization experiments were conducted in order to provide distinguishing information useful for cocktail optimization. Except for cocktail phage in the Podoviridae family, the similarity of host ranges, lysis curve patterns, and adsorption rate constants among phages in the same family suggests redundancy among cocktail phages in the Myoviridae and Siphoviridae

families. Although the largest adsorption rate constants were found for Siphoviridae and Podoviridae families (Table 3.9), Myoviridae phages exhibited lytic activity and plaque-forming ability on a wider range of *Salmonella* serovars in my panel (Table 3.5). In light of these results, I decided to include one phage from each family. Although similar findings were observed for Myoviridae phage (S7, S10, and S13), S7 was selected based on slightly higher (although not statistically significant) EOPs on *Salmonella* serovars in my panel and its lytic activity against *E. coli* O157:H7 (Table 3.7). A high degree of similarity was also observed for Siphoviridae phages (S56 and S57), however, additional analysis of Siphoviridae EOPs, S56 was found to produce significantly more ($p \leq 0.05$) plaques on SE isolates than S57 (Table 3.8 and data not shown) and was selected for inclusion in the cocktail. Although several differences were found among the characteristics of Podoviridae phages (S11 and S25), S11 was selected based on stronger lysis of bovine SE broth cultures (Figures 3.7 and 3.9), and larger host range (Table 3.5).

3.6 Chapter 3 References

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3.7 Tables and Figures

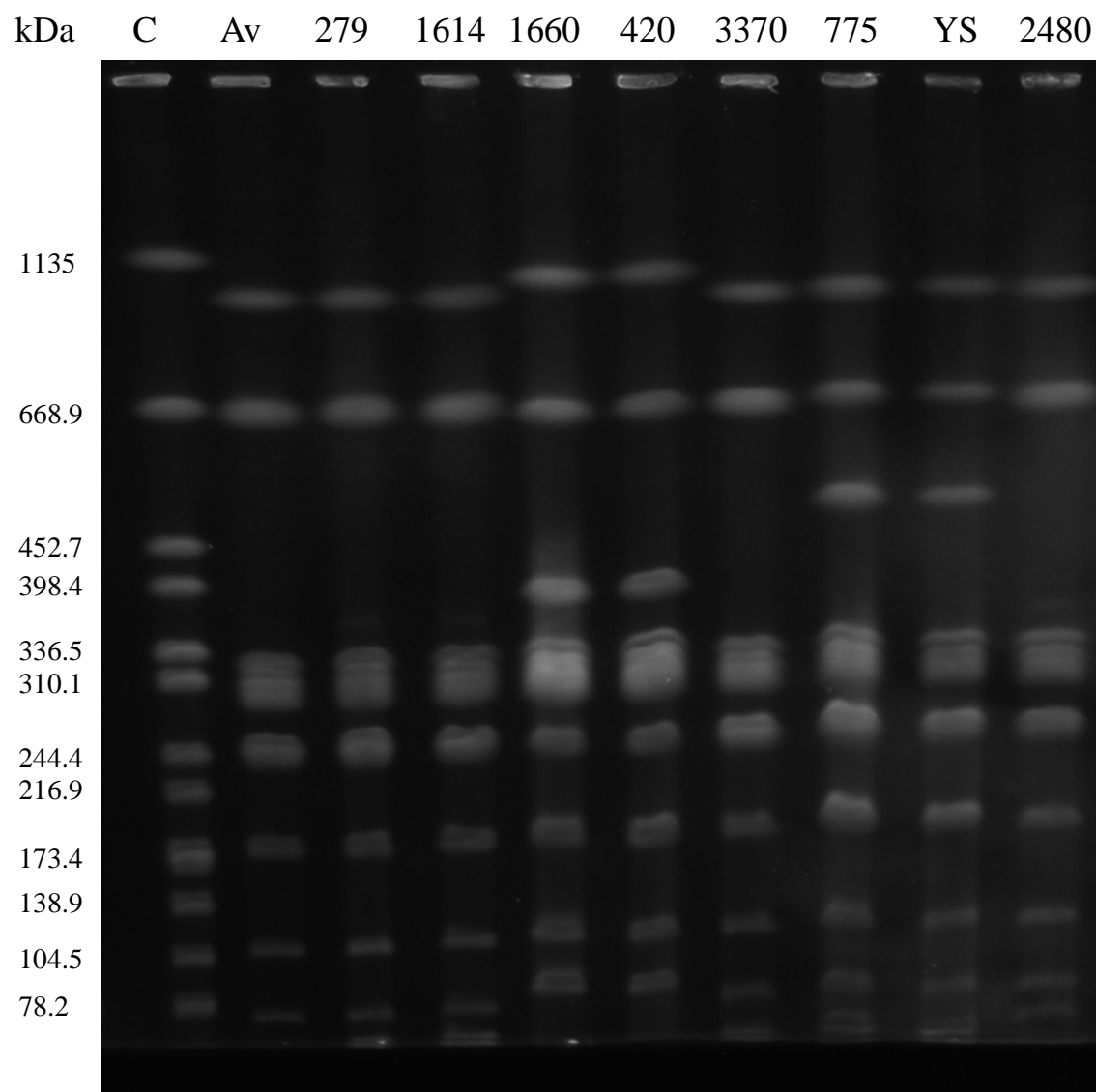


Figure 3.1. Pulsed-field gel electrophoresis of SE isolates. *Xba*I digests of plugs were resolved in 1.2% SeaKem® Gold Agarose gel and stained with GelRed™. Molecular weight standards (C) correspond to *Xba*I digest of *S. Branderup* H9812.

Table 3.1. Phage Typing and PFGE of SE Isolates

Isolate	Host / Source	Phage Type ^a	PFGE Group ^b
Bov	Bovine	RDNC ^c	A
Av	Avian	8	A
279	Equine	RDNC	A
2480	Ovine	RDNC	A
3370	Feline	8	A
420	Equine	9a	B
1660	Canine	RDNC	B
YS	Chicken Egg	13	C
775	Equine	13a	C

^a National Veterinary Diagnostic Lab.

^b Pulsed-field Gel Electrophoresis (Preliminary Results).

^c Reaction Does Not Conform.

