IDENTIFYING AND CHARACTERIZING BACTERIOPHAGES CAPABLE OF INACTIVATING *Salmonella* ISOLATED FROM ENVIRONMENTAL SAMPLES AS A PRE-HARVEST ANTIMICROBIAL INTERVENTION

A Dissertation

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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December 2018

Major Subject: Animal Science

ABSTRACT

Bacteriophages previously found in the feedlot environment may play a role in the ecology of *Salmonella* in the feedlot environment and also prove useful as a means of controlling this pathogen in beef. The ability of a phage to infect and lyse the target bacterial strain is generally agreed to be a basic requirement for successful phage therapy. The objective of this work was to 1) determine phage host range and virulence in a microtiter-plate based liquid assay using the features of an automated plate reader to monitor culture optical density over time in an incubating, aerated environment; 2) perform phage characterizations, including morphological identification, growth kinetics, genomic analysis and 3) conduct antimicrobial efficacy testing of phages in *ex-vivo* models and study relationship between phage efficiency in bacterial reduction and phage characterization.

Host range scores obtained by two methods were compared to each other and to results from a study using phages to decontaminate cattle hides inoculated with *S.* Anatum, in order to determine the ability of the two host range methods to predict antimicrobial efficacy of phages in an *ex vivo* model. The host ranges of the tested phages were highly variable, ranging from infecting 10% to 85% of the tested *Salmonella* strains. Phage Melville was found to have the broadest host range, capable of infecting 85% (17/20) in both methods. Results obtained by the microtiter plate liquid method were found to have higher discriminatory power between bacterial strains. The ability of phages to reduce *Salmonella* loads on cattle hides were correlated with the results obtained by the microtiter plate method developed in this study but not with the traditional agar overlay method, implying that the microtiter method is a more sensitive predictor

of antimicrobial capacity of phages compared to traditional agar overlay methods. The microtiter plate liquid assay could potentially serve as a more advanced alternative of characterizing phages that yields data on both host range and virulence. Bacteriophage capable of significantly reduce *Salmonella* population in cattle hide and soil proved the possible function as an intervention against *Salmonella* to prevent pathogen transmission in feedlot environment and colonization of cattle lymph nodes.

ACKNOWLEDGEMENTS

To my committee chair Dr. Gill, and my committee members, Dr. Taylor, Dr. Gehring and Dr. Lawhon, thank you for your guidance and assistant throughout my Ph.D program at Texas A&M A&M. Special appreciation and thanks to Dr. Gill, my advisor - you have been a tremendous mentor to me, thanks for encouraging and challenging me during my research as well as giving me the flexibility that allowed me to enjoy science. Without the direction and support from my committee every step along the way, I will never make it this far.

To my fellow graduate students, thank you for assistance and companionship. It was you that I spent the majority of my graduate student life with in the lab alongside me. Special thanks to Abby Korn and Denish Piya, my ultimate lab mates – it was always you to whom that I looked to for help.

To my families and friends, words would never be able to express my gratitude. Thank you for your encouragement and support in all my pursuit. To my grandparents, your prayers was the reason why I stayed strong and encouraged. To my parents, it was always you who supported me emotionally and financially and encouraged me to explore every possibility in my life. To my best friend Yang Su, thanks for your support during our 13-years friendship.

Last, to my newly wedded husband, Kevin (Qingquan Chen), I could never image how colorless my graduate student life would be without you. Thank you for all of the support and love throughout these years.

CONTRIBUTORS AND FUNDING SOURCES

This work was supported by a dissertation committee consisting of Drs. Jason Gill (chair), Matthew Taylor, Kerri Gehring of the Department of Animal Science and Dr. Sara Lawhon of the Department of Veterinary Pathobiology.

Cattle hides used for this research were obtained from Rosenthal Meat Science and Technology Center and supported by North American Meat Institute (NAMI). Hide puncher device was provided by Passport Food Safety Solutions (W. Des Moines, IA). Genomic sequencing of bacteriophage sequencing was supported by Dr. H. Morgan Scott's lab from the Department of Veterinary Pathobiology and bioinformatic analysis was supported by Center for Phage Technology at Texas A&M University. Genomic annotation of phage Sw2 was conducted by Quang Xuan Nguyen. Genomic annotation of phage Munch was conducted by Stephen Crosby and Chandler O'Leary.

Graduate research was financially supported by Texas A&M AgriLife Research, National Cattlemen's Beef Association and Texas Beef Council.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Salmonella and Salmonellosis

Bacteria of the genus *Salmonella* are facultatively anaerobic, Gram-negative, non spore-forming rod-shaped enteric bacteria belonging to the family *Enterobacteriaceae* [1]. Although *Salmonella* spp. are generally motile with peritrichous flagella, non-flagellated and non-motile strains with dysfunctional flagella do exist [2]. *Salmonella* are able to utilize a wide range of organic compounds and metabolize nutrients via respiratory and fermentative pathways [2]. Isolates generally catabolize glucose with production of acid and gas. Most strains are unable to ferment lactose, though several mutants that have ability to utilize lactose have been identified [3]. *Salmonella* are oxidase negative, catalase positive, indole negative, Voges-Proskauer negative, methyl red positive, and are able to grow on citrate as a sole carbon source [1]. They produce hydrogen sulfide, decarboxylate lysine and ornithine, and do not produce urease [2].

The genus *Salmonella* is divided into two species: *S. enterica* and *S. bongori*, with *S. enterica* divided into six subspecies (*enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, and *indica*) [4]. The majority of human *Salmonella* infections are caused by *S. enterica* subsp. *enterica* [1]. Subspecies are divided into more than 2,600 serotypes based on their serological reaction to somatic lipopolysaccharide (O), flagellar (H) and capsular antigens according to the Kauffmann-White Scheme [4]. Ninety-nine percent of serotypes were found within the species *S. enterica*, of which 59% belong to subspecies *enterica*. The Kauffmann-White Scheme for

Salmonella serotyping is maintained by World Health Organization (WHO) Collaborating Center for Reference and Research on *Salmonella* at Institut Pasteur, Paris, France [5].

Salmonella are mesophilic and can grow within a temperature range of 5.2 to 46.2 °C, with the optimal temperatures for growth from 35 to 43 °C [6]. Many factors may enhance heat resistance of *Salmonella*: low water activity, high fatty acid content, and sub-lethal stimulation could increase the heat resistance of this organism [2]. Freezing can be detrimental to *Salmonella,* but it does not guarantee destruction [2]. A 0.2 $\log_{10}$ CFU/ml reduction of *S.* Typhimurium was observed on chicken carcasses during crust freezing [7]. However, *Salmonella* are able to survive longer under frozen conditions compared to ambient and refrigeration temperature [6]. The optimal water activity (a_w) for the growth of *Salmonella* is 0.99 and the minimal a_w limit is 0.93. *Salmonella* are capable to survive for months in foods with low a_w such as nuts and spices [6]. *Salmonella* can grow in a broad pH range from 3.8 to 9.5, with an optimum pH range for growth of 7.0 to 7.5. However, the capacity for growth in extreme pH condition is dependent on other factors such as the presence of chemicals, temperature and a_w [2].

Salmonellosis is a human gastrointestinal (GI) illness caused by exposure to non-typhoidal *Salmonella* spp. Outcomes of non-typhoidal *Salmonella* infection can range from asymptomatic colonization of the GI tract to acute gastroenteritis, septicemia, bacteremia or chronic conditions such as arthritis [8]. The pathogenesis of *Salmonella* is complex and the severity displayed during infections in human is variable depending on genotype involved and health status of individuals [1,9]. After the organism is ingested via a cross-contaminated food, the organism multiplies in the small intestine, colonizing and invading the intestinal tissue,

producing a heat-stable enterotoxin that causes an inflammatory reaction [1]. The inflammatory reaction causes symptoms typical of gastroenteritis including watery diarrhea, persistent and spiking fever, abdominal pain, headache, nausea, prostration, and a rash of rose-colored spots on the shoulders, thorax, or abdomen [10]. *Salmonella* could also achieve internalization via type III secretion system [10]. Approximately 5% of individuals with gastroenteritis caused by non-typhoidal *Salmonella* infection develop bacteremia, which occurs when *Salmonella* spp. enter the bloodstream [8]. Bacteremia is associated with fever greater than 39 ^{o}C, lethargy, abdominal and chest pain, chills and anorexia [1]. Reactive arthritis, a chronic sequelum, may occur after *Salmonella* infection and is estimated to happen in 12 cases per 1,000 *Salmonella* infections [11]. The incubation period for typical *Salmonella* infection is 6 to 72 hours and illness generally lasts 2 to 7 days [1,8]. The infectious dose was reported large (10^5 – 10^{10}) in volunteer study, but data from outbreaks indicated that the infectious dose could be as low as a few cells [1,12]. Factors that affect the determination of infectious dose include immunological heterogeneity within human populations, virulence of infecting strains and chemical composition of contaminated food [2]. Infants, the elderly, and immunocompromised individuals are more susceptible to *Salmonella* infections than adults [2]. Foods with high fat content are more likely to be associated with low-dose *Salmonella* infection. Organisms may be protected against the bactericidal action of gastric acidity by being encapsulated within hydrophobic lipid micelles [2].

Epidemiological and Economic Impacts of *Salmonella*

Salmonella is a leading cause of bacterial foodborne illness in the United States. Foodborne illnesses caused by *Salmonella* are estimated to be more than 1.2 million each year in

the United States, with more than 23,000 hospitalizations and 450 deaths [13]. During 2017, 24,484 foodborne infections were identified by culture-confirmed or culture-independent diagnostic tests (CIDs) via the FoodNet network [14]. The second most common cause of foodborne bacterial illness identified by both culture or CIDs, *Salmonella* accounted for 8,172 infections (34% of total foodborne infections), 2,255 hospitalizations (28% of *Salmonella* infections) and 40 deaths (0.5% of *Salmonella* infections) were identified through FoodNet during 2016 [15]. The incidence of salmonellosis increased from 14.51 per 100,000 population in 2016 to 16 in 2017, and it has remained stable with minor fluctuation over the long term from 2000 (Figure 1) [13,14,16-18] .

During 2009 to 2015, *Salmonella* was the leading cause of bacterial foodborne illness outbreaks in the United States, with 896 outbreaks (30%) out of 2,442 outbreaks identified. Outbreaks caused by *Salmonella* resulted in 23,663 illnesses, 3,168 hospitalizations (65% of total hospitalizations associated with outbreaks) and 29 deaths [19]. Among the top 5 pathogen-food category pairs resulting in outbreak-associated illness, *Salmonella* was associated with 4 pairs, including eggs, seeded vegetables, chicken and pork [19]. In addition, *Salmonella* caused the largest multistate foodborne illness outbreak among 177 cases reported, in which 1,939 persons were infected in 10 states beginning in 2010 [19,20].

Figure 2 from the United States Department of Agriculture (USDA) Economic Research Service (ERS) indicates that *Salmonella* imposes the greatest cost of 14 major foodborne pathogens investigated in the United States [21,22]. In 2010, the estimated annual economic cost of illness caused by *Salmonella* was $3.4 billion. This estimate is for all cases of salmonellosis

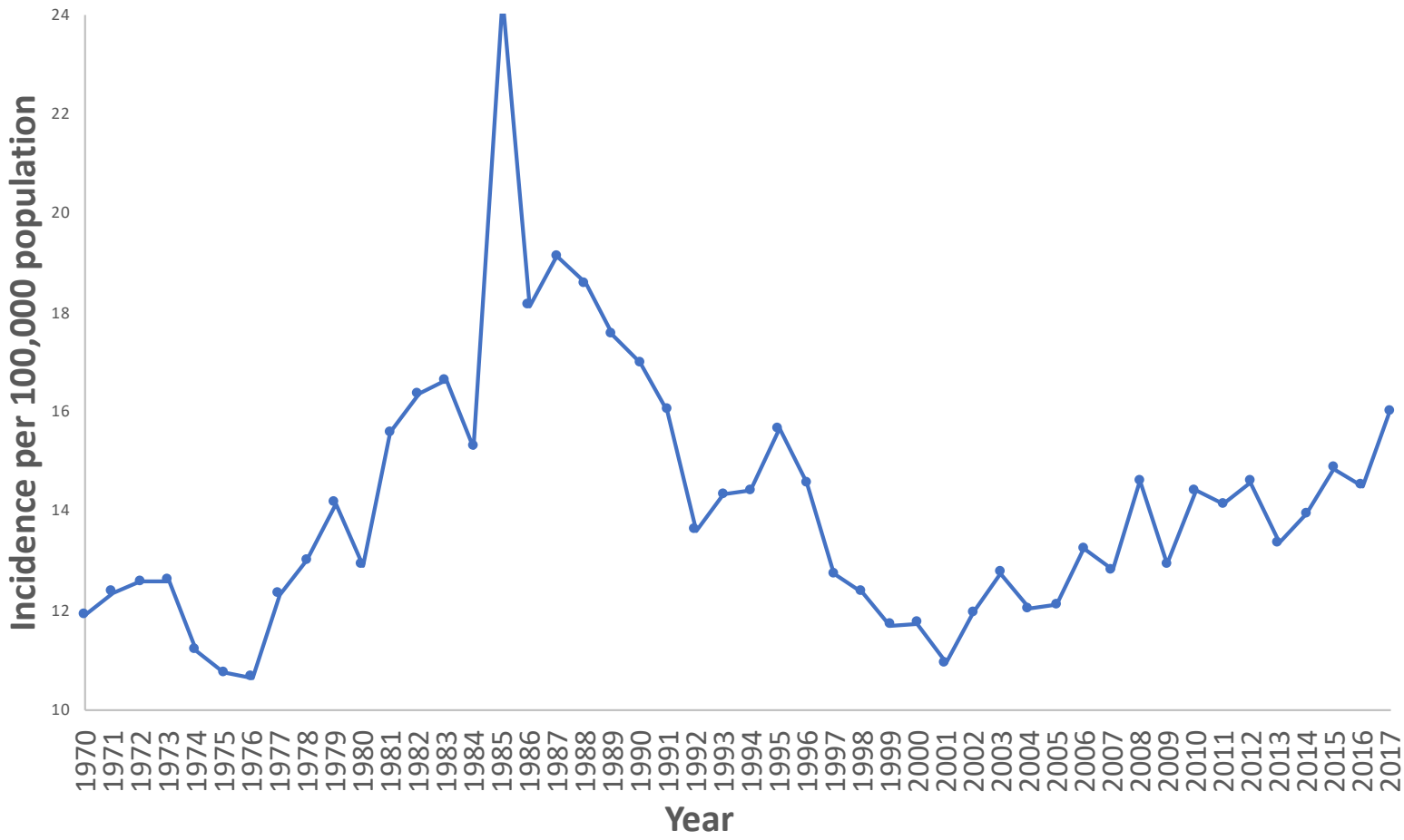

Figure 1. Incidence rate of *Salmonella* infections in the U.S. Incidence rate of laboratory-confirmed human non-typhoidal *Salmonella* infection in the U.S. from 1970-2016 [14,18].

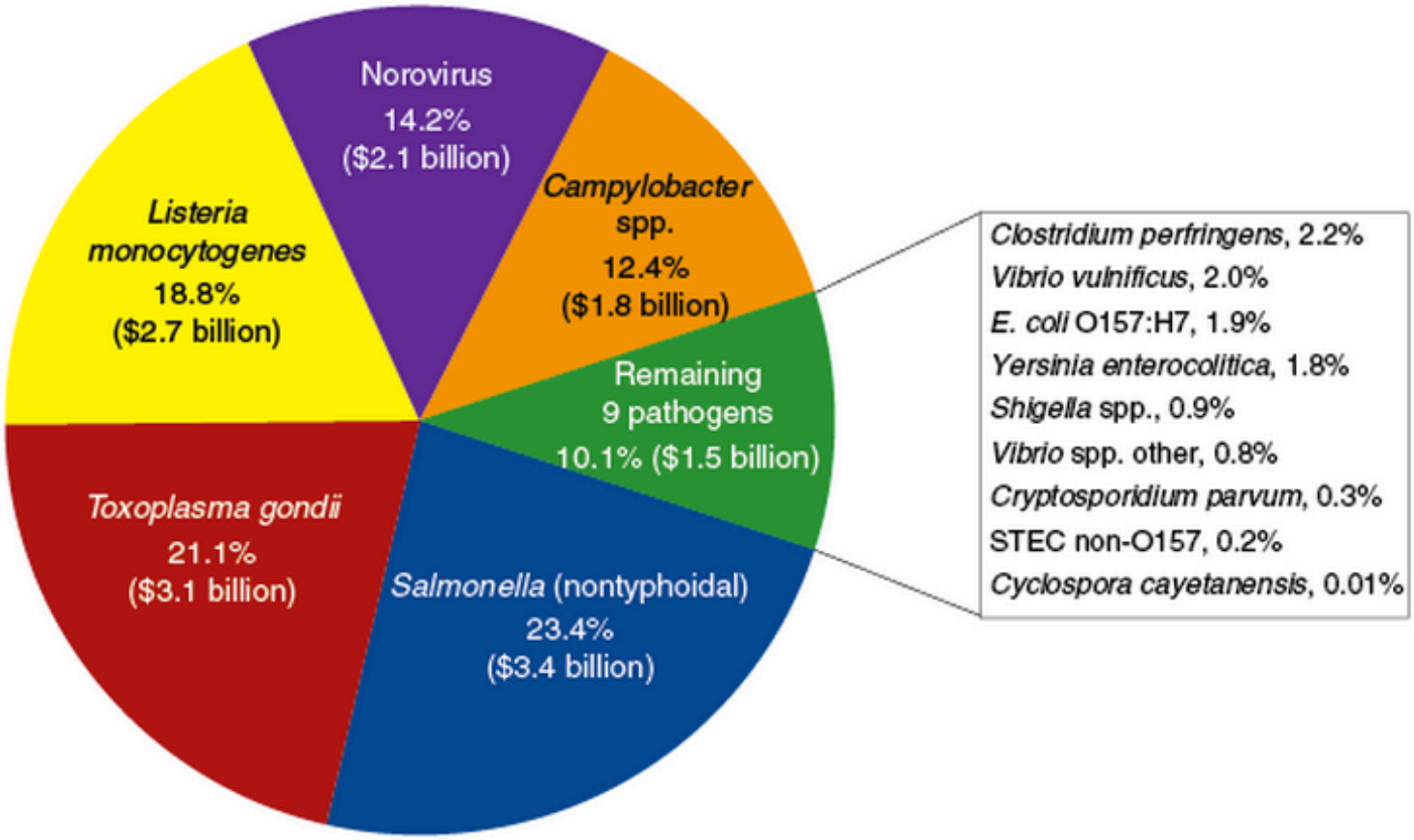

Figure 2. Annual cost and percentage cost estimates in 2010 U.S. dollars based on disease incidence estimates published 2010. *Salmonella* imposes the greatest cost of 14 major foodborne pathogens investigated in the United States [21].

and included medical costs due to illness, the cost of time lost from work due to nonfatal illness, and the cost of premature deaths [21].