Table 3.2. Transmission Electron Microscopy of *Salmonella* Bacteriophage

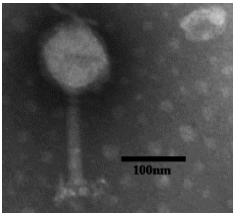
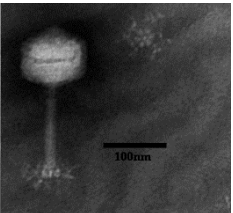
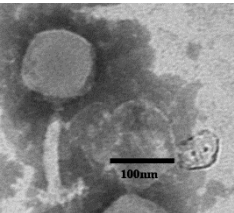
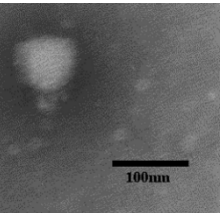
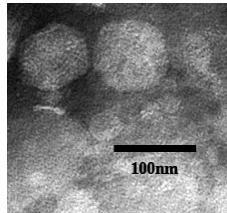
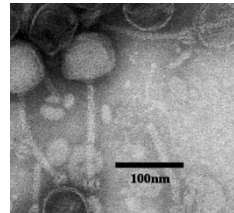
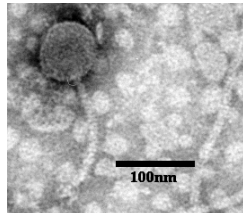
	Bacteriophage						
	vB_SenM-S7	vB_SenM-S10	vB_SenM-S13	vB_SenP-S11	vB_SenP-S25	vB_SenS-S56	vB_SenS-S57
TEM Micrograph							
Family	Myoviridae	Myoviridae	Myoviridae	Podoviridae	Podoviridae	Siphoviridae	Siphoviridae
Nucleic Acid	dsDNA	dsDNA	dsDNA	dsDNA	dsDNA	dsDNA	dsDNA
Chloroform	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant
Similarity	Vi I-like virus	Vi I-like virus	Vi I-like virus	P22-like virus	T7-like virus	Vi II-like virus	Vi II-like virus
Tail Type	Contractile	Contractile	Contractile	Non-Contractile	Non-Contractile	Non-Contractile	Non-Contractile

Table 3.3. Bacteriophage Spot Test on *Salmonella* Serovars

Serovar	Bacteriophage						
	S7	S10	S13	S11	S25	S56	S57
<i>S. Agona</i>	2 ^a	2	2	4	2	3	2
<i>S. Anatum</i>	0	0	0	0	0	0	0
<i>S. Bardo</i>	0	0	0	1	0	0	0
<i>S. Choleraesuis</i>	3	3	3	4	2	4	4
<i>S. Dublin</i>	3	3	3	4	3	4	3
<i>S. Enteritidis</i>	4	4	4	4	3	4	4
<i>S. Heidelberg</i>	3	3	3	3	3	3	3
<i>S. Javiana</i>	4	4	4	3	3	3	3
<i>S. Melagris</i>	0	0	0	0	0	0	0
<i>S. Montevideo</i>	0	0	0	0	0	0	0
<i>S. Muenster</i>	0	0	0	0	0	0	0
<i>S. Kentucky</i>	0	0	0	0	0	0	0
<i>S. Newport</i>	0	0	0	4	0	0	0
<i>S. Typhimurium</i>	4	3	3	4	3	3	2
<i>S. Uganda</i>	0	0	0	0	0	0	0
<i>S. Worthington</i>	0	0	0	0	0	0	0
<i>S. Typhimurium</i> DT104	4	3	3	4	3	4	4
<i>E. coli</i> EK250	4	4	0	0	0	0	0

^a Spot clearings were scored according to a scoring scheme described by Turner *et al.* (2012): 4=confluent lysis, 3=slight turbidity, 2=heavy turbidity, 1=individual plaques, 0=no lysis.

Table 3.4. Bacteriophage Spot Test on SE Isolates

SE Isolate	Bacteriophage						
	S7	S10	S13	S11	S25	S56	S57
Av	3 ^a	4	3	4	3	4	4
Bov	4	4	4	4	3	4	4
YS	4	3	3	3	3	4	4
279	4	3	3	4	3	4	4
420	4	3	3	3	3	4	4
775	4	4	4	3	3	4	4
1660	4	3	3	4	3	4	4
2480	4	3	4	3	3	4	4
3370	4	3	3	4	3	4	4

^a Spot clearings were scored according to a scoring scheme described by Turner *et al.* (2012): 4=confluent lysis, 3=slight turbidity, 2=heavy turbidity, 1=individual plaques, 0=no lysis.

Table 3.5. Bacteriophage Lytic Activity and Plaque Formation on *Salmonella* Serovars

Serovar	Serogroup	Bacteriophage						
		S7	S10	S13	S11	S25	S56	S57
<i>S. Agona</i>	B	(+) ^a	(+)	(+)	+ ^b	(+)	(+)	(+)
<i>S. Heidelberg</i>	B	+	+	+	(+)	+	(+)	(+)
<i>S. Typhimurium</i>	B	+	+	+	+	(+)	(+)	(+)
<i>S. Typhimurium</i> DT104	B	+	+	+	+	(+)	(+)	(+)
<i>S. Choleraesuis</i>	C ₁	+	+	+	+	(+)	(+)	(+)
<i>S. Montevideo</i>	C ₁	- ^c	-	-	-	-	-	-
<i>S. Bardo</i>	C ₂	-	-	-	+	-	-	-
<i>S. Kentucky</i>	C ₂	-	-	-	-	-	-	-
<i>S. Newport</i>	C ₂	-	-	-	+	-	-	-
<i>S. Dublin</i>	C3	+	+	+	+	+	(+)	(+)
<i>S. Enteritidis</i>	C3	+	+	+	+	+	+	+
<i>S. Javiana</i>	C3	+	+	+	+	+	+	+
<i>S. Anatum</i>	E ₁	-	-	-	-	-	-	-
<i>S. Melagrisdis</i>	E ₁	-	-	-	-	-	-	-
<i>S. Muenster</i>	E ₁	-	-	-	-	-	-	-
<i>S. Uganda</i>	E ₁	-	-	-	-	-	-	-
<i>S. Worthington</i>	G	-	-	-	-	-	-	-
<i>E. coli</i> EK250	N/A	+	+	-	-	-	-	-

^a Lysis without plaque formation^b Plaque formation.^c No lytic activity.

Table 3.6. Bacteriophage Lytic Activity and Plaque Formation on SE Isolates

SE Isolate	Bacteriophage						
	S7	S10	S13	S11	S25	S56	S57
Av	^a	+	+	+	+	+	+
Bov	+	+	+	+	+	+	+
YS	+	+	+	(^b)	+	(+)	(+)
279	+	+	+	+	+	+	+
420	+	+	+	+	+	+	+
775	+	+	+	(+)	+	(+)	(+)
1660	+	+	+	+	+	+	+
2480	+	+	+	+	+	+	+
3370	+	+	+	+	+	+	+

^a Plaque formation

^b Lysis without plaque formation

Table 3.7. Efficiency of Plating (EOP) on *Salmonella* Serovars Relative to Bov SE Isolate

Serovar	Bacteriophage						
	S7	S10	S13	S11	S25	S56	S57
<i>S. Enteritidis</i> (Bov)	1.00 ^a	1.00	1.00	1.00	1.00	1.00	1.00
<i>S. Agona</i>	- ^b	-	-	0.09*	-	-	-
<i>S. Bardo</i>	-	-	-	<0.01*	-	-	-
<i>S. Choleraesuis</i>	0.96	0.73*	0.88	<0.01*	-	-	-
<i>S. Dublin</i>	1.16	0.92	0.96	0.74*	0.51*	-	-
<i>S. Heidelberg</i>	0.26*	0.10*	0.10*	-	0.04*	-	-
<i>S. Javiana</i>	0.92	0.65*	0.77*	1.08	0.76*	1.58*	1.54*
<i>S. Newport</i>	-	-	-	0.15*	-	-	-
<i>S. Typhimurium</i>	1.08	0.86	0.81*	0.09*	-	-	-
<i>S. Typhimurium</i> DT104	0.96	0.87	1.06	0.12*	-	-	-
<i>E. coli</i> EK250	0.04*	0.01*	-	-	-	-	-

^a EOP = (average #plaques)/(Bov average #plaques).