Antimicrobial Resistance of *Salmonella*

In the United States, severe non-typhoidal salmonellosis is commonly treated with fluoroquinolone and third-generation cephalosporin antibiotics [23]. Recently recognized decreases in susceptibility to ceftriaxone indicates a possible pattern of increasing resistance to 3rd generation cephalosporins [24]. Other antibiotics such as macrolides, penicillins and trimethoprim- sulfamethoxazole may be of clinical importance [23]. The Centers for Disease Control and Prevention (CDC; Atlanta, GA) National Antimicrobial Resistance Monitoring System (NARMS) reported 3% of tested clinical non-typhoidal *Salmonella* isolates were resistant to ceftriaxone and nalidixic acid [23] in 2012. Nine percent of non-typhoidal *Salmonella* isolates tested were resistant to three or more antibiotic classes [23]. Compared to NARMS-reported data from 2003-2007, *Salmonella* isolates resistant to three or more antibiotic classes was lower in 2012 than in 2003–2007 (12% vs. 9%). However, the differences in resistance to nalidixic acid and ceftriaxone between 2003-2007 and 2012 are not significant (Figure 3, 4) [23]. Crump *et al*. [25] summarized antimicrobial resistance among invasive non-typhoidal *Salmonella* submitted to NARMS from 1996 to 2007 and found that 19.8% were resistant to ampicillin, 11.1% to chloramphenicol and 2.5% to trimethoprim-sulfamethoxazole; 2.7% isolates were resistant to nalidixic acid and 2.5% to ceftriaxone. Previous research has shown that patients with antimicrobial resistant non-typhoidal *Salmonella* infection are more likely to

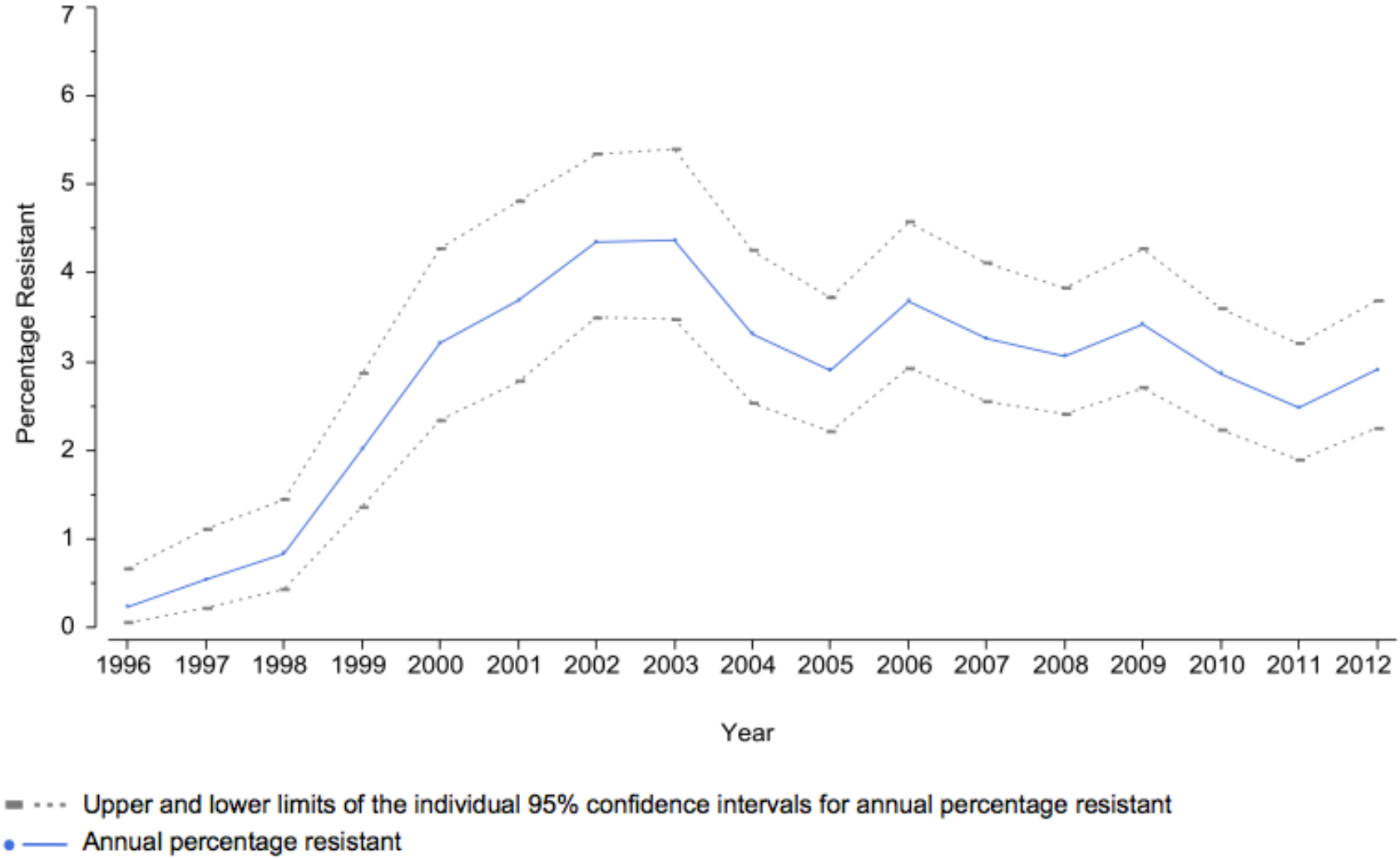

Figure 3. Percentage of non-typhoidal *Salmonella* isolates resistant to ceftriaxone, by year, 1996–2012 [23].

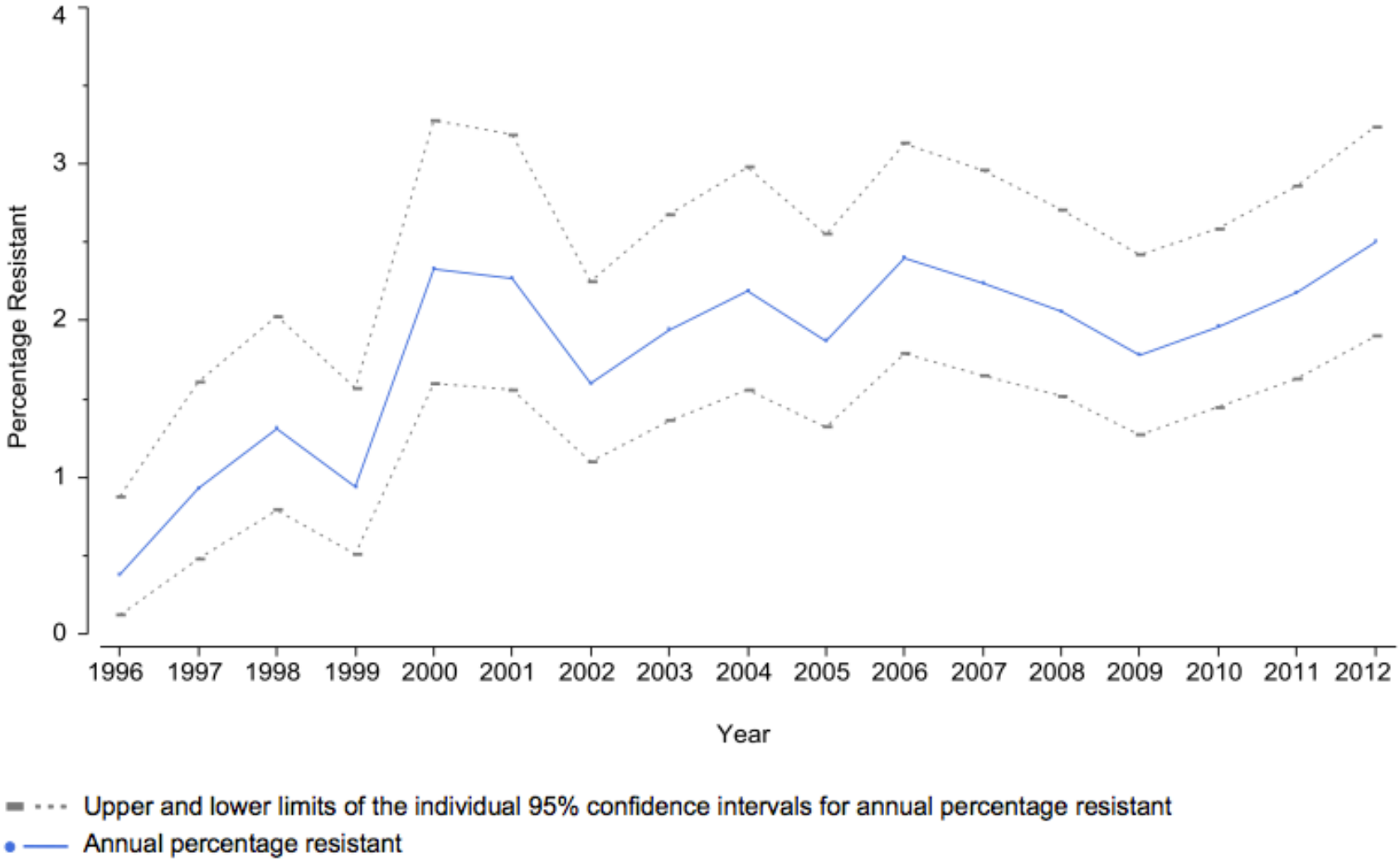

Figure 4. Percentage of non-typhoidal *Salmonella* isolates resistant to nalidixic acid, by year, 1996–2012 [23].

develop bloodstream infection (bacteremia) compared to patients with pan-susceptible *Salmonella* infection [26].

In the United States, there are 1.2 million estimated *Salmonella* annual infections, 100,000 cases and 40 deaths estimated to be caused by drug-resistant *Salmonella* [27]. From 1984 to 2002, among non-typhoidal *Salmonella* outbreaks in which antimicrobial resistance information is available, 28% of outbreaks were caused by antimicrobial-resistant strains [28]. Outbreaks caused by antimicrobial-resistant non-typhoidal *Salmonella* resulted in greater hospitalization rates and a greater portion of deaths [26,28].

Antibiotic-resistant bacteria can be spread from person to person, and from non-human sources in the environment such as food [27]. The over-prescription of antibiotics in food animals may contribute to the emergence of antibiotic-resistant bacteria, which is concerning because people can develop infections by consuming food contaminated with antibiotic-resistant bacteria from these animals, resulting in reduced efficacy of antimicrobials for disease therapy [27]. Therefore, antibiotics must be cautiously used in animal food production and antimicrobial substitutes have to be developed to prevent the emergence and spread of antibiotic-resistant bacteria.

Salmonella **Carriage in the Lymph Nodes of Beef Cattle**

In the U.S., commercial ground beef was reported in 2009 to be contaminated with *Salmonella* at a rate of 4.2% [29], despite the adoption of antimicrobial interventions on the carcass such as sodium hydroxide, sodium sulfide, chlorine, lactic acid and acetic acid solutions [30]. Antimicrobial interventions have been demonstrated to reduce *Salmonella* prevalence on

beef carcasses from 50.2% to 0.8%, but have little effect on ground beef made from edible beef trimmings [31]. The carriage of *Salmonella* in the lymph nodes of beef cattle may contribute to the transmission of the pathogen to consumers via ground beef. Gragg *et al.* [32] reported that *Salmonella enterica* was found in asymptomatic cattle lymph nodes at slaughter, which means in a low dose infection, beef cattle could serve as *Salmonella* carriers without being detected. Without showing any clinical symptoms of *Salmonella* infection or carriage, detection of beef cattle carrying *Salmonella* is difficult. Therefore, there risks of releasing *Salmonella*-contaminated ground beef to consumers.

Major lymph nodes are reported to have relatively high *Salmonella* prevalence in beef cattle. Mandibular, mesenteric, mediastinal, and sub-iliac nodes, which may be removed during evisceration, reportedly may harbor *Salmonella* at rates of 55.9% (95% CL: 43.7 to 67.4%), 91.2% (81.6 to 96.0%), 7.4% (3.1 to 16.2%), and 76.5% (64.8 to 85.2%), respectively [33]. Flank- and chuck-associated nodes, which have been reported to bear *Salmonella* at 3.86% and 0.35%, respectively, are not removed during carcass dressing and may be present in trimmings destined for use in ground beef [34]. Brichta-Harhay*, et al.* [35] indicated that several *Salmonella* serovars, including *S.* Anatum, Dublin, Cubana, Typhimurium, and Montevideo were recovered from lymph nodes and node-surrounding adipose tissue. A similar study showed that among serotypes found in lymph nodes, *S.* Montevideo (44.0%) and *S.* Anatum (24.8%) made up the majority of recovered *Salmonella* [33]. This same group also examined *Salmonella* related to lymph nodes and environmental samples and found that *S.* Kentucky (32.2%), *S*. Anatum (29.9%) and *S*. Reading (17.2%) made up the majority of recovered *Salmonella* serovars [33]. In the same study, antimicrobial resistance of *Salmonella* isolates recovered from lymph nodes (*n* =

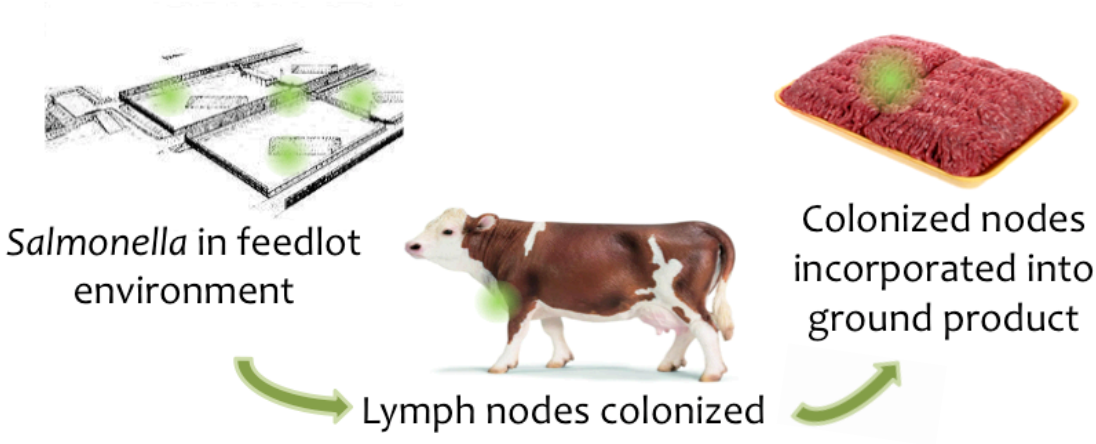

Figure 5. A model of *Salmonella* transmission from feedlot to ground beef. *Salmonella* in the feedlot environment colonizes lymph nodes. Lymph nodes contaminated with *Salmonella* that are not removed during fabrication end up in fat trim and eventually become part of ground beef.

56), feces (n = 18), and hide samples (n = 17) was characterized. Thirty (53.6%), three (16.7%), and four (23.5%) isolates of *Salmonella* from these sample types, respectively, exhibited resistance to one or more antibiotics [33]. Beef cattle hides and feces were found to have a *Salmonella* prevalence of 100% and 94.1%, respectively [33].

The differences of *Salmonella* presence and serotypes across multiple lymph nodes within the same animal indicate that multiple routes of entry into the lymphoidal system for *Salmonella* may exist [33]. Gragg *et al.* suggested a transmission route from the environment via skin abrasion based on the substantial within-animal diversity of *Salmonella* [33]. This hypothesis was further examined by Edrington *et al*. via a transdermal *Salmonella* challenge model [36]. Calves were inoculated with *Salmonella* intra- and/or trans-dermally by applying a skin-allergy instrument over various ventral regions of skin [36]. *Salmonella* was subsequently recovered from the drainage of region-specific lymph nodes and the positive result was persistent for eight days post-challenge [36]. Another study assessed this transdermal transmission by infesting cattle with horn flies that were previously fed with a blood meal containing *S.* Senftenberg [37]. The result from this study indicated that the prolonged exposure to *Salmonella* containing flies had a significant impact on the percentage of culture-positive lymph nodes, as 8% lymph nodes were *Salmonella* positive after 5-day exposure, whereas 50% were positive after 11-day exposure [37].

In addition to the transdermal transmission route of *Salmonella* in cattle feedlots, Edrington *et al.* [38] explored the hypothesis of transmission routes via oral uptake. During a fourteen-day experiment period, calves were orally inoculated either with a single high dose of *S.* Montevideo at (~10^{10} CFU) or daily low dose of 7.1×10^4 CFU and peripheral lymph nodes were

collected on day 14 for *Salmonella* identification. The percentage of *Salmonella* positive lymph nodes were greater ($P < 0.05$) for calves treated single high dose (62.5%) compared to daily low dose (12.5%) [38]. However, deprivation of food and water was not shown to increase translocation of *Salmonella* to the lymph nodes [38]. This study provided evidence for a dose-dependent oral transmission route of *Salmonella* in the peripheral lymph nodes of cattle [38].

Although these hypothesized *Salmonella* transmission routes have not yet been comprehensively described, *Salmonella* within the feedlot environment seems to serve as the origin of *Salmonella* in the lymph nodes of beef cattle (Figure 5). The impact of feedlot environmental *Salmonella* prevalence on transmission during different feed stages was explored [39]. In this study, calves at weaning stage (stage 1) continued to be fed at two feedlots with positive or negative *Salmonella* prevalence [40,41]. Calves were harvested for the examination of *Salmonella* prevalence in lymph nodes at the end of stage 2 (stocker), 3 (60 days on feed) and 4 (120 days on feed). With *Salmonella* not detecting in lymph nodes of cattle from both treatment groups at stage 1, *Salmonella* prevalence in lymph nodes of cattle fed in *Salmonella*-positive feedlot increased as cattle moved into later stages of feeding, at 22%, 78% and 94.4% for feeding stages 2, 3 and 4 while cattle fed in the *Salmonella*-negative feedlot remained negative during all feeding stage, implying that environmental *Salmonella* prevalence during feeding operation has influences on prevalence of *Salmonella* in lymph nodes of cattle at harvest [39].

Other findings indicate variable *Salmonella* prevalence in cattle feedlots within the same geographic region could also differ, possibly due to animal management practices or animal origin [40] . Further studies exploring possible antimicrobial interventions on multiple drug-

resistant *Salmonella* in cattle lymph nodes and post-harvest environments could also be conducted to reduce *Salmonella* issues in beef industry.

Ground Beef Consumption in the U.S. and *Salmonella* in Ground Beef

Figure 2 from USDA-ERS indicates that ground beef holds the largest market share for all identifiable beef products [42]. A 2012 report from National Cattlemen's Beef Association (NCBA) showed that although the market share of ground beef consumption has decreased in recent decades, it still accounts for 42% of total beef consumption in the United States [43]. This translates into the average American consuming 28 pounds per year [42,43]

Consuming raw or undercooked ground beef is a risk factor for contracting a foodborne illness, including *Salmonella* infection. In one survey, 18% of persons who consumed ground beef in the home indicated that they consumed pink/undercooked ground beef [44]. Among all citizens, children younger than 18 years old consumed ground beef product most frequently (82% of total respondents), in which 7.9 % reported consuming undercooked ground beef. Senior age group (> 65 years of age) consumed ground beef least frequently; however, this age group reported higher rates of consumption of undercooked ground beef (18.5 % of total respondents) [44]. Since younger children are more susceptible to *Salmonella* [45], and children are consuming ground beef more frequently than other age groups [44], public education on safe food handling practices and antimicrobial interventions on pre- and post-harvest for beef are required to prevent *Salmonella* transmission.

In the United States, despite efforts to prevent *Salmonella* contamination in foods, *Salmonella* incidence rates remain stable and outbreaks caused by *Salmonella* have continued to

occur [13,45]. From 1973 to 2011, of 1,965 *Salmonella* foodborne outbreaks reported to CDC, beef was the implicated food vehicle in 96 outbreaks [46]. These 96 outbreaks caused 3,684 illnesses, 318 hospitalizations and five deaths [46]. Ground beef accounts for 23% of total beef related outbreaks with median size of 36 illnesses per outbreak [46]. Fifty-nine percent of ground beef attributed outbreaks were caused by serotypes Typhimurium and Newport. Ground beef emerged as the predominant transmission vehicle to beef related outbreak in 2002. From 2002 to 2011, ground beef was reported responsible for 45% of beef related outbreaks in the United States. Among 14 beef-transmitted outbreaks where antimicrobial resistance data were available, ground beef accounted for 3 outbreaks [46]. Although *Salmonella* prevalence in commercial ground beef is low, a survey showed that about 67% *Salmonella*-positive commercial ground beef samples were contaminated with MDR *Salmonella* [47].

Reducing use of antibiotics for agricultural production efficiency and developing interventions targeting MDR *Salmonella* are becoming urging needs to prevent ground beef attributed *Salmonella* outbreaks, particularly MDR *Salmonella* outbreaks.

Bacteriophage: Structure and Infection Pathways

Bacteriophages (phage) are viruses that infect bacteria, and are the most abundant form of life on earth, estimated to number some 10^{31} to 10^{32} organisms in total [48,49]. Phage are ubiquitous in natural environments such as such soil, fresh water, open ocean, and are present in plants and animals as a part of their normal flora. Phages are non-pathogenic to humans and are normal residents of the human microbiome [48,49].

A phage virion consists of a single-stranded or double stranded DNA or RNA genome and a protein or lipoprotein coat [50,51]. Over 96% of described phages belong to the order *Caudovirales* or tailed dsDNA phages [50,51]. The heads of *Caudovirales* are icosahedral in shape and in some cases elongated. *Caudovirales* consists three major families with distinct tail morphologies: *Myoviridae* with contractile tails, *Siphoviridae* with flexible non-contractile tails and *Podoviridae* with short stubby tails [52].

Phage infection starts with adsorption of the phage to its host, where specialized adsorption structures, such as tail fibers and spikes, bind to specific surface receptor on the target bacteria such as outer membrane proteins, flagellum, and liposaccharides [50]. Phage DNA is ejected into the host bacterium following the initial attachment. Based on their infection cycles, *Caudovirales* are divided into two major groups: virulent and temperate [50]. Virulent phages obligately undergo a lytic life cycle upon DNA injection while temperate phages, also known as lysogenic phages, are capable of incorporating their genomes into that of the host cell and coexisting with the host in a quiescent state [52].

After its injecting DNA into the bacterial cell, virulent phages prevent bacterial transcription and replication and direct the host cell to produce more copies of the phage genome and capsid components (Figure 6) [53]. After phage genome replication and protein synthesis, phage particles are assembled to new virions. New virions are released upon disrupting the bacterial cell wall by the combined action of an inner membrane pore-forming holin protein, a peptidoglycan-degrading endolysin and an outer membrane-disrupting spanin [54] .

In contrast, when temperate phages infect the cell a lysis-lysogeny decision is made. If it follows the lysogenic pathway, the phage turns off viral transcription, and its DNA is inserted into the host chromosome via a phage-encoded integrase or exists separately as an episomal element and replicates together with the host chromosome as the host cell divides (Figure 7) [50]. When a temperate phage undergoes its lytic cycle in a process called induction, the phage replicates within the host cell to produce new progeny and lyse the cell upon release of new virions in the same manner as virulent phage (Figure 7) [50].

Bacteriophage as an Antimicrobial Intervention in Agriculture

The antimicrobial activity of bacteriophage was discovered independently by Frederick Twort in 1915 and Felix d'Herelle in 1917 [55]. The therapeutic application for treating human bacterial infections was hypothesized by Felix d'Herelle during an outbreak of severe *Shigella* hemorrhagic dysentery among French troops stationed at Maisons-Laffitte [55]. He filtered the bacterium from patients' fecal samples and combined the filtrates with *Shigella* strains isolated from the patients. After incubation, a clear area was observed on the agar culture, which were later called plaques [55]. d'Herelle also ran a small clinical trial treating young children suffering from bacterial dysentery with phage preparations and observed recovery within 24 hours [56]. Phage therapy attracted interest from pharmaceutical companies, including Eli Lilly and Company, E.R. Squibb & Sons, and Swan-Myers/Abbott Laboratories, who produced phage

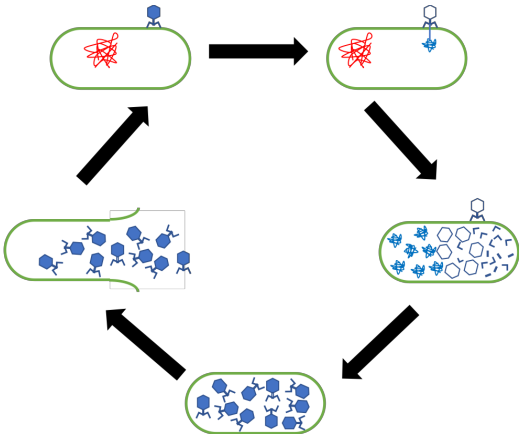

Figure 6. Life cycle of virulent phages. Lytic phage adsorbs to its host and ejects its DNA (blue) into the cell. The host metabolism is redirected to the replication of new virion components. New phage virions are assembled and released upon the lysing the host.

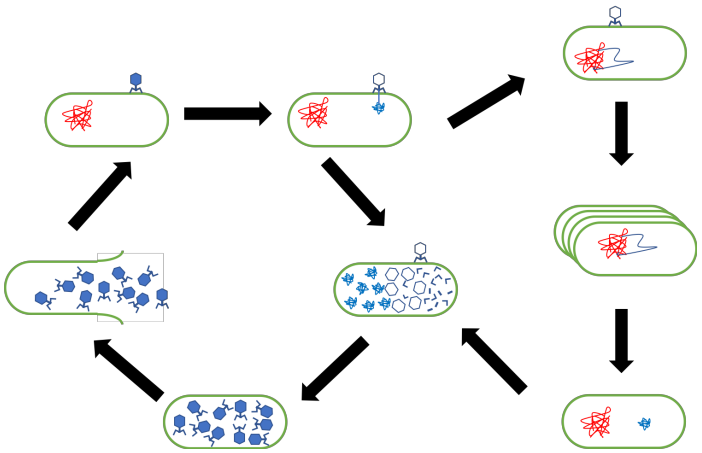

Figure 7. Life cycle of temperate phages. A decision between the lytic cycle and lysogenic cycle is determined by the phage shortly after infecting the cell. In the lytic cycle (black arrows), new phage particles are produced and released upon lysis of the host cell. In the lysogenic cycle (green arrows), the phage DNA (blue) is incorporated into the host chromosome (red) and replicated together with the cell chromosome when cell division occurs.

preparations for clinical applications [56]. However, in the 1940s and 1950s, due to the discovery and later use of antibiotics, along with doubts on efficiency of phage therapy, bacteriophage therapy research discontinued in the West [57]. Nevertheless, phage therapy continued to develop in Eastern Europe and the former Soviet Union, including work at several institutions such as the Eliava Institute of Bacteriophage, Microbiology and Virology (Tbilisi, Georgia) and the Hirszfeld Institute of Immunology and Experimental Therapy (Wroclaw, Poland) [57].

The spread of antibiotic resistant bacteria has become a worldwide threat, which has renewed the interest in exploring bacteriophage as a potential alternative to control pathogenic bacteria in Western countries [58]. Garcia *et al.* [57] suggested that phage can be addressed to all stages of food production, from "farm to fork", to prevent foodborne infections due to advantages that phages offered as biocontrol agents [59]. First, being able to target bacterial hosts with high specificity by recognizing unique bacterial surface structure, antimicrobial treatments with phages leave the remaining microbiota unharmed. This property favors phages over broad-spectrum antimicrobials that could cause collateral damage to the microbial floral [59]. Second, purified phages do not alter flavor, color, aroma and nutritional content of food product and have little inherent toxicity due to their natural composition of nucleic acids and proteins. In addition, phage-containing products are considered "clean label" because of their wholesomeness of ingredients and lack of artificial ingredients or allergens [60].

Several phage-based commercial products have gained approval for use by the U.S. Department of Agriculture (USDA), Food and Drug Administration (FDA) and Environmental Protection Agency (EPA) Regulations and are now available for food producers as an

antimicrobial intervention. EcoShield™, a bacteriophage preparation produced by Intralytix Inc., obtained regulatory approval from the FDA through a "Food Contact Notification" (FCN No. 1018) for use on raw meat cuts and trim to control *E. coli* O157:H7 prior to grinding [61]. The FDA has also approved ListShield™, another phage-based product from Intralytix Inc., for application on ready-to-eat (RTE) meat and poultry products for *Listeria monocytogenes* reduction [58]. SalmoFresh™ from the same company gained approval with generally recognized as safe (GRAS) for application on poultry products for reducing *Salmonella enterica* population (GRN No. 435.). AgriPhage™, a phage cocktail produced by Phagelux Inc. (previously OmniLytics Inc.), gained approval from the Environmental Protection Agency for application on growing produce in the field to reduce plant pathogenic bacteria [57]. Finalyse®, produced by Passport Food Safety Solutions, Inc., was approved for application to the hides of beef cattle to reduce *E. coli* O157:H7 prior to slaughter [59]. This product was recommended to be applied via a walk-through misting system designed to fully cover cattle before harvest. Micreos Food Safety (Wagentingen, Netherlands) has launched two products, PhageGuard Listex™ and PhageGuard S™, which has been approved as GRAS and certified as useful in organic food production. Similar to ListShield™, PhageGuard Listex™ is used as a processing aid for control of *L.* monocytogenes. According to Microes, PhageGuard S™ is intended to be used as a spray on food post-harvest at points of contamination during processing and is expected to achieve 1.0 – 3.0 $\log_{10}$ reduction of salmonellae.

Prevalence and antimicrobial activities of phages in the pre-harvest environment has been studied. Callaway *et al.* has shown that phages targeting *E. coli* O157:H7 were widely distributed in beef cattle feedlots [62]. This research group also conducted studies on utilizing phage

antimicrobial interventions against *E. coli* O157:H7 and demonstrated their efficacy for eliminating *E. coli* O157:H7 in the ceca and rectums of sheep [63,64]. Phage have also been shown to be effective in controlling a wide variety of pathogens in other animal models, including *Salmonella* in poultry and *E. coli* O157:H7 [65] in cattle [63]. After inoculation of *E. coli* O157:H7 via rectal application, Sheng *et al.* administrated 25 mL bacteriophage mixture of SH1 and KH1 at concentration 10^{10} PFU/mL via direct anal application with a syringe and via additional oral intake from drinking water at average concentration ~2.4 PFU/mL [63]. The administration of mixed phages were able to achieve a reduction of 2.4 $\log_{10}$ CFU/swab immediately after the treatment on day 1 and continued to suppress bacterial population throughout the 16-day sample period [63]. Sukumaran *et al.* [65] conducted a study on reduction of *Salmonella* on chicken meat that provided peer review on SalmoFresh$^{TM.}$ (Instralytix Inc., USA). In this study, the commercial bacteriophage preparation at concentration 9$\log_{10}$ PFU/mL was able to achieve 2$\log_{10}$ CFU/mL reduction in diluent homogenized with samples during a 7-day refrigerated storage period [65].

Lytic *Salmonella* Phages

After reviewing the literature up to 2005, Abedon and Ackermann described 170 named *Salmonella* phages [53,66]. Among phages in order *Caudovirales*, 44 Myoviridae, 65 Siphoviridae and 63 Podoviridae were summarized in this list [53,66].

In the family of *Myoviridae*, lytic *Salmonella* phages mainly fall into four genera, *Viunalikevirus* (Vi-like phages)*, Felixounalikevirus* (Felix O1-like phages)*, V5likevirus* (v5-like

phages) and *T4likevirus* (T4-like phages), with an exception, phage SPN3US, that is uncategorized [53].

The first phenotypic characterization of Vi phage was conducted by Ackermann el al. in 1970 and it was further characterized at the molecular level by Pickard in 2010 [67,68]. Genome sizes of ViI-like phages are approximately 157 kb with a GC content of 44.5% [69]. Morphology of *Viunalikevirus* features icosahedral heads of 90 nm and contractile tails of 110 x 18 nm [69]. A unique characteristic of ViI-like phages is that this genus contains a conserved maturation-adhesion tail spike protein that recognizes the Vi exopolysaccharide capsule as the receptor [68]. Up to 2014, there were seven *Salmonella* phages of this genus that have been sequenced and the genome identities of these phages revealed >59% identity [53].