^b Does not form plaques.

*EOP is significantly different from EOP on Bov SE ($p \leq 0.05$)

Table 3.8. Efficiency of Plating (EOP) on SE Isolates Relative to Bov Isolate

Isolate	PT ^a	Group ^b	Bacteriophage						
			S7	S10	S13	S11	S25	S56	S57
Bov	RDNC ^c	A	1.00 ^d	1.00	1.00	1.00	1.00	1.00	1.00
Av	8	A	1.02	1.00	0.99	0.99	1.07	1.69*	1.15
3370	8	A	0.92	0.94	1.04	0.92	1.06	1.16	1.04
279	RDNC	A	0.99	1.01	1.12	0.93	1.12	1.29	1.05
2480	RDNC	A	0.91	0.97	1.10	0.97	1.22*	1.11	0.93
420	9a	B	0.99	0.99	0.70*	0.97	1.17*	2.81*	1.95*
1660	RDNC	B	0.97	1.08	0.96	0.99	1.17*	2.06*	1.81*
YS	13	C	1.14	1.01	0.83*	- ^e	1.01	-	-
775	13a	C	0.81*	0.85	0.81*	-	1.05	-	-

^a National Veterinary Diagnostic Lab.

^b Pulsed-field Gel Electrophoresis (Preliminary Results).

^c Reaction Does Not Conform

^d EOP = (average #plaques)/(Bov average #plaques).

^e No plaque formation

*EOP is significantly different from EOP on Bov SE ($p \leq 0.05$)

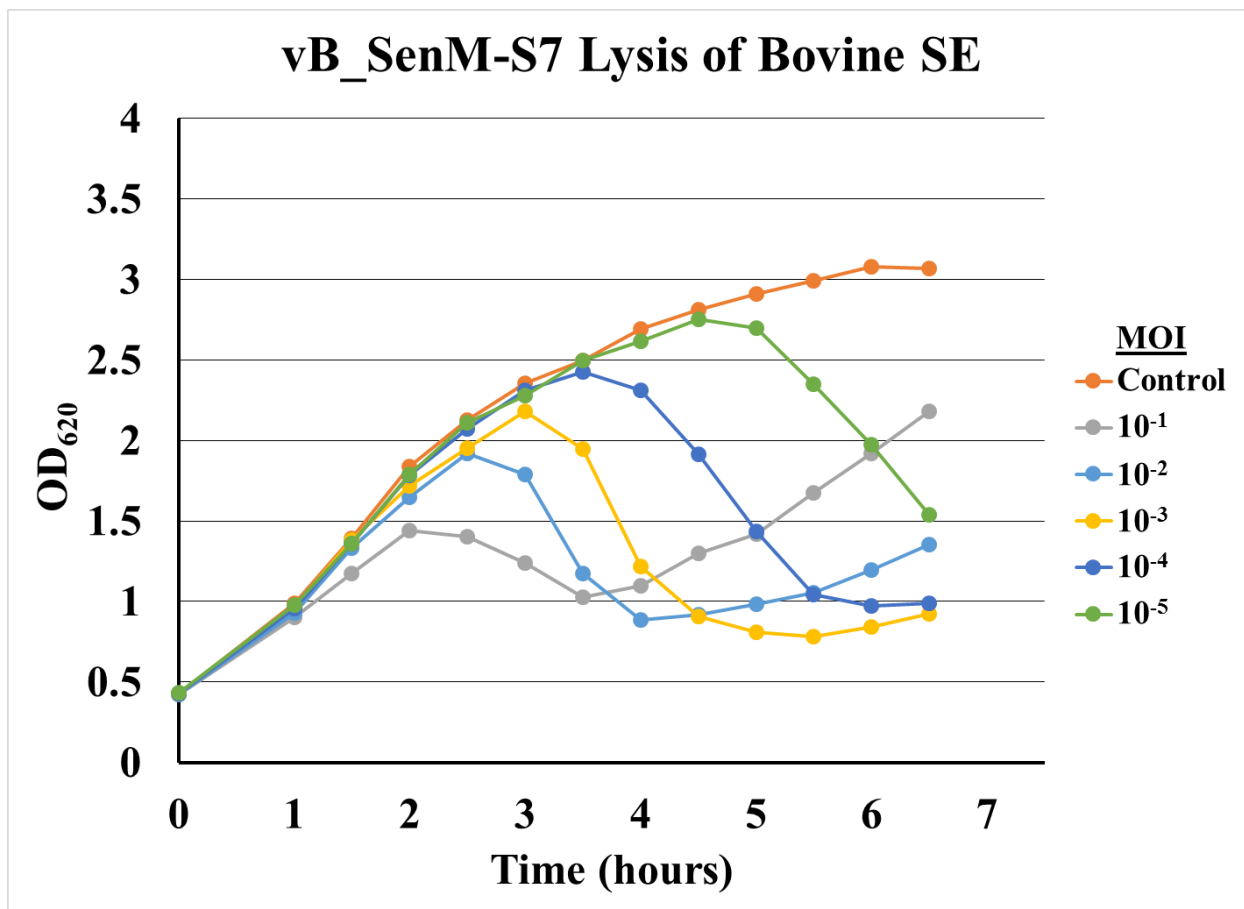


Figure 3.2. S7 lysis of bovine SE LB broth cultures. Optical densities at 620 nm (OD_{620}) were determined for phage to SE MOIs ranging from 10^{-1} to 10^{-5} in addition to a control culture to which no phage was added. OD_{620} readings were collected for 6.5 hours.