Felixounalikevirus were first known as phage O1 that were originally isolated by Felix and Callow [70]. Phage FelixO1 has an icosahedral head of 73 nm and a contractile tail of 17 x 113 nm that contains six straight tail fibers folded along the tail [71]. Within this genus, seven phages capable of infecting *Salmonella* are sequenced, and their genomic characteristics include a genomic size of 86 kb and a GC content of 39%. In addition, they were found to have >20 tRNAs and homing endonuclease in their genomes [71]. Broad host ranges are generally found in this genus, with phage FelixO1 reportedly capable of infecting 98.2 % (640/652) tested *Salmonella* strains at concentration 10^{12} PFU/mL [72].

V5likevirus was proposed as a genus with two members targeting *Salmonella*, five targeting *E. coli* and one targeting *Cronobacter sakazakii.* V5-like phages are found to have genomic size ranging from 136 to 148 kb and GC content ranging from 37.4% to 46.3% [53]. *Salmonella* phage PVP-SE1, which is a member of this genus, has an icosahedral head of 85 nm

and contractile tail of 120 x 18 nm. The receptor of phage PVP-SE1 was recognized as the inner core of LPS, and this relatively conserved receptor region explains the broad host range of this phage [73].

Phages in the genus of *T4likevirus* are related to coliphage T4, but they are generally diverse ecologically, morphologically, genomically, and proteomically [74,75]. Two *Salmonella* phages within this genus, STML-198 [76] and S16 [77], were previously sequenced while molecular level analysis was conducted only in phage S16. This phage shares 60% homologous proteins with T4 and it has a genome of 160kb with GC-ratio of 36.9% [77]. Morphologically, its head is elongated, 117 nm in length and 91 nm in width [77]. This phage was found to have broad host range, especially in *Salmonella* species, infecting 76% (96/126) of isolates tested while resistant other species within the family of *Enterobacteriaceae* tested [77]. Authors identified *Salmonella* outer membrane protein OmpC as the primary receptor and LPS as the secondary receptor of phage S16 [77].

Within the family of *Myoviridae*, a jumbo phage SPN3US was sequenced and showed little homology to any other phages in the NCBI database [78]. The majority of the genome (>79%) was annotated as hypothetical protein and the putative functions that were annotated in phage S16 were related to phage structure (capsid, tail, terminase), replication and transcription (helicase, RNA polymerase), and lysis (endolysin) [78].

In the family of *Siphoviridae*, five major genura were identified among *Salmonella* phages that were sequenced and published [53].

Salmonella phages SPC35 [79] and EPS7 [80] are members of the genus *T5likevirus*, in which phage T5, a well-characterized Siphoviridae infecting *E. coli*, is the type species of this

genus [81]. *Salmonella* phages in the family of *T5likevirus* generally have genome of ~110kb with GC-ration of 39%. They are generally found to have more than 20 tRNAs in their genome [53]. They typically have an icosahedral head of 70 nm in diameter and long noncontractile tails of 185 nm in length [81]. Both phages SPC35 and EPS7 were found to utilize *Salmonella* outer membrane protein BtuB as their receptors [79,80].

Salmonella phages within the family of *Jerseylikeviruses* were found to be related to phage Jersey, a tying phage that has been used in the S. *Paratyphi* B tying scheme [82]. Genomic characteristics of this genus include a genome size of 42kb and a GC-ration of 49%. *Jerseylikeviruses* generally have icosahedral heads of 64 nm, a long noncontractile tail of 120x7 nm and a baseplate of 20 nm with spikes [83]. *Salmonella* phages in this genus were found to have high level of nucleotide identity up to 93%, which is a distinct feature of phages of this genus [83].

Salmonella phages in the family of *Tunalikevirus* are related to phage T1, a phage that was isolated by Milislav Demerec and later intensively study by Max Delbrück [84]. Due to the ability of T1 to form aerosol, T1 contaminations continue to be a problem in laboratory working with *E.coli* [53]. Morphological characteristics of *Tunalikevirus* include a icosahedral head of 60 nm and a long noncontractile tail of 200 nm that are terminated by four fibers [53]. T1-like phages commonly have a genome size of 50.7 kb and a GC-ratio of 45.6% [85]. The genome is terminally redundant and circularly permuted [85].

The original phage Chi in the family of *Chilikevirus* was isolated in 1935 by Sertic and Boulgakov [86]. The most distinct property of phages in this family is its specificity to motile bacteria in the family of *Enterobacteriaceae*, including *Salmonella*, *Serratia* and *E. coli* [86].

Schade *et al.* suggested that phage Chi attaches to the filament of bacterial flagellum by its tail fiber but the actual receptor was located at the base of flagellum [87]. Chi-like phages have icosahedral head with diameter ranging from 65.0 – 67.5 nm and long flexible tail with size 220 – 230 nm x 12.5 – 14 nm [88]. Phage Chi has a genome size of 59.6kb and 56.6% GC content [89]. Phage Chi and its related phage SPN19 and iEPS5 share high level of DNA and protein identity of 90% and 93%, respectively [53].

Unclassified *Salmonella* phages in the family of *Siphoviridae* include a group of closely-related phages isolated by Switt *et al.* [90]. Genomic characteristics of these phages include a genome size of 55kb and GC content of 51%. The most distinct property of this genus is their very narrow host range [90]. Among 25 *Salmonella* serovars tested, these phages were only able to infect one strains from the serovar of *S*. Cerro [90]. They are in the process of proposing a new genus of *Sp03unalikevirus* to ICTV [53].

The family of *Podoviridae* contains *Phieco32likevirus, N4likevirus* and a super-group *Autographivirinae* that includes *T7likevirus* and *Sp6likevirus* [53].

Phages in the subfamily of *Autographivirinae* generally process a genome size of approximately 40kb and a GC-ration of 47% [91,92]. Coliphage T7, the type phage in the genus of *T7likevirus*, is one of the most well-characterized virulent phage and one of the very first phage that were completely sequenced [91]. Another two phages, SP6 and K1-5, process direct terminal repeats of 174bp and 245bp, respectively [92]. The three phages mentioned show low level of homology in their genome, and therefore, suggested by Scholl *et al.*, diverged a long time ago [92].

Phages in the genus of *Phieco32likevirus* process morphological characteristics including a unusual elongated head (145 nm × 44 nm) and a short tail (13 nm x 8 nm) terminated by tail fibers [93]. Up to 2011, there was only one *Salmonella* phage, phage 7-11, that was sequenced and analyzed in this genus [94]. Phage 7-11 possesses a genome size of 89.9kb and a GC-ratio of 44.1%. Although phage 7-11 was classified as a member of *Phieco32likevirus,* its genome showed little homology with phage phiEco32 (32% homologous protein) [94].

In 2013, Switt *et al.* added two *Salmonella* phages, FSL SP-058 and FSL SP-076, to the genus of N4likevirus [90]. Coliphage N4 processes morphological characteristics including a icosahedral head of 70 nm in diameter and short tail fiver between the head and tail [95]. Phages from this genus generally have a genome of 72kb in size and a GC-ration of 39.5% , encoding 10 tRNAs [90]. Phage FSL SP-058 and FSL SP-076 are closely related, with a high protein identity of 89% [90].

Current Status of Phage Isolation

During 2013 – 2016, three phage isolation were performed. Table 1 processes information including their identification, bacterial host, morphology, sequence status, annotation status, phage type and additional information provided. Phage isolated from previous study conducted by Xie *et al.* [41] were describe in two published sources [96,97]. Additional isolation and genomic sequencing of phages performed are updated in Table 1.

Table 1. Bacteriophage information

	Phage ID	Host	Morphology	Sequencing	Annotation	Rename	Phage Type	Note
2013 Fall Waste water, IntestiPhage	Intesti 1	*S.* Typhimurium LT2	Myo	Yes	Yes	Mushroom	Felix O1	Genome Submitted
	Intesti 3	*S*. Typhimurium LT2	Sipho	Yes	Yes	Shivani	T5-like	Genome Submitted
	pk2	*S.* Typhimurium LT2	Sipho	Yes	Yes	Seelong	Chi-like	
	Intesti3(E.coli)	*E. coli* K12	Podo	Yes	Yes	Peach	T7-like	
	Intesti1(E.coli)	*E. coli* K12	Myo	Yes	Yes	Mishka	N/A	
Spring/Summer 2014 Three South Texas Cattle Feedlots NCBA Funded	3	*S.* Anatum FC1033C3	Sipho	Yes	Yes	Sasha	9NA-Like	Genome Submitted
	5	*S.* Anatum S2029C2	Sipho	Yes	Yes	Season5	9NA-Like	
	6	*S.* Typhimurium LT2	Sipho	Yes	Yes	Season6	Chi-Like	
	9	*S*. Anatum FC1033C3	Sipho	Yes	Yes	Sergei	9NA-Like	Genome Submitted
	12	*S.* Newport USDA2	Sipho	Yes	Yes	Season12	Chi-Like	
	13	*S.* Typhimurium LT2	Sipho	Yes	Yes	Season13	Chi-Like	
	15	*S*. Montevideo USDA3	Myo	Yes	Yes	Munch	Novel Big Head Myo	
	17	*S.* Anatum S2028C1	Myo	Yes	Yes	Minion	Novel Big Head Myo	
	21	*S.* Muenchen FD1001A1	Myo	No	Yes	Mecon		Cannot be sequenced
	24	S. Anatum USDA4	Sipho	Yes	Yes	Season24	9NA-Like	
	25	*S.* Anatum S2028C1	Myo	Yes	Yes	Morel	Novel Big Head Myo	
	27A	*S.* Anatum FC1033C3	Sipho	Yes	Yes	Season27A	Chi-Like	
	28	*S*. Montevideo USDA3	Myo	Yes	No		Novel Big Head Myo	Cannot be recovered from parent stock
Summer 2016 Samples from Cattle Harvet Facility (Michgan) Texas Feedlot (Cummings) Waste Water (Austin, College Station)	MIS1-NP1	*S.* Newport USDA2	Myo	No	No			
	MIS1-LT2	*S.* Typhimurium LT2	Sipho	Yes	Yes		Novol phage type	
	MIS3-3117	*S.* Heidelberg 3117	Myo	Yes	Yes	Meda	Felix O1	
	MIS4-UE	*S*. Enteritidis UE	Sipho	Yes	No		FSL-SP-101	Similar to Moerno Switt phage
	SL-Monte2	*S.* Anatum S2029C2	Sipho	Yes	No		9NA-like	
	SE-Anatum	*S.* Anatum S2028C1	Sipho	Yes	No		FSL-SP-031	Similar to Moerno Switt phage
	SL-Ken	*S.* Anatum FC1033C3	Sipho	Yes	Yes		9NA-like	
	1ww-UE	*S*. Enteritidis UE	Myo	No	No			
	2ww-3119	*S.* Newport 3119	Myo	Yes	Yes	Melville	S16-like	
	5ww-LT2	*S.* Typhimurium LT2	Sipho	Yes	Yes		Chi-like	
	Sw1-3003	*S.* Typhimurium 3003	Myo	Yes	Yes		T4-like (mostly structural)	Silimiar to one of EcoShield phage
	Sw2-Ken	*S.* Anatum FC1033C3	Sipho	Yes	Yes		T5-like	
	Sw2-Monte2	*S.* Anatum S2029C2	Myo	No	No			
	Sw2-NP2	*S.* Newport 10-014	Myo	No	No			
	SW4-UEa	*S*. Enteritidis UE	Myo	Yes	No		T5-like??	Needed to be resequenced
	SW1-LT2	*S.* Typhimurium LT2	Sipho	Yes	No		Chi-Like	
	Sw-UH	*S.* Heidelberg UH	Myo	Yes	No		T4-like (mostly structural)	Silimiar to one of EcoShield phage

CHAPTER II*

DEVELOPMENT AND VALIDATION OF A MICROTITER PLATE-BASED ASSAY FOR DETERMINATION OF BACTERIOPHAGE HOST RANGE AND VIRULENCE

Introduction

The growing threat of antibiotic resistance has led to increased calls for new antimicrobials to control bacterial pathogens and treat infectious diseases [98-100]. Bacteriophages are the natural predators of bacteria and were used to treat bacterial infections in the pre-antibiotic era [101,102]. Phages have re-emerged as an attractive alternative to combat antibiotic resistant bacteria in recent decades [103]. The abundance of phages in natural environments makes the discovery and isolation process rather simple [104,105], but screening and selecting the right phages is crucial for achieving successful therapeutic outcomes. One advantage of phages as therapeutics is their host specificity [106,107] and the ability of most phages to infect only a relatively narrow range of closely related bacterial strains limits its impact on normal bacterial flora [106,108]. The drawback to host specificity is the limitation on treatment outcomes prior to identifying bacterial susceptibility of particular phages [106].

The ability of a phage to infect and lyse th[1]e target bacterial strain is generally agreed to be a basic requirement for successful phage therapy [109-111]. Phage virulence, which is defined as the ability of a phage to control the growth of its host in culture, may also be an indicator of

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phage utility [112-114]. The phage host range is affected by a number of factors [115,116]. The phage must be able to adsorb to the cell surface in order to initiate infection and the absence or masking of a compatible cell surface receptor will prevent this initial interaction. With successful phage adsorption, the entry of phage DNA to a bacterial cell could be blocked by superinfection exclusion systems or the absence of required accessory proteins. Restriction-modification and CRISPR systems block infection by degrading phage DNA shortly after it arrives in the cell cytoplasm and abortive infection systems that are triggered on phage infection result in host cell death before new phages can be produced. All of these factors together limit the host ranges of naturally recurring phages and can affect the results of experiments designed to determine the phage host range. Given the increased interest in phage therapy, there is a need for in vitro assays that can be used to help determine the suitability of phages for in *vivo* application [114,117].

The definitions and outcomes of the phage host range vary between testing methodologies [107,118]. To claim a host is "sensitive" to a phage may mean that the phage is able to infect, produce progeny, and lyse its host or simply that the host cell dies following phage infection. The spot test is often used to determine the host range by measuring bacterial killing by applying high-titer phage lysates to agar lawns inoculated with host bacteria [119]. Applying phage only at high titer can fail to distinguish between the ability of phage to replicate within or simply kill the test strain since a similar result could be produced through phage infection and lysis, abortive infection, or lysis from without [107]. Spotting single dilutions of lower phage concentrations has also been a common host range method [120,121]. In this approach, the phage is diluted to a routine test dilution (RTD), which is typically defined as the lowest phage dilution that still forms a zone of lysis on its propagation host. This method is more sensitive than

spotting a high-titer phage lysate since a level of productive phage infection is required to produce a signal. Another commonly used method for testing the host range is measuring phage efficiency of plating (EOP), which counts the number of plaques formed by a phage on a test strain relative to its titer observed on its original host [107,119]. Plaque formation is a better indicator of productive phage infection since it is the result of multiple rounds of infection, lysis, and release of progeny. Mirzaei and Nilsson [122] compared the results from spotting high titer lysates and measuring EOP in phages infecting *E. coli* and *Salmonella*. The researchers determined that the high-titer spotting method often overestimated the phage host range and virulence. While the production of clearing zones in bacterial lawns following application of high-titer phage lysate may overestimate phage sensitivity, the inability of a phage to form visible plaques in a bacterial lawn does not necessarily mean a lack of productive infection. Plaque formation is a dynamic process and differences in phage latent period, burst size, diffusion rate, and growth of the host can all affect plaque size and visibility [107,123]. Observation of phage infection in a liquid culture could serve as an alternative method for measuring the phage host range and virulence for phages that are incapable of making observable plaques [107]. Henry, Lavigne and Debarbieux [112] analyzed phage virulence in *Pseudomonas aeruginosa* broth culture and were able to differentiate between phages based on differences in the culture optical density over time. In this case, all phages studied were already known to infect the host strain by EOP-type assays. However, performing liquid culture assays in traditional culture flasks is time and labor intensive and limits the throughput of the method.

Minimum inhibitory concentration (MIC) assays are typically used to simultaneously test multiple chemical antimicrobials against target organisms in a high throughput format such as

the standard 96-well microtiter plate [124]. In this approach, a culture of the test organism is inoculated at a low density (~10^5–10^6 CFU/mL) into wells containing graded concentrations of antimicrobial compounds in broth medium. The presence or absence of bacterial growth is scored following a fixed period of incubation. The culture containing the lowest concentration of antimicrobial that inhibits bacterial growth is termed the MIC of the compound for that organism. This approach has been expanded to estimate the performance of other biocidal compounds in food safety applications [125,126]. Endpoint MIC-like assays have been adopted to measure the host range and virulence of phages infecting enterohemorrhagic *E. coli* [127] and phages of *S. aureus*, *E. coli*, and *Salmonella* [128]. However, such endpoint assays can yield false-negative results if phage resistance acquired during the course of the assay results in culture turbidity at the time of end-point determination [107]. Phages that are capable of infecting the host but do not replicate well in liquid culture could fail to clear the culture. This also leads to a false negative at end point determination [107]. The Bioscreen-C growth analysis system has been used to obtain real-time measurement of culture optical density in the presence of phages to determine phage virulence [129] and host range [130-132]. The Bioscreen-C system can provide high-resolution monitoring of culture optical densities over time and these assays have been evaluated qualitatively or by measuring the inflection of the growth curve at set time points. The measurement of bacterial respiration in the presence of phage in liquid culture has also been used to simultaneously determine phage host range and virulence in a high-throughput, 96-well format [133]. In this system, the production of a colorimetric signal by reducing a tetrazolium dye is measured instead of the culture's optical density [133].

In the current study, a liquid culture-based host range method is developed, which continuously monitors bacterial growth in the presence of phage in a standard 96-well format and this method is compared to the results from conventional agar overlay spot assays. The intent of this methodology is to determine the phage host range, virulence, and bacterial resistance development in a single high-throughput format by using the features of an automated plate reader to monitor the culture optical density over time in an incubating, aerated environment. Growth responses are quantified by integrating the growth curve over the entire experiment, which allows them to be directly compared. This microtiter plate host range assay is expected to serve as an alternative host range method and can potentially be a more sensitive predictor of virulence of phages by providing more information on bacterial inhibition with high resolution between bacterial strains.

Materials and Methods

1. Bacterial Strains and Culture Conditions

A panel of 20 *Salmonella* strains from various sources representing 11 serovars were used in this study, which is shown in Table 2. All bacteria and phages were cultured in tryptic soy broth (TSB) (Becton-Dickinson, Franklin Lakes, NJ, USA) or tryptic soy agar (TSB plus 1.5% *w/v* Bacto agar (Becton-Dickinson)) aerobically at 37 °C.

2. Bacteriophage Strains and Culture Conditions

Phage FelixO1 was obtained from the *Salmonella* Genetic Stock Center (University of Calgary, Calgary, AB, Canada) and propagated on the *S.* Typhimurium strain LT2. Twelve of the phages used in this study were isolated in 2014 from a set of 72 phage-enriched beef feedlot

Table 2. Bacterial strains used in this study and their origins. Reprinted from Xie *et al*., 2018 [96].

Strains	Serovars	Sources/References
USDA4	Anatum	T. Edrington (USDA)/[41]
FC1033C3	Anatum	Cattle Feedlot Environment/[41]
S2029C2	Anatum	Cattle Feedlot Environment/[41]
S2028C1	Anatum	Cattle Feedlot Environment/[41]
FD1001A1	Muenchen	Cattle Feedlot Environment/[41]
H2006-1	Cerro	Cattle Feedlot Environment/[40]
08-022	Dublin	S. Lawhon (Texas A&M Veterinary Medicine)
SGSC 2475	Enteritidis	*Salmonella* Genetic Stock Centre/(University of Calgary, CA)/[134]
3115	Enteritidis	T. M. Taylor (Texas A&M University)
SGSC 2480	Heidelberg	*Salmonella* Genetic Stock Centre (University of Calgary, CA)/[134]
3117	Heidelberg	K. Cummings (Texas A&M Veterinary Medicine)
USDA3	Montevideo	T. Edrington (USDA)/[41]
H1042-3	Montevideo	Cattle Feedlot Environment/[40]
USDA2	Newport	T. Edrington (USDA)/[41]
10-014	Newport	S. Lawhon (Texas A&M Veterinary Medicine)
330-1	Reading	S. Lawhon (Texas A&M Veterinary Medicine)
USDA1	Typhimurium	T. Edrington (USDA)/[41]
3116	Typhimurium	T. M. Taylor (Texas A&M University)
LT2	Typhimurium	American Type Culture Collection/ATCC 19585
USDA5	Kentucky	T. Edrington (USDA)

environmental samples described previously [41]. The 72 enrichments were pooled by pen, feedlot, and enrichment strains to produce 18 composite samples and enriched again against the same two sets of mixed bacterial hosts, which was described previously [41]. Two additional phages known as Sw2 and Melville were isolated in 2016 from a municipal wastewater influent sample enriched for phage using the same mixed-host panel as above. Each enrichment was serially diluted and plated to soft agar lawns inoculated with each individual enrichment host. Additionally, individual plaques were picked and purified by subculturing three times. Plaques picked from the third subculture of each bacteriophage were used to produce high-titer phage stocks by using the confluent plate lysate method [135]. Soft agar overlays were prepared as described below. Phage stocks were stored and diluted in phage buffer (100 mM NaCl, 25 mM

Tris-HCl pH 7.4, 8 mM $MgSO_4$, 0.01% *w/v* gelatin) at 4 °C. Phages used in this study and their propagation hosts are summarized in Table 3.

Table 3. Bacteriophages used in this study, their propagation hosts, and their origins. Reprinted from Xie *et al*., 2018 [96].

Phages	Propagation Host	Source
Sasha	FC1033C3	Cattle Feedlot Environment/[41]
Season5	S2029C2	Cattle Feedlot Environment/[41]
Season6	LT2	Cattle Feedlot Environment/[41]
Sergei	FC1033C3	Cattle Feedlot Environment/[41]
Season12	USDA2	Cattle Feedlot Environment/[41]
Season13	LT2	Cattle Feedlot Environment/[41]
Munch	USDA3	Cattle Feedlot Environment/[41]
Minion	S2028C1	Cattle Feedlot Environment/[41]
Mecon	FD1001A1	Cattle Feedlot Environment/[41]
Season24	USDA4	Cattle Feedlot Environment/[41]
Morel	S2028C1	Cattle Feedlot Environment/[41]
Season27A	FC1033C3	Cattle Feedlot Environment/[41]
Sw2	FC1033C3	Municipal wastewater influent, TX
FelixO1	LT2	*Salmonella* Genetic Stock Centre (University of Calgary, CA)
Melville	USDA2	Municipal wastewater influent, TX

3. Phage Host Range Agar Overlay Spot Assay

Fresh (~18 h) overnight cultures of bacterial strains were prepared in TSB, subcultured 1:100 in fresh TSB, and grown to OD_{550} ~0.5. Agar overlays were prepared by inoculating 4 mL of molten top agar (10 g L^{-1} Bacto tryptone (Becton-Dickinson), 10 g L^{-1} NaCl, 5 g L^{-1} Bacto agar) with 100 µL of OD_{550} ~0.5 host culture and then poured over TSA plates. Lysates of each phage were ten-fold serial diluted and 10 µL of each dilution was spotted onto the overlay inoculated with the original host of each phage, dried, and incubated at 37 °C overnight [119]. In

this study, the routine test dilution (RTD) [120] was defined as the first dilution at which the phage produced countable plaques on a lawn of their propagation host. Each phage lysate was adjusted with phage buffer to achieve the RTD and 100× RTD, spotted on the overlays of the panel of 20 *Salmonella* strains, and incubated at 37 °C overnight. Spot assay results of each phage-bacterium combination were scored using the following parameters. Production of >50% of the plaques formed on the propagation host at RTD = 4; production of <50% of the number of plaques formed on the propagation host at RTD = 3; production of a confluent zone of lysis but no individual plaques at 100× RTD = 2; production of individual plaques at 100× RTD = 1; and no plaque formation at either dilution was scored as 0.

4. Methodology Development for the Microtiter Plate Host Range Assay

A subset of four bacterial strains (*S.* Anatum strain FC1033C3, *S.* Newport strain USDA2, *S.*Typhimurium strain USDA1, and *S.* Enteritidis strain SGSC 2475) and four phages (Sasha, Season12, Munch, and Sw2) were selected to develop the parameters for the microtiter plate liquid-culture host range assay. Different initial bacterial inoculum levels were tested in combination with phages at starting concentrations of 10^6 to 10^8 PFU/mL. The low inoculum condition (~10^5 CFU/mL) was achieved by adjusting fresh overnight cultures OD_{550nm} ~0.5 and diluting 1000-fold in TSB. For the high inoculum condition, fresh overnight cultures were adjusted with TSB to OD_{550nm} ~0.1 to achieve a concentration of ~10^8 CFU/mL. Phage lysates were titered and adjusted to concentrations of 10^7, 10^8, and 10^9 PFU/mL with phage buffer. For each assay, 180 µL of adjusted bacterial inocula in TSB were mixed with 20 µL of phage in sterile, untreated Falcon (Corning) 96-well transparent plates to achieve final phage concentrations of 10^6 PFU/mL, 10^7 PFU/mL, and 10^8 PFU/mL. The plates were incubated at 37

°C with double orbital shaking in a Tecan Spark 10 M plate reader (Tecan Group Ltd., Männedorf, Switzerland) and growth was monitored by measuring OD_{550nm} at 30-min intervals for 12 h, which results in 25 total time points including the initial (time 0) measurement. Growth curves were obtained by plotting OD after baseline adjustment against time. All assays were performed with three biological replicates.

5. Analysis of Microtiter Plate Host Range Assay Data

Based on the results obtained from the above pilot experiments, the high bacterial inoculum (~10^8 CFU/mL) condition with phages at 10^6 and 10^8 PFU/mL was used to assess the phage host range and virulence for the rest of the collection. Preparation of bacterial inoculum, phage, and measurement parameters were as described above in triplicate. To facilitate data analysis, the growth patterns observed in each assay were distilled into single numerical values by measuring the area under each curve for both positive control and phage treatments by using the equation below.

$$Area\ under\ the\ curve = \sum_{i=1}^{24} \frac{OD_{i+1} + OD_i}{2} \quad (1)$$

where *OD* were measured at 550 nm at 30-min time points *i.* For example, the OD_{550nm} measured at time timepoint $i = 3$ (60 min into the experiment) is added to the OD_{550nm} measured at $i = 4$ (90 min) and divided by 2 to give the OD_{550nm} value for the center of the interval. Each of these values are combined over the entire experiment to give a total area under the growth curve. Since all time intervals are equal in this procedure, time is not explicitly required in this calculation. The areas under the curve calculated in Equation (1) are normalized as percentages of the area under the curve of the positive control by Equation (2).

$$Liquid\ assay\ score = \frac{Area_{positive\ control} - Area_{phage\ treatment}}{Area_{positive\ control}} \times 100 \qquad (2)$$

where the $Area_{positive\ control}$ and $Area_{phage\ treatment}$ are the areas under each curve obtained from Equation (1). The *liquid assay score* is equal to the area between the positive control curve and phage treatment curve, divided by the total area below positive control curve, and multiplied by 100. An illustration of the derivation of the assay score is shown in Figure 8. In this way, the assay scores represent how well phages are able to suppress bacterial growth during the 12-h experiment. The average values calculated by Equation (2) across triplicate biological replicates (n = 3) were used as the assay scores for all phage-host combinations.Comparisons between the microtiter plate assay and spotting assay results for each phage-host combination were carried out by normalizing the results of both methods to the result obtained for the phage propagation host and calculating the difference using the equation below.

$$Difference\ score = \frac{Spot\ assay\ score}{Spot\ assay\ score\ obtained\ on\ its\ host} \times 100 - \frac{Liquid\ assay\ score}{Liquid\ assay\ score\ obtained\ on\ its\ host} \times 100 \qquad (3)$$

The calculated values of difference demonstrate the agreement or disagreement between two host range methods with greater positive values, which indicates higher phage sensitivity measured by the spot assay, and greater negative values indicate higher sensitivity measured by the microtiter assay. In each case, one spot assay score was compared to two microtiter assay

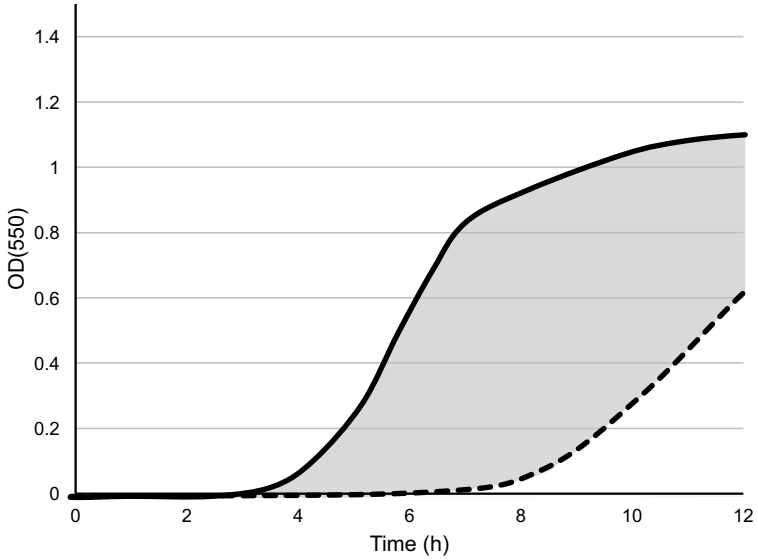

Figure 8. An illustration of the liquid assay score is derived by Equation (2). Reprinted from Xie *et al*., 2018 [96].The equation calculates the area (grey) between the positive control (solid line) and the phage-inoculated culture (dashed line) and expresses this as a percentage of the total area under the positive control curve. No inhibition of bacterial growth (solid and dashed lines overlap) results in a score of zero and completes the absence of growth in the phage-inoculated culture (dashed line follows X-axis) would result in a score of 100.

scores at different phage concentrations to determine if one condition yielded higher agreement with the spotting assay.

6. Statistical Analysis

Statistical analyses were performed using JMP Pro v12 (SAS Inst. Inc., Cary, NC, USA). The Shapiro-Wilk Test was performed to determine the normality of distribution for microtiter assay scores by concentrations across all tested bacterial strains. Significantly differing assay scores by concentrations were separated by using the Wilcoxon/Kruskal Wallis Test ($p < 0.05$).

Results and Discussion

1. Measurement of Phage Host Range by Traditional Spot Assay

The host ranges of 15 *Salmonella* phages against a panel of 20 *Salmonella* strains, which is measured by a traditional spot assay, are shown in Figure 9. In this method, phages adjusted to a consistent routine test dilution (RTD) were spotted to agar overlays inoculated with each bacterial test strain and observed for the formation of plaques. A scoring system was used to summarize plaque formation by spotting the phage lysates at the RTD and at 100× RTD. A score of 4 corresponds to phage efficiency of plating (EOP, the number of plaques observed on the test strain divided by the number of plaques observed on the phage host) of ~0.5 to 1, a score of 3 represents an EOP of ~0.05–0.5, a score of 2 represents an EOP of ~0.01–0.05, and a score of 1 represents an EOP of ~0.0002–0.01. Scores of 0 represent phage EOP of less than ~0.0002. The *Salmonella* strain panel used in this study was diverse and represents 11 serotypes that are commonly associated with human disease or animal carriage [134]. The host ranges of the tested phages were highly variable, which infects from 10% to 85% of the tested *Salmonella* strains.

Phage \ Strains	USDA4 Anatum	FC1033C3 Anatum	S2029C2 Anatum	S2028C1 Anatum	FD 1001A1 Muenchen	H2006-1 Cerro	08-022 Dublin	SGSC 2475 Enteritidis	3115 Enteritidis	SGSC 2480 Heidelberg	3117 Heidelberg	USDA3 Montevideo	H1042-3 Montevideo	USDA2 Newport	10-014 Newport	330-1 Reading	USDA1 Typhimurium	3116 Typhimurium	LT2 Typhimurium	USDA5 Kentucky
Sasha	4	4	4	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Season5	4	0	4	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Season6	0	3.3	0	0	0	0	0	2.3	0	0	0	0	0	0	4	0	2	0	4	0
Sergei	4	4	4	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Season12	0	4	0	0	0	2	0	2.7	0	0	0	0	0	4	4	0	2	0	4	0
Season13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	2	0	4	0
Munch	4	4	4	3.3	0.3	0	4	4	4	3.7	4	4	1.7	2	4	2	2	0	0	0
Minion	4	2	4	4	0	0	0	3.7	0	0	0	2	0	0	0	2	0	0	0	0
Mecon	0	0	0	0	3.7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
Season24	4	0	4	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Morel	2.3	1	4	3	0	0	0	3	0	0	0	2	1.7	0	4	2	0	0	0	0
Season27A	0	4	0	0	0	2	0	3.7	0	0	0	0	0	0	4	0	2	2	4	0
Sw2	4	3.3	4	4	0	0	4	3	2	1.3	4	0	0	0	0	3.3	0	0.7	3	0
FelixO1	0	0	0	0	0	0	1	0	2	2	1.3	3	0	4	4	0	2	4	4	3.7
Melville	3	0	3	2.7	3	0	4	4	2	2	3.7	4	4	4	4	1	4	3.3	3	0

Figure 9. The host range of 15 *Salmonella* phages against 20 *Salmonella* strains were measured by spotting on soft agar overlays. Reprinted from Xie *et al.,* 2018 [96]. Phages were plated at the routine test dilution (RTD and determined as the first tenfold serial dilution of phage lysate that formed countable plaques on lawns of its own host) and 100× the RTD. Phage were tested and scored on the following criteria: phage forming >50% of the number of plaques formed on its host strain at its RTD = 4; phage forming 5% to 50% of the number of plaques formed on its host strain at its RTD = 3; phage forming a zone of confluent lysis but no individual plaques at 100× RTD = 2; phage forming individual plaques at 100× RTD = 1; and no plaque or clearing formation at either dilution = 0. The scores from three replicate experiments were averaged. To aid the reader, cells are shaded with stronger color intensity indicating a greater score. Boxed cells indicate the initial isolation and propagation host of the phage. For clarity, all values are displayed to one significant figure unless a non-zero value is present at the first decimal place.