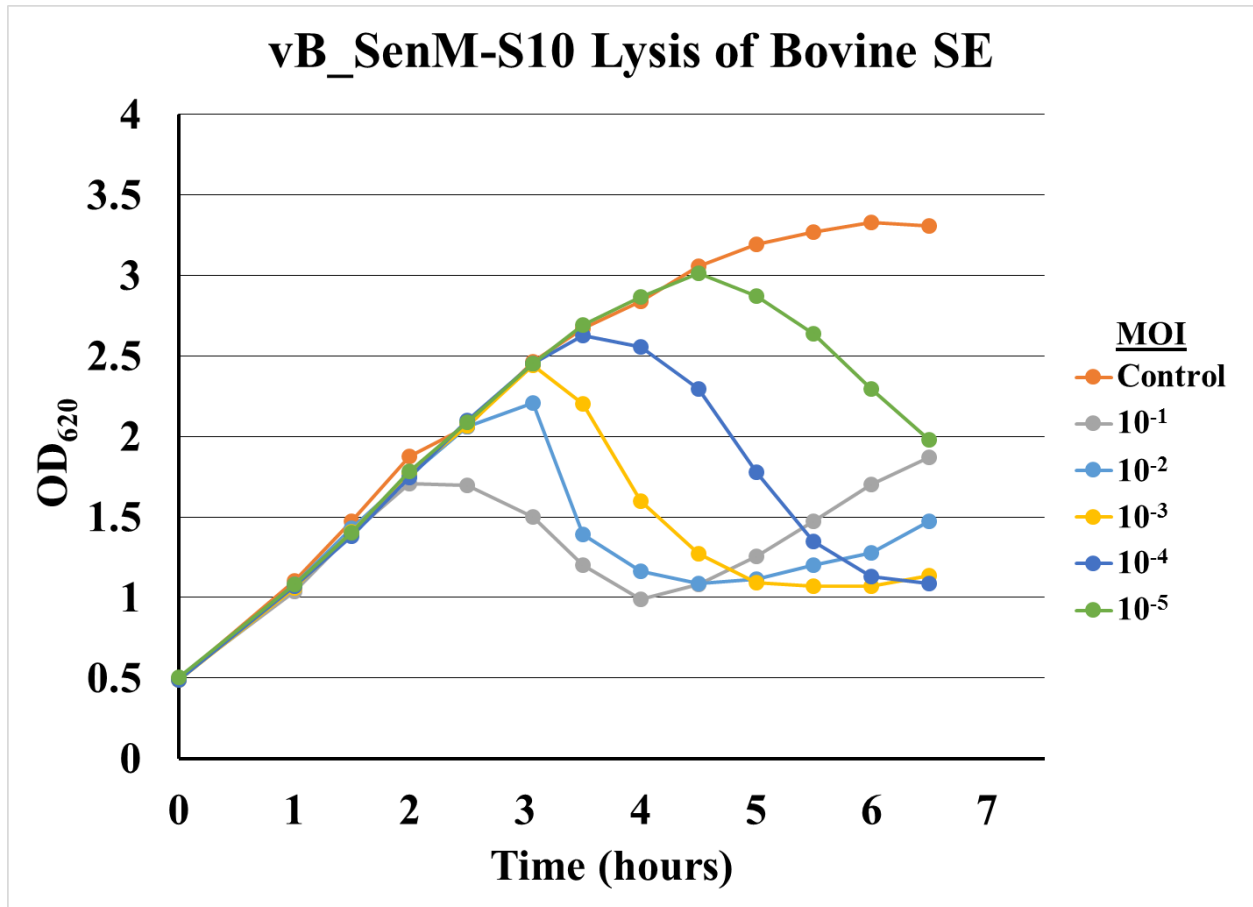


Figure 3.3. S10 lysis of bovine SE LB broth cultures. Optical densities at 620 nm (OD_{620}) were determined for phage to SE MOIs ranging from 10^{-1} to 10^{-5} in addition to a control culture to which no phage was added. OD_{620} readings were collected for 6.5 hours.

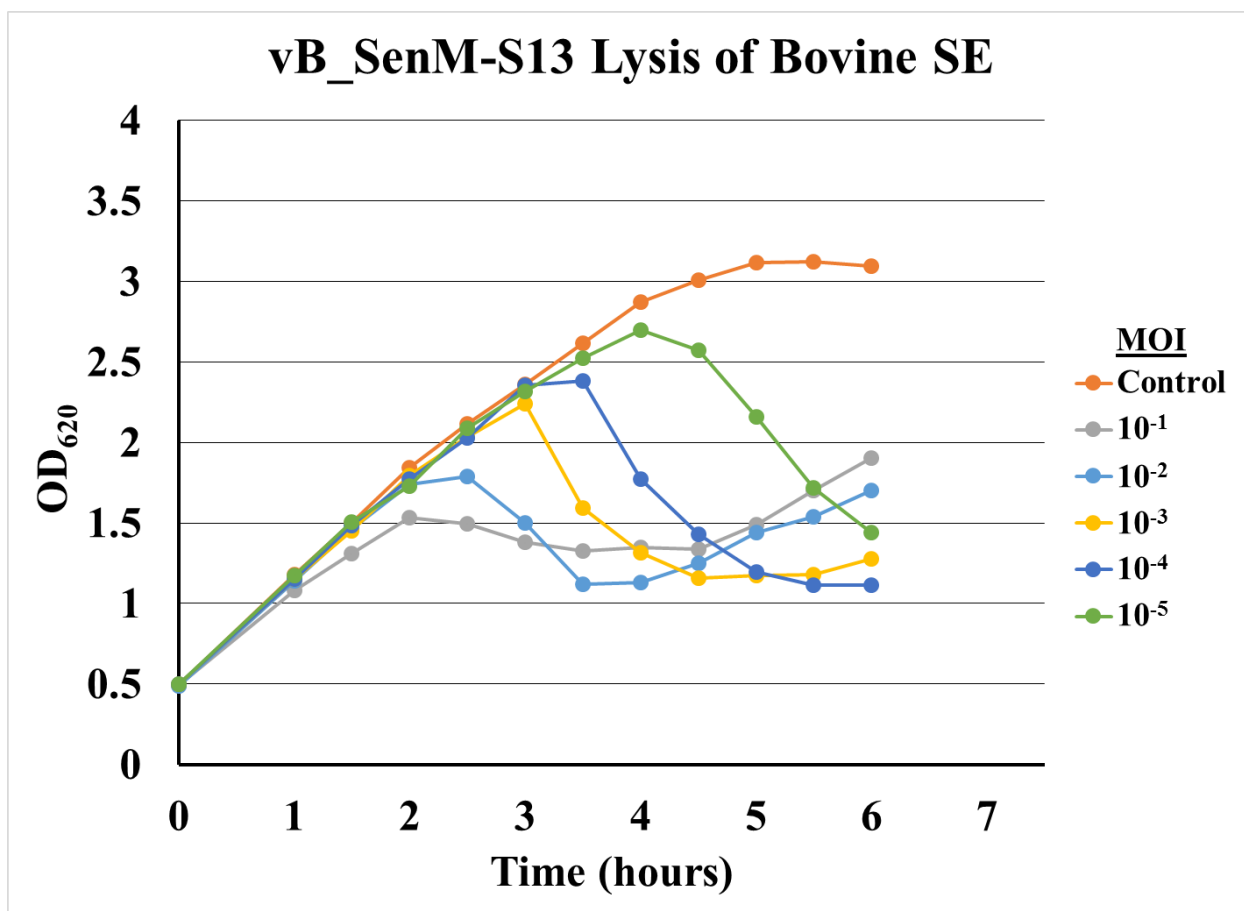


Figure 3.4. S13 lysis of bovine SE LB broth cultures. Optical densities at 620 nm (OD_{620}) were determined for phage to SE MOIs ranging from 10^{-1} to 10^{-5} in addition to a control culture to which no phage was added. OD_{620} readings were collected for 6.0 hours.

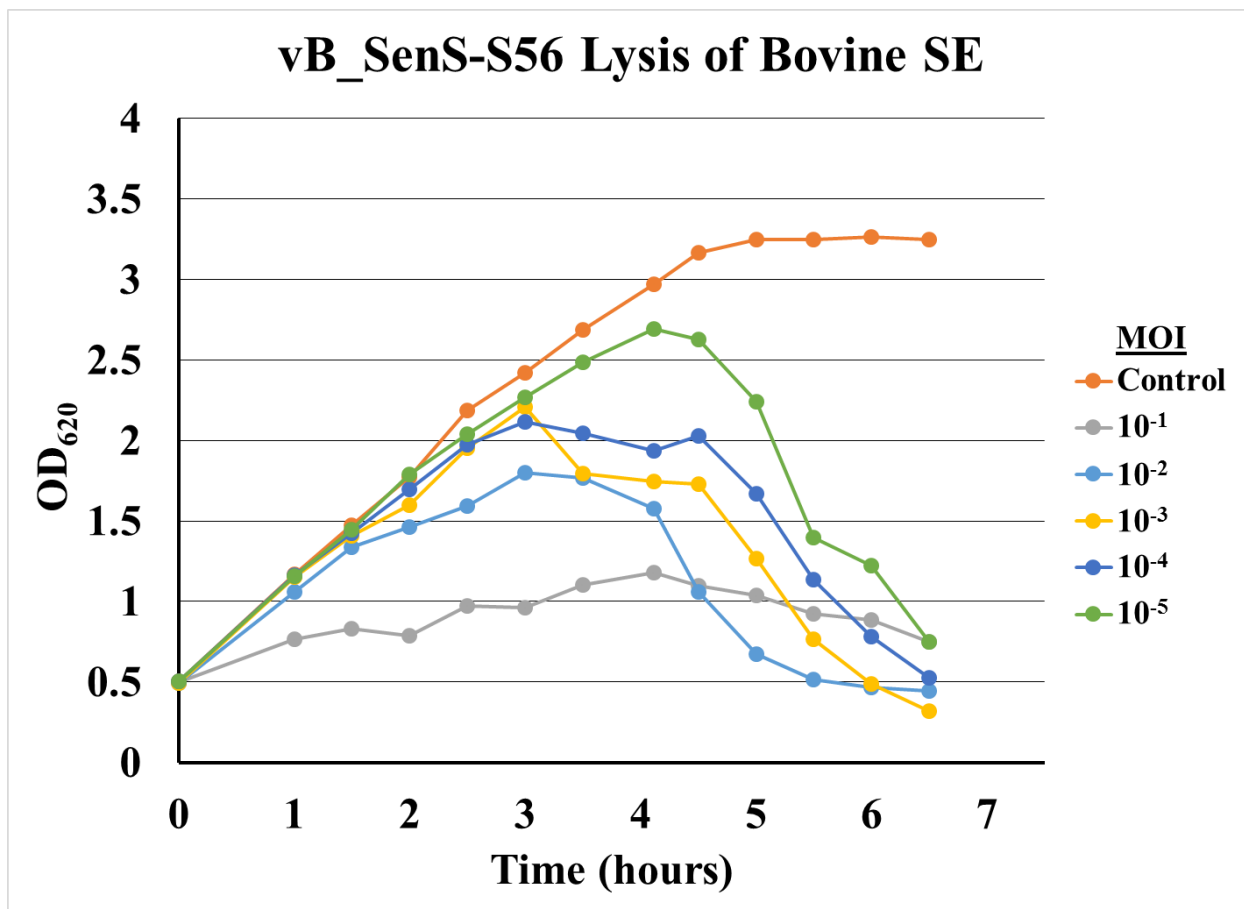


Figure 3.5. S56 lysis of bovine SE LB broth cultures. Optical densities at 620 nm (OD_{620}) were determined for phage to SE MOIs ranging from 10^{-1} to 10^{-5} in addition to a control culture to which no phage was added. OD_{620} readings were collected for 6.5 hours.

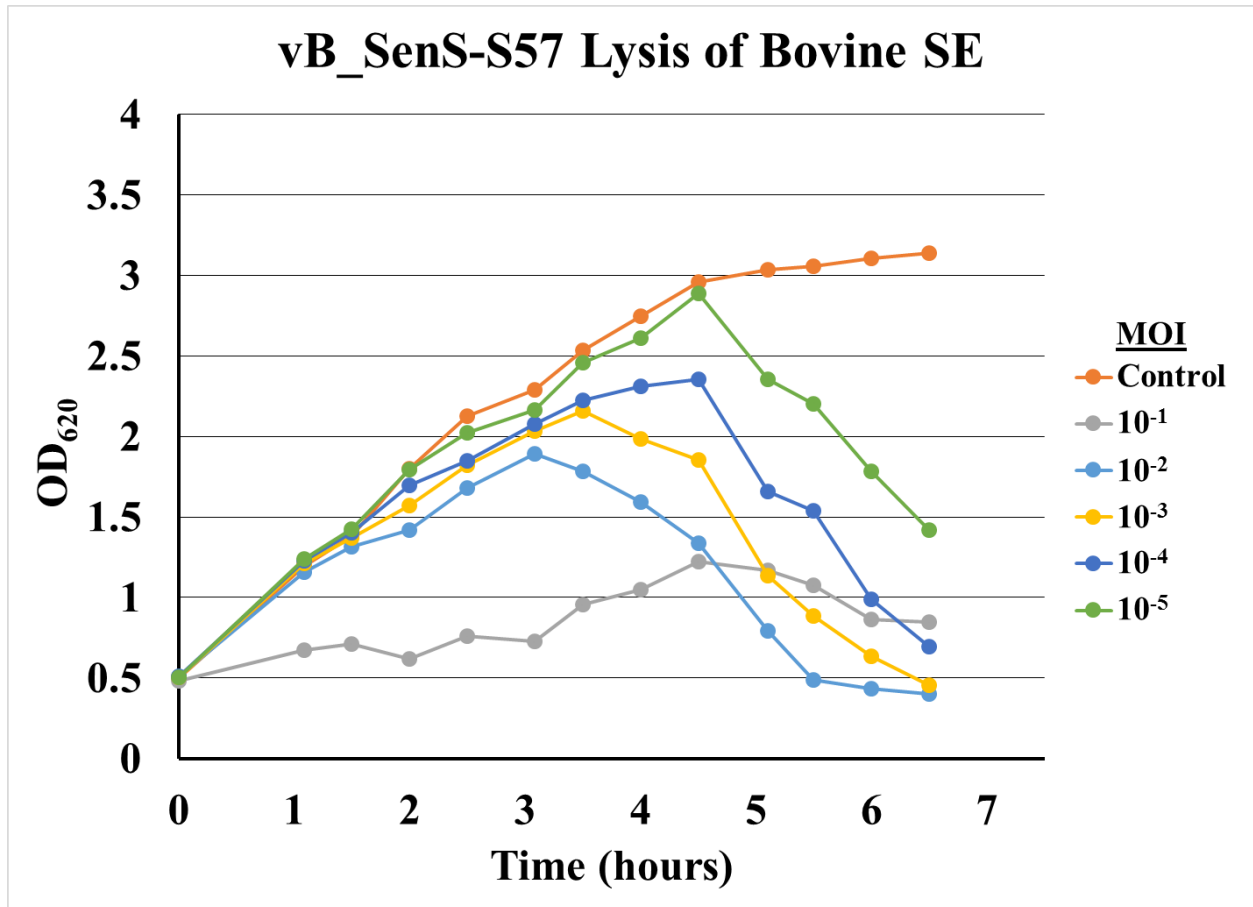


Figure 3.6. S57 lysis of bovine SE LB broth cultures. Optical densities at 620 nm (OD_{620}) were determined for phage to SE MOIs ranging from 10^{-1} to 10^{-5} in addition to a control culture to which no phage was added. OD_{620} readings were collected for 6.5 hours.