Phage Melville was found to have the broadest host range since it is capable of infecting 85% (17/20) of strains tested and able to efficiently infect (defined here as a score >3, which corresponds to an EOP of ~0.05–0.5) 13 strains. Phage Mecon was found to have the narrowest host range by using this method, which capable of efficiently infecting only its own host and lysing one other strain.

Phage Felix O1, a well-studied broad host range phage, was previously shown to lyse 85.3% (191/224) of *Salmonella* strains and 5.9% of *Escherichia coli* strains when tested by a spot assay at a concentration 6×10^{10} PFU/mL [72]. In the present study, this phage was able to infect only 55% (11/20) *Salmonella* strains tested when applied at concentrations of ~10^5 PFU/mL or less. This difference illustrates the importance of assay parameters in estimating the host ranges of phages since application of high phage titers can tend to overestimate bacterial sensitivity to phage [119]. Consistent with this observation, Welkos *et al.* (1974) further showed that spot testing Felix O1 at 10^{12} PFU/mL resulted in an increase of the apparent sensitivity of *Salmonella* strains to 98.5%.

Defined as the highest dilution for the phage to produce countable plaques on its propagation host, the phage titer of the RTD in this study is slightly lower than the method described by Wilson and Artkinson [120] in which RTD was defined the lowest serial dilution for the phage to produce a confluent zone of lysis. By using both RTD and 100× RTD, this method was designed to test the ability of phages to replicate on a host strain without the false positive results due to abortive infection and lysis. Without that, it can be seen when high phage titers are applied [107] while also providing a simplified score for of EOP that is restricted to a

~2 $\log_{10}$ range [107,119]. The scoring system used in this study also helps in methodology comparison by providing a numerically consistent, semi-quantitative result.

2. Determination of Microtiter Assay Parameters

Based on the results of the spot host range assay, pilot experiments that explored the parameters for a liquid culture-based host range assay were performed. Phage-host combinations representing sensitive, intermediate, and resistant phenotypes were selected to evaluate the performance of different levels of bacterial and phage inocula in a 96-well microplate format assay (see Figure 10).

Figure 10 A,D, which show the growth of the *S.* Anatum strain FC1033C3 in the presence of phage Sasha at low (~10^5 CFU/mL) and high (~10^8 CFU/mL) bacterial inoculum levels, represent the scenario of high bacterial sensitivity to phage with a score of 4 (EOP ~ 1) in the spot host range assay (see Figure 9). The initial bacterial inoculum level had an effect on the shape of the bacterial growth curve, but in both experiments, the phage was able to produce strong control of growth in liquid culture. The level of phage inoculum had only a minor effect on the bacterial growth phenotype. In the low-inoculum condition (see Figure 10A), phages were able to suppress bacterial growth up to approximately nine hours while in the high-inoculum condition (Figure 10D), inhibition of bacterial growth was not observed until 1–2 h of the experiment with OD_{550} reaching a minimum at five hours followed by bacterial regrowth at the end of the experiment. In the low inoculum condition, the input multiplicity of infection (MOI) is relatively high (~10–10^3 PFU:CFU). Therefore, the shape of the growth curve phage likely reflects an initial killing of most of the bacterial population followed by the regrowth of a phage-insensitive mutant population after 9–10 h. On the other hand, the input MOI in the high

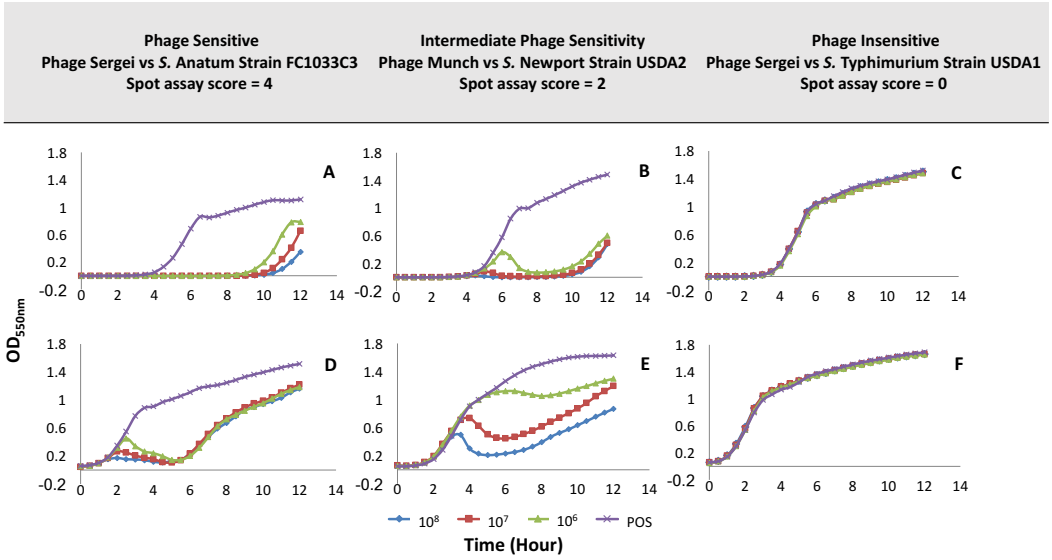

Figure 10. Sample growth curves illustrating different phage sensitivity phenotypes in the low- and high-inoculum experimental setups. Reprinted from *Xie et al.*, 2018 [96]. The X-axis indicates the OD_{550nm} and the Y-axis represents the time in hours. Panels (**A**–**C**): experiments run with the low bacterial inoculum condition (~10^5 CFU/mL); panels (**D**–**F**): experiments run with the high bacterial inoculum condition (~10^8 CFU/mL). Both conditions were challenged with phage at 10^8 PFU/mL, 10^7 PFU/mL, and 10^6 PFU/mL; POS: positive culture control with no phage. Phage-host pairs were selected based on their scores from the spot host range assay, which was shown in Figure 9.

inoculum condition is relatively low (~0.01–1 PFU:CFU), so significant lysis of the bacterial population cannot occur until the input phages have undergone multiple replication cycles to reach high enough concentrations to infect the majority of cells in the culture. When the bacterial host is highly sensitive to the phage, the low inoculum condition appears to mainly show the time until the arisal of a phage-resistant population while giving little information on factors such as the rate of phage replication. However, by using the high inoculum method, the phage must be able to replicate fast enough to outnumber, infect, and lyse a large proportion of the bacterial population in order to observe a significant reduction in OD_{550}, which provides more information on phage virulence.

A scenario of intermediate bacterial sensitivity to phage was evaluated using *S.* Newport strain USDA2 and phage Munch (spot assay score of 2, Figure 9). In the low bacterial inoculum condition (see Figure 10B), growth curves at phage concentrations of 10^7 and 10^8 PFU/mL were similar to those observed for the highly sensitive phage-host pair shown in Figure 10A. At the lowest phage concentration (10^6 PFU/mL, Figure 10B), the OD_{550} rose similar the positive control peaked at six hours and then dropped until the regrowth of the culture at ~10 h. In the high bacterial inoculum condition (see Figure 10E), the bacterial growth curve was qualitatively different from the sensitive phage-host pair (see Figure 10D), which produces a dose-dependent reduction in bacterial growth that was similar to the sensitive host at high phage concentration but weaker at the lower phage concentrations. This combination represents a situation in which the phage is able to infect its host, replicate, and produce progeny, but it may not be able to accomplish this as efficiently as observed in the Sasha/FC1033C3 phage-host pair. This

observation demonstrates that the high inoculum condition provides greater discriminatory power between intermediate phage sensitivity phenotypes.

Phage Sasha and *S.* Typhimurium strain USDA1 (see Figure 10C and Figure 10F), which represents bacterial insensitivity to phage with a spot assay score of zero (see Figure 9), yielded no observable effects of phage on bacterial growth under any condition. This would be the result expected in the case of true phage resistance where the phage is unable to interact with the bacterium and has no effect on its growth.

Based on the observations of bacterial growth inhibition in Figure 10, the high bacterial inoculum condition (~10^8 CFU/mL) with phage inoculation at 10^8 PFU/mL and 10^6 PFU/mL (corresponding to multiplicities of infection of ~1 and 0.01, respectively) were selected for conducting microtiter host range assays for the remainder of the phage collection. These parameters appeared to provide the greatest discriminatory power between high and intermediate phage sensitivity phenotypes, which was illustrated by the differences between Figure 10D and Figure 10E. The high bacterial inoculum condition demonstrated dosage effects of phage treatment with a higher observable effect on growth than was observed in the low inoculum condition. Phage concentrations of 10^8 PFU/mL and 10^6 PFU/mL were selected for further assays in order to simplify the method since the 10^7 PFU/mL condition did not appear to add any additional discriminatory power.

3. Measurement of Phage Host Range and Virulence by Microtiter Plate Assay

Using the high inoculum condition and two phage concentrations as described above, the remaining 297 phage-host combinations were tested in a liquid culture-based host range assay in the 96-well microtiter plate format. To simplify the display of this data and facilitate

comparisons, each growth curve was transformed (as shown in Equation (2) and Figure 9) into a single value representing the difference under the growth curves between the phage treatment and the positive control (see Figure 11). Since the largest standard deviation observed in any individual assay was 10.89, an assay score of greater than 10.9 was used as a cutoff to distinguish a legitimate signal from noise. Using this simple cutoff, 186 individual assays produced a positive signal in this system. Similar to the results found in the spot host range assay (see Figure 9), phage Melville displayed the broadest host range capable of infecting 16/20 (80%) of hosts tested (see Figure 11) with the highest score of 80 against *S.* Reading strain 330-1 at a phage inoculum of 10^8 PFU/mL.

A phage dosage effect was observed across this assay with greater bacterial growth suppression observed in the high phage concentration (10^8 PFU/mL) condition. As an example, phage Felix O1 was able to suppress bacterial growth in 25% (5/20) strains at a phage concentration 10^6 CFU/mL and the observed suppression expanded to 60% (12/20) of strains when the phage concentration was increased to 10^8 CFU/mL. The greatest difference in bacterial growth between the two tested phage concentrations was in the combination of phage Felix O1 against *S.* Montevideo strain H1042-3 in which the low phage concentration produced a score of 1 (bacterial growth almost identical to the no-phage control) and the high concentration produced a score of 71 (strong suppression of bacterial growth). Across all assays, the 10^6 PFU/mL phage inoculum produced 80 positive results (score >10.9) while the 10^8 PFU/mL inoculum produced 106 positive results. The overall distribution of microtiter assay scores was statistically greater in the assays with higher phage concentration (Wilcoxon/Kruskal Wallis Test, $p = 0.0141$). This dosage effect is similar in principle to the observations of Welkos *et al.* [72] in the traditional

Phage	Initial phage titer (PFU/ml)	*Salmonella* strains																				
		USDA4 Anatum	FC1033C3 Anatum	S2029C2 Anatum	S2028C1 Anatum	FD 1001A1 Muenchen	H2006-1 Cerro	08-022 Dublin	SGSC 2475 Enteritidis	3115 Enteritidis	SGSC 2480 Heidelberg	3117 Heidelberg	USDA3 Montevideo	H1042-3 Montevideo	USDA2 Newport	10-014 Newport	330-1 Reading	USDA1 Typhimurium	3116 Typhimurium	LT2 Typhimurium	USDA1 Kentucky
Sasha	10^8	62	53	60	62	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	10^6	55	48	48	55	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Season5	10^8	65	0	58	59	0	2	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
	10^6	55	0	47	54	1	4	2	1	0	0	0	0	0	0	0	0	0	3	0	0	0
Season6	10^8	1	15	0	1	2	21	0	27	4	6	7	0	0	10	11	0	5	6	42	4	0
	10^6	0	21	0	0	1	21	0	23	0	1	0	0	0	10	15	0	8	2	42	0	0
Sergei	10^8	65	55	57	63	0	2	6	0	0	0	0	0	0	0	0	0	0	1	0	0	0
	10^6	58	51	52	56	0	2	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Season12	10^8	13	10	0	0	25	80	1	49	7	13	11	4	1	79	79	2	12	12	25	3	0
	10^6	2	32	0	0	3	46	0	34	0	0	1	0	1	76	79	0	8	2	21	0	0
Season13	10^8	0	0	0	0	0	15	0	0	0	8	7	0	0	4	8	0	10	0	47	0	0
	10^6	0	0	0	0	0	0	0	0	0	0	13	1	0	6	10	0	9	0	46	0	0
Munch	10^8	9	27	9	9	19	0	17	60	27	29	0	48	45	56	17	7	31	0	0	0	0
	10^6	6	0	8	7	0	0	21	45	7	0	0	47	39	23	17	9	47	0	0	0	0
Minion	10^8	65	24	68	68	0	0	0	43	6	0	0	13	14	13	19	9	0	0	1	0	0
	10^6	50	0	53	54	0	0	0	41	1	0	0	10	12	2	16	10	0	0	1	0	0
Mecon	10^8	0	0	0	0	74	0	14	33	3	0	0	2	0	52	0	5	2	0	13	32	0
	10^6	0	0	0	0	65	0	0	5	0	0	0	1	0	16	0	0	0	0	0	0	0
Season24	10^8	53	4	54	53	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
	10^6	47	1	49	47	0	0	0	0	0	0	0	1	0	0	0	1	0	2	0	2	0
Morel	10^8	20	26	5	9	0	0	0	34	0	1	0	6	7	5	7	0	0	0	0	0	0
	10^6	34	1	9	10	0	0	0	35	0	1	0	7	8	1	10	5	0	1	0	0	0
Season27A	10^8	0	23	0	0	3	14	0	22	3	12	8	0	0	9	9	2	11	2	32	3	0
	10^6	0	31	0	0	0	14	3	19	0	2	0	0	0	11	14	1	3	2	28	0	0
Sw2	10^8	77	86	77	78	0	2	21	21	0	13	27	0	4	0	0	21	8	35	19	0	0
	10^6	73	79	68	72	1	0	21	22	0	15	21	0	1	0	0	21	0	0	21	0	0
FelixO1	10^8	0	0	0	0	10	19	65	6	4	28	18	66	71	38	24	7	27	70	64	73	0
	10^6	0	0	0	0	2	0	29	2	0	2	4	3	1	17	4	0	1	59	23	72	0
Melville	10^8	30	0	26	27	44	0	34	39	2	57	70	38	32	79	51	80	75	70	43	0	0
	10^6	10	0	15	15	38	0	27	37	0	18	68	36	32	73	45	70	65	69	40	1	0

Figure 11. Host range of 15 *Salmonella* phages against 20 *Salmonella* strains is determined by the microtiter plate liquid assay at two initial phage concentrations of 10^6 PFU/mL and 10^8 PFU/mL. Reprinted from Xie *et al.*, 2018 [96]. Values indicate average liquid assay scores calculated by Equation (2) across three replicate experiments. To aid the reader, cells are shaded with stronger color intensity indicating a greater score. Assay score represents the differences in area under the bacterial growth curve with and without phage. Larger numbers indicate a greater suppression of bacterial growth in the presence of phage. Boxed cells indicate the initial isolation and propagation host of the phage. Standard deviations of each experimental unit across this assay ranged from 0.03 to 10.89. Negative values falling within one standard deviation were adjusted to zero for the convenience and for calculating the following comparison between two host range methods.

spot assay where the testing phage at higher titer resulted in an increased apparent sensitivity to phage in *Salmonella*. This observation highlights the importance of the assay parameters on the observed phenotypes and the value of using multiple initial phage concentrations to ascertain phage host range and virulence in such assays.

An advantage of liquid culture assays over the traditional spot assay is that they measure the ability of a phage to control bacterial growth over time, which is a property typically referred to as phage virulence [136]. Measurement of virulence in this sense is an integrated result of a phage's ability to infect, reproduce within, and lyse a bacterial host. Phages with high adsorption rate constants, short latent periods, and large burst sizes would be expected to produce a stronger signal in this type of experiment [137],[138]. In the case of the microtiter assay, multiple comparisons between phage-host pairs can be made because of the standardization of the assay and the mathematical transformation of the resulting bacterial growth curves into single numerical values. This assay can, therefore, determine the ability of a phage to interact with a bacterial strain at a detectable level (the phage "host range") and also measure the ability of a phage to control a bacterial population in a liquid culture (phage "virulence").

4. Comparison Between Two Host Range Methods

The differences between the two host range methods are shown in Figure 12, which was calculated by Equation (3). Difference scores close to zero of either sign indicate high agreement between the two methods and greater deviations from zero indicate greater disagreement. Negative values (blue) indicate a greater response was observed in the microtiter plate assay while positive values (red) indicate a stronger response in the spot assay.