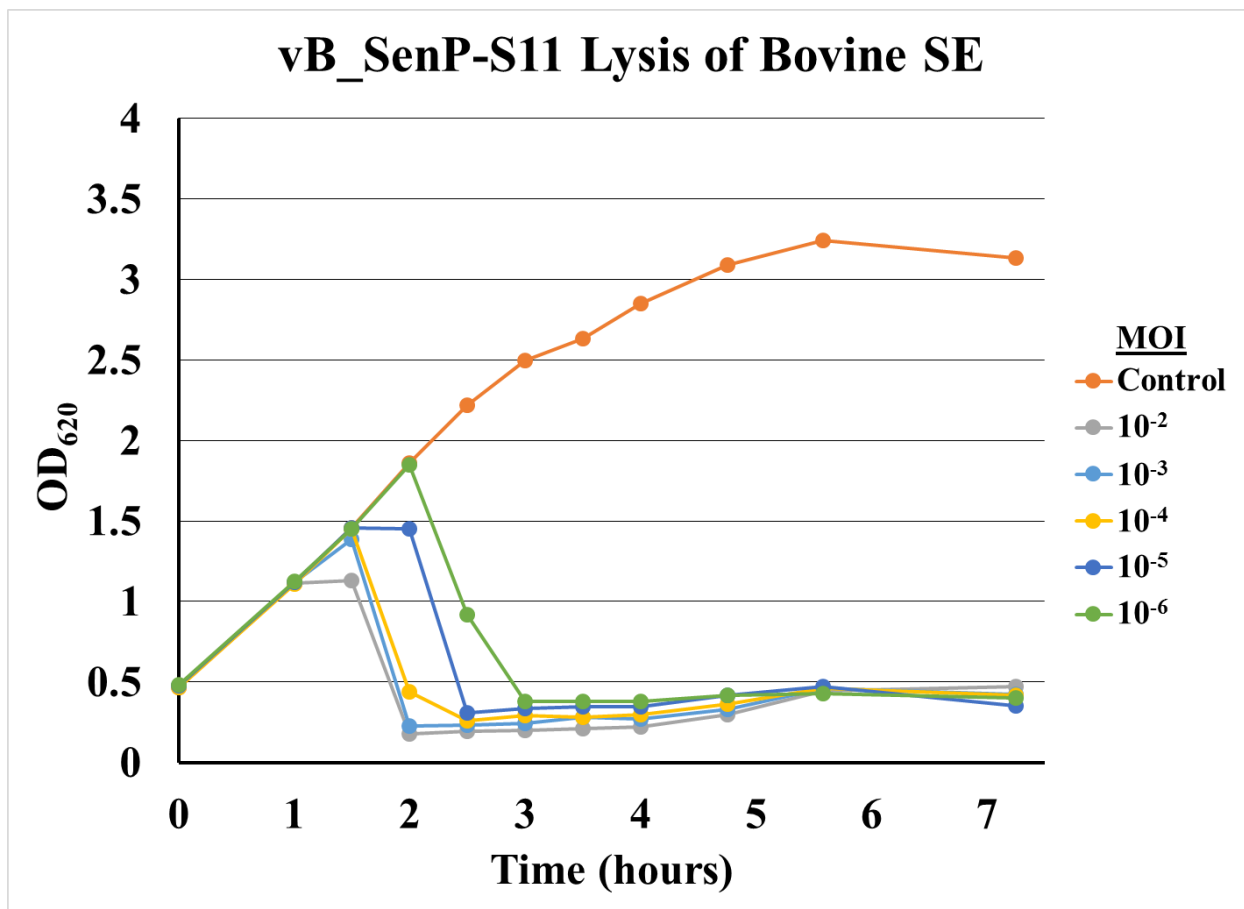


Figure 3.7. S11 lysis of bovine SE LB broth cultures. Optical densities at 620 nm (OD_{620}) were determined for phage to SE MOIs ranging from 10^{-2} to 10^{-6} in addition to a control culture to which no phage was added. OD_{620} readings were collected for 7.0 hours.

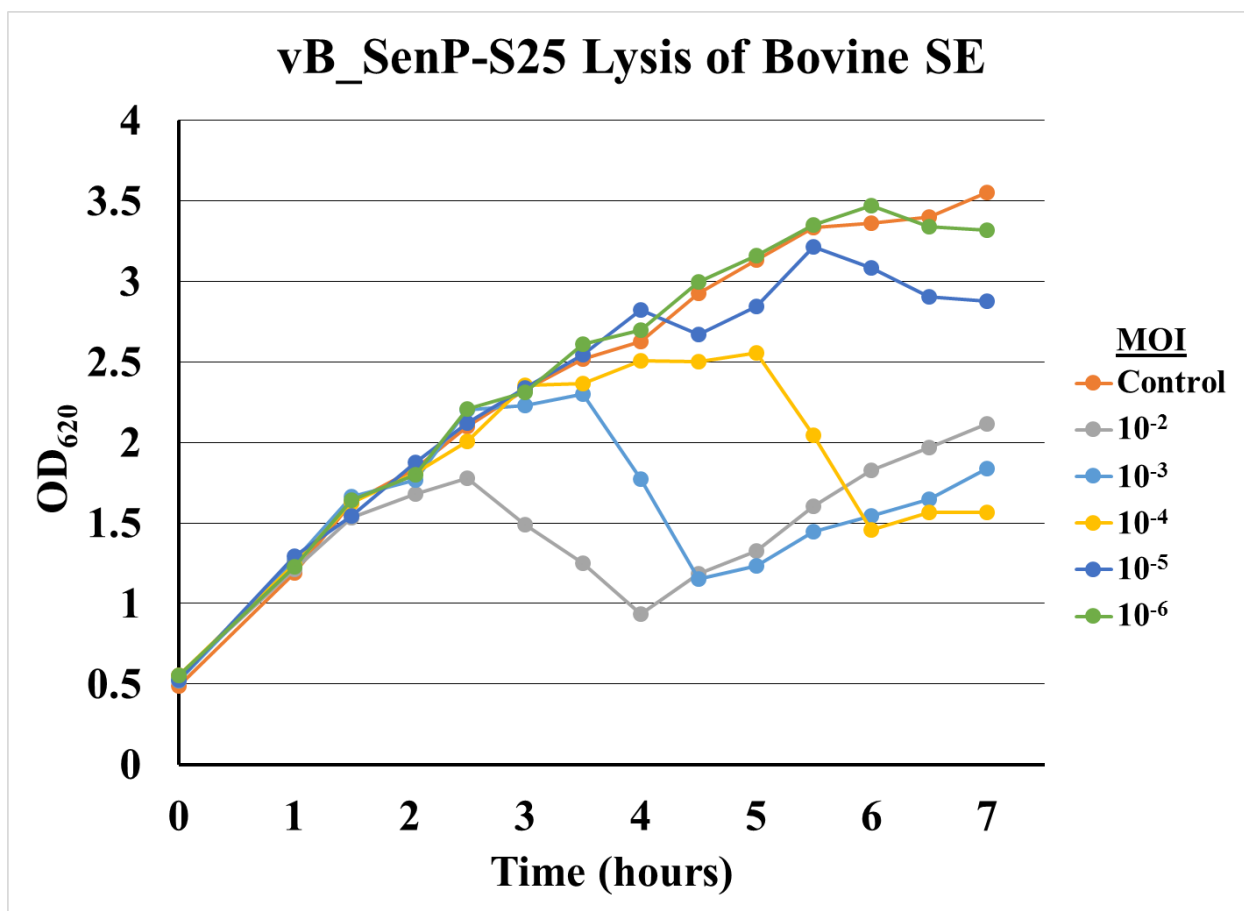


Figure 3.8. S25 lysis of bovine SE LB broth cultures. Optical densities at 620 nm (OD_{620}) were determined for phage to SE MOIs ranging from 10^{-2} to 10^{-6} in addition to a control culture to which no phage was added. OD_{620} readings were collected for 7.0 hours.