Phage	Initial phage titer (PFU/ml)	*Salmonella* strains																			
		USDA4 Anatum	FC1033C3 Anatum	S2029C2 Anatum	S2028C1 Anatum	FD 1001A1 Muenchen	H2006-1 Cerro	08-022 Dublin	SGSC 2475 Enteritidis	3115 Enteritidis	SGSC 2480 Heidelberg	3117 Heidelberg	USDA3 Montevideo	H1042-3 Montevideo	USDA2 Newport	10-014 Newport	330-1 Reading	USDA1 Typhimurium	3116 Typhimurium	LT2 Typhimurium	USDA1 Kentucky
Sasha	10^8	-17	0	-13	-17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	10^6	-15	0	0	-15	0	0	0	0	0	0	0	0	0	0	0	0	0	-2	0	0
Season5	10^8	-12	0	0	-2	0	-3	0	0	0	0	0	0	0	0	0	0	0	-2	0	0
	10^6	-17	0	0	-15	-2	-9	-4	-2	0	0	0	0	0	0	0	0	0	-6	0	0
Season6	10^8	-2	47	0	-2	-5	-50	0	-2	-10	-14	-17	0	0	-24	74	0	38	-14	0	-10
	10^6	0	33	0	0	-2	-50	0	8	0	-2	0	0	0	-24	64	0	41	-5	0	0
Sergei	10^8	-18	0	-4	-15	0	-4	-11	0	0	0	0	0	0	0	0	0	0	-2	0	0
	10^6	-14	0	-2	-10	0	-4	-8	0	0	0	0	0	0	0	0	0	0	0	0	0
Season12	10^8	-16	87	0	0	-32	-51	-1	5	-9	-16	-14	-5	-1	0	0	-3	35	-15	68	-4
	10^6	-3	58	0	0	-4	-11	0	23	0	0	-1	0	-1	0	-4	0	41	-3	72	0
Season13	10^8	0	0	0	0	0	-32	0	0	0	-17	-15	0	0	-9	58	0	29	0	0	0
	10^6	0	0	0	0	0	0	0	0	0	0	-28	-2	0	-13	53	0	30	0	0	0
Munch	10^8	81	44	81	64	-32	0	80	-25	44	32	100	0	-51	-67	80	42	-15	0	0	0
	10^6	87	100	83	68	8	0	55	4	85	93	100	0	-40	1	80	31	-50	0	0	0
Minion	10^8	4	15	0	0	0	0	0	29	-9	0	0	31	-21	-19	-28	37	0	0	-1	0
	10^6	7	50	2	0	0	0	0	17	-2	0	0	31	-22	-4	-30	31	0	0	-2	0
Mecon	10^8	0	0	0	0	0	0	-19	-45	-4	0	0	-3	0	-61	0	-7	-3	0	-18	11
	10^6	0	0	0	0	0	0	0	-8	0	0	0	-2	0	-25	0	0	0	0	0	54
Season24	10^8	0	-8	-2	-25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-2
	10^6	0	-2	-4	-25	0	0	0	0	0	0	0	-2	0	0	0	-2	0	-4	0	-4
Morel	10^8	-139	-256	78	0	0	0	0	-278	0	-11	0	0	-21	-56	56	67	0	0	0	0
	10^6	-257	23	43	0	0	0	0	-250	0	-10	0	-3	-23	-10	33	17	0	-10	0	0
Season27A	10^8	0	0	0	0	-13	-11	0	-3	-13	-52	-35	0	0	-39	61	-9	2	41	-39	-13
	10^6	0	0	0	0	0	5	-10	31	0	-6	0	0	0	-35	55	-3	40	44	10	0
Sw2	10^8	32	0	32	31	0	-2	97	66	61	30	90	0	-5	0	0	76	-9	-23	69	0
	10^6	29	0	21	30	-1	0	95	63	61	20	95	0	-1	0	0	73	0	18	64	0
FelixO1	10^8	0	0	0	0	-16	-30	-77	-9	44	6	4	-28	-111	41	63	-11	8	-9	0	-21
	10^6	0	0	0	0	-9	0	-101	-9	50	41	15	62	-4	81	83	0	46	-157	0	-220
Melville	10^8	40	0	42	33	19	0	61	51	47	-22	4	52	59	0	40	-76	5	-6	21	0
	10^6	61	0	54	47	23	0	63	49	50	25	-1	51	56	0	38	-71	11	-12	20	-1

Figure 12. Agreement levels between two host range methods. Reprinted from Xie *et al*., 2018 [96]. Equation (3) was used to determine agreement levels between two host range methods. One spot assay score representing one bacteria-phage combination (see Figure 9) was compared to two liquid assay scores at two different phage concentrations (see Figure 11) to determine if one phage concentration in the liquid assay yielded a difference in agreement levels. Values closer to zero indicate greater agreement between the two methods and values closer to 100 indicate disagreement. Negative values indicate greater bacterial sensitivity to phages observed in the liquid assay and positive values indicate greater sensitivity observed in the spot assay. Color intensity indicates greater divergence from zero in either direction, with blue shades indicating negative values and red shades indicating positive values. Boxed cells indicate the initial isolation and propagation host of the phage.

Among these 600 points of comparison (fifteen phages against twenty bacterial strains at two concentrations), the majority of the assay results (74%, 444/600) showed high agreement in which their difference scores ranged between an arbitrary limit of −20 to +20 while only 4.7% (28/600) showed high disagreement with difference scores of +/−80 or more. Among 300 phage-host combinations, over half (171/300, or 57%) of the total results were cases where both methods produced a result of phage insensitivity (score of 0 in the spot assay and less than 10.9 in the microtiter assay), which indicates that the two methods tend to support each other in answering the question of whether a bacterial strain is sensitive or insensitive to a given phage.

In 19 phage-host combinations, the spot assay indicated phage insensitivity (score = 0) but the microtiter assay produced a detectable response (score > 10.9). At the same time, 19 other phage-host combinations showed no response (score < 10.9) in the microtiter assay but a detectable response (score > 0) in the spot assay. This supports the finding that both methods are generally equally likely to detect phage sensitivity across phage-host pairs, but any given phage-host pair could show a false-negative result (compared to the other method) approximately 6% of the time in either assay. Differences in detecting phage sensitivity between the two methods were not evenly distributed across phage-host pairs. In the spot assay, 16/19 (84%) of false-negative results (where the spot assay score was 0 but the microtiter assay score was >10.9) were confined to the results of five phage isolates. In the microtiter assay, 11/19 (58%) of false-negative results (where the microtiter assay score was less than 10.9 but the spot assay score was >0) were confined to only two phages. This indicates certain phage isolates may fail to provide a response in either method but this can only be determined empirically. For example, phage Munch was capable of producing a response on 16 of 20 tested strains in the spot assay (see Figure 9), but

this was modulating bacterial growth in the microtiter assay in 11/20 strains at the 10^8 PFU/mL inoculum and in 7/20 strains at the lower 10^6 PFU/mL inoculum (see Figure 12). While the lack of signal does not definitively rule out the ability of a phage to interact with the test strain, it does indicate that the phage cannot efficiently infect and lyse the strain, which is a primary concern when selecting phages for potential use in therapeutic applications.

When disagreements arose between the two methods, the spot assay tended to indicate higher levels of phage sensitivity. Of the 156 difference scores greater than 20 or less than −20 (see Figure 12), 108 (69%) were positive, which shows a greater response in the spot assay than in the microplate assay. In the level of high disagreement (difference scores greater than ±80), 19 out of 28 difference scores (68%) were positive, which shows that the ability of a phage to form plaques or clear zones on agar plates does not always confer the ability to suppress bacterial growth in liquid culture. For example, phage Munch, which showed high efficiency of plating against four *S.* Anatum strains in the spot assay with scores of 3.3 to 4 (see Figure 9), largely failed to inhibit bacterial growth of those strains in the microtiter assay (see Figure 11). The disagreement between the two methods also appeared to be associated with broad host-range phages and with slightly more than half (87/156) of all disagreement scores ≥ ±20 associated with only four phages: Munch, Sw2, FelixO1, and Melville.

The ability of a phage to suppress bacterial growth in liquid culture is largely due to the integrated result of its adsorption rate, latent period, and burst size, which may be modulated by the physiological state of the host culture. Plaque formation is an analogous but not identical process. The spatial structure imposed by the soft agar overlay and its effects on phage diffusion play significant roles in the formation of plaques that can be observed by the unaided eye

[123,139]. Phages with lower adsorption rates can actually produce more robust plaques, which is observed in the case of coliphage lambda PaPa and produces larger and more visible plaques due to the loss of its side tail fibers [140].

Strongly negative difference scores (i.e., less than ~−100) tended to be produced when a phage was more effective against a test strain than on its own host in the microtiter assay since all scores were normalized to the result on their propagation host. This result produces an over-unity microtiter assay score that can exceed even the highest possible score from the spot assay, which turns into a strongly negative difference score. In the case of most intermediate negative difference scores (i.e., from −20 to ~−100), the phage produced only a weak signal in the spot assay but was able to strongly control bacterial growth in the microplate assay. Hyman and Abedon [107] suggested that liquid culture-based host range methods can be used to determine the host ranges of phages with poor ability to form plaques on solid media. Phages with poor diffusion through soft agar overlays, very high adsorption rates, or long latent periods would be expected to produce smaller plaques with a potential impact on plaque-based measurements of phage sensitivity [123].

Conclusion

The majority of host range results produced by two methods agreed with one another, and the microtiter host range assay was generally able to determine phage host range at the same level of sensitivity as the conventional agar overlay spot method. This result indicated that the microtiter plate method developed here could serve as an alternative to the conventional agar overlay spot method for basic determination of phage host range. The evaluation of phage host

range in the liquid culture-based microtiter assay provides several advantages over the traditional spot assay: it is repeatable, it eliminates the need for (often subjective) visual inspection of plaques, and provides information on both phage host range and phage virulence in a single assay. If desired, the large amounts of data generated by this method may be transformed into a single numerical value for inter-assay comparisons. Dosage effects were observed across the panel of phage-host combinations in the microtiter assay, in which testing phages at higher titers resulted in an increased apparent bacterial sensitivity to phage. This observation demonstrates the importance of assay parameters on the observed phenotypes. Conducting such assays with at least two phage concentrations is beneficial for determining phage host range and virulence characteristics.

CHAPTER III

CHARACTERIZATION OF *SALMONELLA* BACTERIOPHAGES AND APPLICATION TO REDUCE *SALMONELLA* ON CATTLE HIDE AND IN SOIL FROM CATTLE FEEDLOTS

Introduction

In the United States, foodborne illnesses caused by *Salmonella* are estimated to number more than 1.2 million each year in the United States, with more than 23,000 hospitalizations and 450 deaths [13]. The United States Department of Agriculture (USDA) Economic Research Service (ERS) indicates that *Salmonella* imposes the greatest cost among 14 major foodborne pathogens investigated in the United States, with an estimated annual economic cost of $3.4 billion caused by *Salmonella* illness [21,22]. *Salmonella* is associated with a wide range of food commodities and beef was identified as a transmission vehicle in 5% (96/1,965) of *Salmonella* outbreaks during 1973 – 2011, in which ground beef contributed to 23% of beef-related *Salmonella* outbreaks [141]. Previous study indicated that commercial ground beef in the United States is contaminated with *Salmonella* at 4.2% [29]. While applying antimicrobial interventions, such as sodium hydroxide, sodium sulfide, chlorine, lactic acid and acetic acid, have been reported to reduce *Salmonella* prevalence on beef carcasses from 50.2% to 0.8%, there is little effect on ground beef made from edible beef trimmings [30,31]. Lymph nodes, which commonly present in lean trimmings destined for ground beef productions, could harbor *Salmonella* in cattle without displaying clinical symptoms of illness [32]. This finding implies that asymptomatic animal carriage of *Salmonella* may contributes to contamination of ground beef [29].

Two major theories of *Salmonella* colonization of lymph nodes have been proposed in previous studies: 1) *Salmonella* is transmitted to lymph nodes via cattle intake of contaminated water or feeds and 2) transdermal transmission in which *Salmonella* is introduced via insect bites or skin abrasions occurs. Pullinger *et al.* demonstrated the possibility of translocation of *Salmonella* from the distal ileum through lymphatics via type III secretion 1 (T3SS-1) [142]. This hypothesis was later tested in a *Salmonella* oral challenge study by Brown *et al.* [38], in which calves were orally inoculated either with a single high dose (~10^{10} CFU) of *S.* Montevideo or daily low dose (average $7.1x10^4$) during a fourteen-day experiment. Peripheral lymph nodes collected and examined for *Salmonella* prevalence at the end of this experiment showed both single high dose and subsequent low dose inoculation resulted in colonization of peripheral lymph nodes of calves at harvest, with greater percentage of *Salmonella* positive lymph nodes (62.5%) observed compared to daily low dose (12.5%) [38]. This same team conducted research on the hypothesis of transdermal *Salmonella* transmission via two challenge models. In the first study, *S.* Senftenberg was inoculated to calves intra- and/or trans-dermally by applying a skin-allergy instrument over various ventral regions of skin, resulting in the predictable recovery of *Salmonella* from draining of region specific lymph nodes [36]. The *Salmonella* positive result was persistent for eight days post challenge in this study [36]. The following experiment assessed the concept of transdermal transmission by challenging cattle with horn flies that were previously fed with a blood meal containing *S.* Senftenberg [37]. The result showed that 8% lymph nodes were *Salmonella* positive after 5-day exposure, whereas 50% were positive from 11-day exposure. This study implied that prolonged exposure to *Salmonella* containing flies has a significant impact on percentage of positive culture of sampled lymph nodes [37].

Although theories of *Salmonella* transmission and colonization of lymph node have not been conducted in combination systematically, exposure to *Salmonella* within beef cattle feedlot environment throughout the feeding operation seems to serve as the origin despite the various transmission routes. Researchers explored the impact of feedlot environmental *Salmonella* prevalence on transmission during different feed stages [39]. In this study, calves at weaning stage (stage 1) were separated at two feedlots, one with positive and one with negative *Salmonella* prevalence historically [40,41]. Calves were harvested at the end of stage 2 (stocker), 3 (60 days on feed) and 4 (120 days on feed), and lymph nodes were collected for the examination of *Salmonella* prevalence. Results from this study indicated that prevalence of lymph nodes of cattle fed in *Salmonella*-positive feedlot increased as cattle moved into later stages of feeding, at 22%, 78% and 94.4% for feeding stages 2, 3 and 4, respectively, while cattle fed in the *Salmonella*-negative feedlot remained negative during all feeding stage. With negative *Salmonella* prevalence in lymph nodes of cattle from both treatment groups at stage 1, this result implied that environmental *Salmonella* prevalence during feeding operation has influences on prevalence of *Salmonella* in lymph nodes of cattle at harvest [39].

Bacteriophages (phage) are viruses that infect bacteria, and are the most abundant form of life on earth, estimated to number some 10^{31} to 10^{32} organisms in total [48,49]. Phages are ubiquitous in natural environment as well as in plants and animals as a part of their normal flora. Feeding environments of both beef and dairy cattle were previously identified as reservoirs of *Salmonella* phages [41,90,143]. A study on the prevalence of *Salmonella* and *Salmonella* phages in beef cattle feedlots indicated that more than 90% of soil and fecal samples harbor at least phage capable of infecting at least one *Salmonella* host, implying that soil and feces are major

reservoirs of phage among four environmental sample type (soil, feces, feed and water) [41]. Phage isolated from these feedlot environmental samples displayed a diverse host range phenotypes, with phages ranging from infecting 10% to 85% of the tested *Salmonella* strains [96]. Switt *et al.* conducted a research study on genomic characterization of 22 *Salmonella* phage isolated in dairy farms and were able to identify a high level of genomic diversity among phages sequenced [144]. Phages characterized in this study belong to genus including *Viunalikevirus, Felixounalikevirus*, *Sp03unalikevirus, Chilikevirus* and *Jk06likevirus* as well as a new viral genera *Sp062likevirus* that has been proposed to the International Committee on Taxonomy of Viruses (ICTV) [144].

Phages are non-pathogenic to humans and are normal residents of the human microbiome [48,49]. The increasing spread of bacterial resistance to antibiotic has become a worldwide threat, resulting the renewal of interest in exploring bacteriophage as a potential alternative to control pathogenic bacteria in Western countries [58].

The U.S. Department of Agriculture (USDA), Food and Drug Adminstration (FDA) and Environmental Protection Agency (EPA) have approved phage-based commercial products that are now available as antimicrobial interventions in food production. In the category of post-harvest interventions, EcoShield™, a bacteriophage preparation produced by Intralytix Inc., obtained regulatory approval from the FDA through a "Food Contact Notification" (FCN No. 1018) for use on raw meat cuts and trim to control *E. coli* O157:H7 prior to grinding [61]. SalmoFresh™ from the same company gained approval with generally recognized as safe (GRAS) for application on poultry products for reducing *Salmonella enterica* population (GRN No. 435.). Micreos Food Safety (Wagentingen, Netherlands) currently launched two products,

PhageGuard Listex™ and PhageGuard S™, which has been approved GRAS and certified to be used in organic production. Similar to ListShield™, PhageGuard Listex™ is used as a processing aid for control of *L.* monocytogenes. According to Microes, PhageGuard S™ is intended to be used as a spray on food post harvested at points of contamination during processing and is expected to achieve 1.0 – 3.0 $\log_{10}$ reduction of Salmonellae. The pre-harvest intervention, Finalyse®, produced by Passport Food Safety Solutions, Inc., was approved for application on the hides of beef cattle to reduce *E. coli* O157:H7 prior to slaughter [59]. This product was recommended to be applied via a misting system in which cattle walk through the spraying system targeting full coverage of the animal before harvest. In the environmental biocontrol aspect, AgriPhage™, a phage cocktail produced by Phagelux Inc. (previously OmniLytics Inc.), gained approval from the Environmental Protection Agency for application on growing produce in the field to reduce plant pathogenic bacteria [57].