Table 3.9. Phage Adsorption Rate Constants

Phage	Family	k (mL/min) ^a
S7	Myoviridae	6.1×10^{-9}
S10	Myoviridae	7.4×10^{-9}
S13	Myoviridae	5.8×10^{-9}
S11	Podoviridae	9.4×10^{-9}
S25	Podoviridae	9.4×10^{-9}
S56	Siphoviridae	1.3×10^{-8}
S57	Siphoviridae	1.0×10^{-8}

^a Average of three runs.

Table 3.10: Phage UV Inactivation Constants

Phage	Family	Fluence (J/m ²)	k (m ² /J)	# Runs
S7	Myoviridae	64.49	4.2×10^{-4}	4
S10	Myoviridae	64.49	4.4×10^{-4}	1
S13	Myoviridae	64.49	1.7×10^{-4}	1
S11	Podoviridae	64.49	2.4×10^{-4}	1
S25	Podoviridae	64.49	7.8×10^{-4}	1
S56	Siphoviridae	64.49	7.4×10^{-5}	1
S57	Siphoviridae	64.49	7.8×10^{-5}	1

Chapter 4. Discussion

4.1 *S. Enteritidis* Disease in Calves

In the work described in this thesis, I show that SE causes disease and disseminates to peripheral lymph nodes in five to seven week-old calves and that oral administration of a phage cocktail has the potential to reduce disease signs, fecal shedding, and peripheral lymph node contamination in infected calves. To my knowledge, this is the first study to demonstrate SE disease and peripheral lymph node carriage in experimentally infected calves. Calves inoculated with a high dose of a bovine SE isolate ($\sim 10^{10}$) exhibited variable fecal shedding and disease outcomes, similar to previous observations in calves naturally infected with SE (Petrie *et al.*, 1977) and other serovars (Cummings *et al.*, 2009), as well as in calves experimentally infected with doses of *S. Typhimurium* similar to doses used in this work (de Jong and Ekdahl, 1965). Although several factors may play a role in the disease severity of *Salmonella*-infected calves (Santos *et al.*, 2001), it is my opinion that the severe disease observed in three of the calves in my experiments is the result of the high challenge dose.

4.2 Recovery of *S. Enteritidis* from the Peripheral Lymph Nodes

Previous studies have demonstrated that a large dose is necessary to reliably recover *Salmonella* from the peripheral lymph nodes (de Jong and Ekdahl, 1965; Edrington *et al.*, 2013b; Brown *et al.*, 2015a). In my experiments, I recovered my experimental SE isolate from the peripheral lymph nodes of calves with severe, lethal disease similar to previous findings in *S. Typhimurium* infected calves (de Jong and Ekdahl, 1965). Although it is tempting to infer from

these results that peripheral lymph node carriage is mediated by severe systemic disease involving bacteremia, the condition of these animals does not necessarily reflect animals at slaughter. Moreover, *Salmonella* was isolated from the peripheral lymph nodes of healthy, adult cattle in previous reports (Arthur *et al.*, 2008; Brichta-Harhay *et al.*, 2012; Haneklaus *et al.*, 2012; Gragg *et al.*, 2013a, 2013b;) suggesting that severe disease is not required for *Salmonella* peripheral lymph node carriage in cattle at slaughter. Instead, peripheral lymph node carriage in cattle likely results from repeated exposure to lower doses of *Salmonella*, such as those cattle would encounter in feedlots (Brown *et al.*, 2015b).

4.3 Bacteriophage Treatment

Many studies have explored phage treatment as a pre-harvest intervention strategy to reduce *E. coli* in cattle (Rozema *et al.*, 2009; Stanford *et al.*, 2010) and previous work has shown that cocktails of several phages are more effective than single phage treatments in reducing *E. coli* O157:H7 in cattle (Tanji *et al.*, 2004; Tanji *et al.*, 2005; Rozema *et al.*, 2009). However, to my knowledge, this work is the first to explore the potential of phage treatment to reduce *Salmonella* in cattle. In my experiments, I treated a pair of experimentally SE-infected calves with a cocktail of seven lytic phages targeting SE. Although only one calf (T1) treated with phages survived experimental infection with *Salmonella*, the fecal shedding and disease of phage-treated calf T2 was particularly severe and likely beyond treatment, similar to observations in phage treatment of calves experimentally inoculated with *E. coli* (Smith and Huggins, 1983). Nevertheless, I found that phage-treated calf T1 exhibited the mildest disease and lowest fecal *Salmonella* shedding among the calves in these experiments despite receiving the highest challenge dose. This finding suggests that oral phage therapy has the potential to reduce disease signs and fecal shedding in

experimentally infected calves and warrants future experiments involving phage treatment of experimentally infected calves to confirm the results in this calf.

4.3.1 Phage Cocktails and Phage Resistance

The success of phage cocktails over single phage treatments is likely due to the ability of phage cocktails to control or prevent the emergence of phage resistant bacteria (Tanji *et al.*, 2004; Callaway *et al.*, 2008). Successful infection of a suitable host bacterium requires the adsorption of phage to bacterial surface molecule or phage receptors (Katsura 1983; Goldberg *et al.*, 1994; Tanji *et al.*, 2004; Skurnik and Strauch, 2006; Shao and Wang, 2008). Alteration of these receptors resulting from spontaneous mutations confers resistance of the host bacterium to phage adsorption and subsequent infection (Levin and Bull, 2004; Tanji *et al.*, 2004; Callaway 2008). Moreover, phages may utilize common receptors of the host bacterium. Thus, if two different phages bind to the same receptor, alteration of this surface molecule would confer resistance to both phages (Tanji *et al.*, 2004). However, phages are known to attach to a range of bacterial surface molecules such as lipopolysaccharide, outer membrane proteins, capsule and flagella (Levin and Bull, 2004). Thus, the use of cocktail containing phages that recognize different receptors may mitigate, or perhaps prevent, the emergence of phage resistant bacteria (Carvalho *et al.*, 2012). Although I did not identify the phage receptors in my *in vitro* characterization of my seven cocktail phages, the slight variations in their host ranges and the differences of the Siphoviridae adsorption rate constants from Podoviridae and Myoviridae suggest that phages in my experiments attach to different SE surface molecules and this observation warrants future work elucidating the receptors of these phage.

4.3.2 Phage Treatment to Reduce *S. Enteritidis* Carriage in PLN

A growing body of evidence has documented *Salmonella* carriage in bovine peripheral lymph nodes (Arthur *et al.*, 2008; Brichta-Harhay *et al.*, 2012; Koohmaraie *et al.*, 2012; Haneklaus *et al.*, 2012; Gragg *et al.*, 2013a, 2013b; Edrington *et al.*, 2013a; Vipham *et al.*, 2015) and the vast majority of serotypes isolated from the peripheral lymph nodes (Arthur *et al.*, 2008; Ayala *et al.*, 2013; Gragg *et al.*, 2013a, 2013b) have also been isolated from ground beef (Bosilevac *et al.*, 2009). However, only two studies so far have investigated approaches to reduce *Salmonella* in these sites (Edrington *et al.*, 2013b; Vipham *et al.*, 2015) and, to date, nothing has been published on the use of oral phage treatment to reduce *Salmonella* carriage in peripheral lymph nodes. In my phage-treated calves, phages were recovered from two SE-positive peripheral lymph nodes of Calf T2, whereas neither SE nor phage were found in peripheral lymph nodes of Calf T1. The ability to invade and replicate intracellularly, avoid host immune defenses, and cause disseminated disease, are key features of *Salmonella* pathogenicity, irrespective of serovar (Mohler *et al.*, 2009), and such an escape of *Salmonella* into intracellular environments is a potential challenge facing phage therapy of intracellular pathogens (Skurnik and Strauch, 2006). Nevertheless, phage have been shown to rapidly distribute to systemic tissues and organs in vertebrates, and are also capable of disseminating to organs such as the prostate gland, bone, and brain, which are often inaccessible to drugs (Dabrowska *et al.*, 2005). Phage may also infect pathogens prior to intracellular invasion and thereby reduce intracellular bacteria similar to the concept of a Trojan horse (Broxmeyer *et al.*, 2002; Skurnik and Strauch, 2006). Thus recovery of phage from the peripheral lymph nodes of an experimentally infected calf suggests that phage treatment can penetrate the PLN and may reduce *Salmonella* in systemic sites.

Although neither *Salmonella* nor phage were isolated from the lymph nodes of Calf T1, fecal *Salmonella* shedding for this calf was the lowest among all of the calves in this study despite receiving the highest challenge dose. Future experiments in SE infected calves are needed to confirm peripheral lymph node penetration and the reduction of fecal shedding by my phage cocktail.

4.3.3 Fecal Shedding of Bacteriophage

An advantage of phage treatment to reduce pathogens in preharvest cattle is that phages are shed in the feces of treated animals. Previous studies have documented that *E. coli*-infected calves treated with phage shed phage in their feces (Smith and Huggins, 1983; Smith and Huggins, 1987b; Rozema *et al.*, 2009; Stanford *et al.*, 2010). In my study, Calf T1 shed $\sim 10^4$ PFU/g of phages in the feces for days 2-4 post-treatment (Figure 2.5), which is considerably lower than phage sheds in *E. coli*-infected calves and were reported to be $10^6 - 10^{10}$ PFU/g after oral treatment (Smith and Huggins, 1983; Smith *et al.*, 1987b; Rozema *et al.*, 2009). Nevertheless, shedding of phage may have advantages extending beyond pathogen control in cattle at slaughter. In previous work examining the prevalence of both *E. coli* O157:H7 and phages in feedlots, it was found that *E. coli* O157:H7 prevalence was inversely proportional to prevalence of its infecting phages (Oot *et al.*, 2007; Niu *et al.*, 2009). Also, in a study designed to recapitulate feedlot conditions, Rozema *et al.* (2009) suggested that *E. coli* O157:H7-infecting phage can be transmitted among feedlot cattle after detecting phage in the feces of control calves two days after phage administration to the treatment group. Isolation of phage from control cattle environments was also reported in a study examining microencapsulated phage to reduce *E. coli* O157:H7 (Stanford *et al.*, 2010). Similarly, in a study involving phage treatment of *S. Newport*-infected calves, I were able to detect phages in feces from an untreated calf less than 24 h after treatment of a co-housed calf (Hyland

et al., 2016). The transmission of phages among cattle in herds suggests that sufficient control of pathogens could be accomplished by administering phage to bedding, feed, or water troughs without having to administer phage to each individual animal (Rozema *et al.*, 2009). Fecal shedding of phage may also serve to reduce pathogen loads of cattle hides which are considered to be the principle sources of carcass contamination in processing plants (Barkocy-Gallagher *et al.*, 2003; Koohmaraie *et al.*, 2005; Brichta-Harhay *et al.*, 2008; Koohmaraie *et al.*, 2012; Schmidt *et al.*, 2015) in addition to reducing pathogen levels in lairage environments. In my study, Calf T1 shed phage concomitantly with SE in the feces for nine days after initial treatment (Figure 2.5). Taken together, these findings warrant future work investigating the potential for phage treatment to limit environmental deposition and transmission of *Salmonella* among feedlot cattle.

4.4 Bacteriophage Characterization

Bacteriophages are generally thought to infect a narrow range of hosts, often a specific species or strain within a single bacterial genus (Rakhuba *et al.*, 2010), and this had been mentioned as one of the disadvantages of phage therapy (Greer 2005). However, phages capable of infecting a wide range of *Salmonella* serovars and multiple bacterial genera have been isolated and characterized (Bielke *et al.*, 2007). Similarly, phages in my experiments were shown to produce lytic activity against nine of the sixteen *Salmonella* serovars in my panel and against an *E. coli* O157:H7 strain. I also demonstrated that phages in cocktail lyse and/or infect five of the most frequently isolated *Salmonella* serovars implicated in human illnesses in the U.S over the past two decades (Sarwari *et al.*, 2001; Jones *et al.*, 2008; CDC 2009; CDC 2011; Gould *et al.*, 2013; Jackson *et al.*, 2013; Crim *et al.*, 2014; Crim *et al.*, 2015). This finding alone validates the remarkable utility of phage to control *Salmonella* in food and emphasizes the value of future phage therapy work.

Due to the constraints of evaluating the safety and efficacy of seven phages in a calf animal model, phage characterization experiments were conducted in order to provide distinguishing information useful to reduce the cocktail to three phages. Except for cocktail phages in the Podoviridae family, the similarity of host ranges, lysis curve patterns, and adsorption rate constants among phages in the same family suggests redundancy among cocktail phages in the Myoviridae and Siphoviridae families. Although the highest adsorption rate constants were found for my cocktail phages in the Siphoviridae and Podoviridae families, Myoviridae phages exhibited lytic activity and plaque-forming ability on a wider range of *Salmonella* serovars in my panel. In light of these results, I decided to include one phage from each family. Although similar findings were observed for Myoviridae phages (S7, S10, and S13), S7 was selected based on slightly higher (although not statistically significant) EOPs for *Salmonella* serovars in my panel and its lytic activity against *E. coli* O157:H7. A high degree of similarity was also observed for Siphoviridae phages (S56 and S57). However, additional analysis of Siphoviridae EOPs showed that S56 produced significantly more ($p \leq 0.05$) plaques on SE isolates than S57 and therefore was selected for inclusion in the cocktail. Although several differences were found among the characteristics of the Podoviridae phages (S11 and S25), S11 was selected based on its stronger lysis of bovine SE broth cultures, and broader host range.

4.5 Chapter 4 References

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