Previous antimicrobial efficacy studies on *Salmonella* phages demonstrate their capacity in reducing *Salmonella* population in poultry production. Described by Atterbury *et al*., oral administration of phages were able to significantly reduce *Salmonella* colonization, with the one selected phage reducing cecal colonization by ≥ 4.2 $\log_{10}$ CFU and the other by ≥ 2.2 $\log_{10}$ CFU within 24 h [129]. Other research studied the ability of phages to reduce *Salmonella* on chicken skin at 10^9 PFU/mL for 30 min in comparison with chemical decontamination agents (200 mg/L sodium dichloroisocyanurate for 10 min; 100 mg/L peracetic acid for 10 min and 2% (v/v) lactic acid for 90s), resulting in similar reductions of about 1 $\log_{10}$ CFU/cm^2 observed [145]. A study conducted by Grant *et al.* evaluated the antimicrobial efficacy of a commercial available product, Salmonelex™ (Micreos), on reducing *Salmonella* concentration in ground chicken, in which

ground chicken associated *Salmonella* isolates showed reductions of 0.4 Log_{10} CFU/cm^2 and 0.7 Log_{10} CFU/cm^2 reductions after 30 min and 8 h, respectively ($P < 0.05$) [146]. A previous National Pork Board funded research on the effectiveness of oral phage treatment in preventing *Salmonella* infection in pigs and/or reduce *Salmonella* population in infected pigs showed that the administration of 10^{10} phage Felix O1 were able to significantly reduce *Salmonella* in cecum by 1.59 log_{10} PFU per sample [147]. Antimicrobial efficacies of *Salmonella* phages were also explored in the seafood category, in which Galarce *et al*. observed a significant reduction in raw salmon samples on days 3, 6 and 10 incubated at 18°C (from 0.75 to 3.19 log_{10} CFU/g) and at 4°C (from 2.82 to 3.12 log_{10} CFU/g) [148]. In the same study, phage treatment showed a lower reduction in smoked salmon (from 1.02 to 1.96 log_{10} CFU/g at 18°C and from 0.50 to 1.16 log_{10} CFU/g at 4°C) [148]. A post-harvest application of phages as antimicrobial strategy were also conducted in raw beef, in which a significant reduction of 2–3 log_{10} cm^{-2} at 5 °C and >5.9 log_{10} cm^{-2} at 24 °C were achieved [149].

Phages are capable of targeting bacterial hosts with high specificity by recognizing unique bacterial surface structures, leaving the remaining microbiota unharmed. The high level of specificity makes phage treatment more favorable over other broad-spectrum antimicrobials that could cause collateral damage to the microbial flora [59]. Suggested by Garcia *et al*. [57] , phages can be applied to all stage of food production from “farm to fork” to prevent foodborne infections [59]. In the current study, we performed characterization of four lytic *Salmonella* phages with distinct genotypes, including their morphologies, infection kinetics, and genomic analysis. In addition to their basic characteristics, we also examined their cross-resistance in order to formulate phage cocktails capable of overcoming phage resistance. Finally, we

conducted efficacy studies on their antimicrobial capacities using two models, a cattle hide model and a soil model, in order to reduce the transmission of *Salmonella* from the beef cattle feedlot environment to the final product, with intention of improving the microbiological safety of ground beef.

Materials and Methods

1. Bacterial Strains and Culture Conditions

S. Anatum strain FC1033C3 was isolated previously in fecal samples from a cattle feedlot located in south Texas [41]. *S.* Montevideo strain USDA3 and *S.* Newport USDA2 were obtained from T. Edrington (USDA, College Station, TX). A nalidixic acid-resistant *S.* Anatum strain, which was used in efficacy testing models of phages, was obtained by plating an overnight culture of *S.* Anatum strain FC1033C3 on tryptic soy agar (TSA) (TSB plus 1.5% w/v Bacto agar (Becton-Dickinson)) supplemented with 25 mg/l nalidixic acid, incubating at 37 ºC overnight and selecting for surviving colonies. Bacteria and phages were enumerated in tryptic soy broth (TSB) (Becton-Dickinson) or tryptic soy agar (TSB plus 1.5% w/v Bacto agar (Becton-Dickinson)) aerobically at 37 ºC.

2. Bacteriophage Strains and Culture Conditions

Isolation of phage Sergei, Season12 Munch and Sw2 was described in a previous study [96]. High-titer phage stocks were prepared by the confluent plate lysate method [135]. Phage stocks were diluted in phage buffer (100 mM NaCl, 25 mM Tris-HCl pH 7.4, 8 mM $MgSO_4$, 0.01 % w/v gelatin) to achieve concentrations at 10^8 and 10^9 PFU/mL and stored at 4 ºC. Phage

stocks were adjusted in phage buffer to achieve concentrations of 10^8 and 10^9 PFU/mL before use in the hide and soil models.

3. Transmission Electron Microscopy Imaging

Transmission electron microscopy of phages were performed by staining virions with 2% uranyl acetate and imaging in a JEOL 1200 EX transmission microscope operating at an acceleration voltage of 100 kV as previously described [150,151]. Head dimensions and tail length were measured using ImageJ [152,153] and standardized against images of a carbon grating replica of known dimensions (Ted Pella, cat# 607). Virion head width was measured face to face perpendicular to the axis of the tail, and head height was measured from vertex to vertex from the top of the tail to the top of the head.

4. Genomic DNA Extraction, Sequencing and Bioinformatic Analysis

Phage genomic DNA was prepared by using a modified Wizard® DNA Clean-Up System (Promega, Madison, WI) [151,154]. Genomic DNA were stored at 4 ºC before use.

Bacteriophage genomic DNA was sequenced as paired-end 250 bp reads using the Illumina MiSeq platform. FastQC (bioinformatics.babraham.ac.uk), FastX Toolkit (hannonlab.cshl.edu), and SPAdes 3.5.0 [155] were used for read quality control, read trimming, and read assembly, respectively. Genome closure were achieved using PCR and Sanger sequencing of the products. Glimmer3 [156] and MetaGeneAnnotator [157] were used to predict protein coding genes and manually corrected, while tRNA genes were predicted using ARAGORN [158]. Putative protein functions were assigned based on sequence homology detected by BLASTp [159] and conserved domains detected by InterProScan 5 [160]. Analyses were performed via CPT Galaxy [161] and WebApollo [162] interfaces (cpt.tamu.edu).

5. Growth Kinetics

Determination of adsorption rate were performed as previously described [135]. A mid-log culture of *S.* Anatum strain FC1033C3 (OD_{550} ~ 0.25) was infected with phage at a multiplicity of infection (MOI) of ~0.01 and incubated at 37 ºC statically. Aliquots were taken periodically and diluted 100X in phage buffer to stop adsorption. Diluted aliquots were centrifuged at 18,000 g for 5 min and the supernatants that contained unadsorbed phages were titered. The values of log percent free phage at each time point were plotted against time, and the slopes were obtained from a linear regression line generated in Microsoft Excel. The adsorption rate constant *K* for each phage was calculated as (-slope)/(bacterial concentration) [135]. Three biological replications were performed for each phage.

One-step growth experiments were performed using a modified method of Adams [135]. A mid log culture of *S.* Anatum strain FC1033C3 (OD_{550} ~ 0.5) was combined with phage at MOI ~0.01 and allowed adsorption for 10 min, followed by 100X dilution in phage buffer and centrifugation at 18,000 g for 5 min. Supernatants containing unadsorbed phages were removed. Pellets containing phage-infected cells were resuspended in TSB and incubated at 37 ºC. Aliquots were taken periodically and immediately titered by the soft agar overlay method [135]. The phage latent period was defined as the mid-point of the rise period between the end of the initial adsorption period and lysis [163]. Phage burst size was calculated as the average yield of PFU per infected host cell [135]. Three biological replicates were performed for each phage.

6. Characterization of Cross Resistance of Phages

Phage-resistant mutants of *S.* Anatum FC1033C3 were selected by co-culturing the bacterium with each of four phages individually and isolating surviving bacterial colonies from

TSA plate. Efficiency of plating (EOP) of the remaining phages was performed against each phage-resistant strain to determine if resistance to one of the test phages conferred resistance to other phages in the collection [96]. Two phages with independent resistance were combined and evaluated for their efficacy of suppressing *S.* Anatum in *vitro* assay.

Briefly, a standardized inoculum (~10^5 CFU/mL) obtained by adjusting fresh overnight cultures OD_{550nm} ~0.5 and diluting 1000-fold in TSB was placed in 96-well microtiter plates and challenged separately with single phages or phage mixtures at concentrations of 10^8 and 10^6 PFU/mL. The plates were incubated at 37 °C with double orbital shaking in a Tecan Spark 10 M plate reader (Tecan Group Ltd., Männedorf, Switzerland) and growth was monitored by measuring OD_{550nm} at 30-min intervals for 12 h. Growth curves were achieved by plotting OD after baseline adjustment against time. Three biological replicates were performed in this assay.

7. Efficacies of Phages on Reducing *Salmonella* Population in Two Testing Models

Previous work has shown that the feedlot environment and cattle hide are major reservoirs of *Salmonella* that could subsequently contribute to the colonization of lymph nodes of cattle [33,41].Two testing models, cattle hide model and soil model, were used in the current study for efficacy testing of the antimicrobial capacities of phages. Mentioned in multiple previous studies, *S.* Anatum was the most frequently found sevovar in cattle feeding environment and therefore, was selected in this study as the model bacterial strain.

Phage Sergei, Season12, Munch and Sw2, representing four distinct genotypes, were used against the model strain in efficacy studies in two models mentioned above. In addition, two phage combinations, Sergei and Mucnch as well as Sergei and Sw2, were also selected for efficacy testing in two models.

7.1 Cattle Hide Model

Overnight culture of nalidixic acid marked *S.* Anatum strain FC1033C3 was centrifuged, washed three times with peptone water and inoculated into a sterile gelatin-based slurry to mimic the adherent properties of soil and fecal contamination [164]. Cattle hide pieces were obtained from Texas A&M Rosenthal Meat Science and Technology Center during harvest with a circular punch to achieve an average surface size of 70 cm^2. The slurry was then applied to freshly-collected cattle hide pieces and allowed 30 min of contact time, followed by removal of excess material. Inoculated hide pieces were then sprayed with 5 ml of individual phages and phage combination at concentrations of 10^8 or 10^9 PFU/ml and held at 37 °C for one hour. Sham treatments were performed by spraying 5 ml of peptone water onto inoculated hide pieces and held at 37 °C for one hour. Treated hide pieces were placed in filtered stomacher bags with 100 ml of peptone water and homogenized in stomachers for 60 seconds. Homogenized mixtures were centrifuged at 8,000 x g for 10 min and pellets were suspended in peptone water, serially diluted and spread on xylose lysine deoxycholate agar (XLD) supplemented with 25 mg/l nalidixic acid and 0.1% cycloheximide. Plates were incubated at 37 °C for 18 hours and plate counts were performed.

7.2 Soil Model

The bacterial inoculum was prepared as described above. Soil were collected in a cattle feedlot located in College Station, TX. Soil was sterilized by autoclaving at 121 °C for 30 minutes for three times, and 10 g aliquot of sterilized soil was placed into a standard 90 mm petri-dish. Inoculation of soil was achieved by spraying 3mL of inoculum on the soil aliquot in the Petri-dish, followed by a 30 min attachment at 37 °C. Inoculated soil samples were then

sprayed with 3 mL of individual phages and phage combinations at concentrations of 10^8 or 10^9 PFU/ml and held at 37 °C for 1 hour or 24 hour treatment periods. Treated soil samples were placed in filtered stomacher bags with 100 ml of peptone water and homogenized in stomachers for 60 seconds. Homogenized mixtures were centifuged at 8,000 x g for 10 min and pellets were suspended in peptone water, serially diluted and spread on xylose lysine deoxycholate agar (XLD) supplemented with 25 mg/l nalidixic acid and 0.1% cycloheximide. Plates were incubated at 37 °C for 18 hours and phage plaque counts were performed.

In order to understand the dynamic of reduced efficacies of phage treatments observed in the soil model, a standard sand (MilliporeSigma, Burlington, MA) model was performed on one selected phage, Sw2, with the same experimental method. This additional experiment in sand was intended to provide an inert condition to study the interaction between bacteria and phage in a fine particle matrix with high total surface area that mimics the physical property of soil.

8. Statistical Analysis

Bacterial survival from different phage treatments in two testing models were analyzed for differences between treatments by one-way analysis of variance (ANOVA) at $\alpha = 0.05$ via JMP v12.1.0 (JMP® Statistical Discovery™ From SAS, Cary, NC). Significantly differing bacterial concentrations were separated by Student's t-test ($P < 0.05$).

Results

1. Bacteriophage Morphology

Images of four phages via TEM are shown in Figure 13. Phages Sergei, Season12 and Sw2 belong to the family *Siphoviridae* with flexible non-contractile tails, while Munch appears

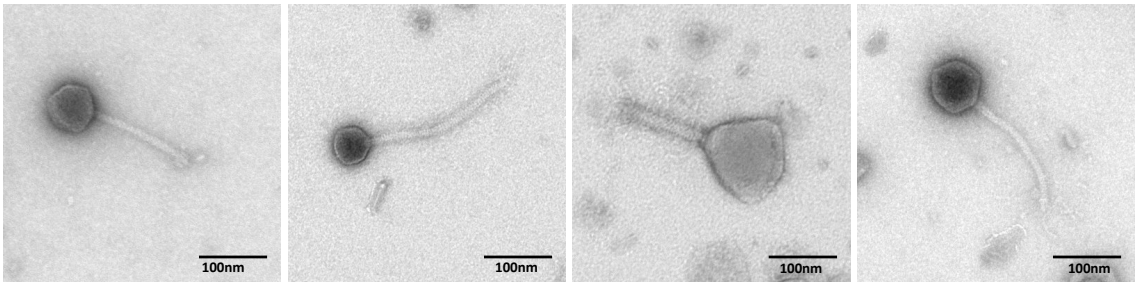

Figure 13. TEM images of four phages. TEM of phages were performed by staining the virions with 2% uranyl acetate and imaging in a JEOL 1200 EX transmission microscope operating at an acceleration voltage of 100 kV as previously described [150,151]. Black lines indicate 100 nm. Four images from left to right were obtained from Sergei, Season12, Munch and Sw2, respectively.

to be a *Myoviridae* with a contractile tail. Morphologically, phage Sergei has an icosahedral head of 64.4 nm in diameter and a tail of 156.2 x 10.6 nm in size; phage Season12 has an icosahedral head of 52.9 nm in diameter and a tail of 231.1 x 11.8 nm in size; phage Munch has a slightly elongated head with 118.8 nm in length and 98.5 nm in width, alone with a tail of 125.3 x 23.2 nm in size; phage Sw2 has an icosahedral head of 79.5 nm in diameter and a tail of 185.2 x 8.3 nm in size.

2. Genomic Analysis

Genome maps of Season12, Munch and Sw2 are shown in Figure 14A. 14B and 14C, respectively. Genome map of Sergei presented by Zeng *et al.* [165].

Phage Sergei belongs to a group of closely related phages that was proposed to be grouped as a new virus genus *9NAlikeviruse* by Zeng *et al.* [165]. Genomic characteristics of this phage include a genome size of 56,051 bp with a GC-ratio of 43.5%. The assembly of this phage runs into a single circular contig, implying direct termini contained in the virion genome [165]. Annotation predicts 91 protein coding genes in Sergei, in which (59/91) 64.8% are hypothetical proteins with no assignable function. No tRNA genes were found in the genome of Sergei [165].

Phage Season12 is closely related to phage Chi (KM458633) the type phage in the genus of *Chilikevirus* [89]. The genome of Season12 is 59,059 bp in length with a GC content of 56.5%. The assembly of this genome was a single linear contig. At the left (5') end of the chromosome of phage Season 12, it has 12-bp predicted 5'-overhanging cohesive (*cos)* ends with the sequence 5'-GGTGCGCAGAGC that is conserved with phage Chi and other Chi-like phages [89,166]. There are 76 protein coding genes predicted in the genome of Season12, in which 50 are hypothetical proteins. No tRNA genes were detected in the chromosome of Season12. Three

1 kb tandem repeats were identified from 33834 – 36947 bp located downstream of the tail fiber protein.

Phage Munch is a so-called “jumbo” phage with a large genome size of 350,103 bp and a relatively low GC content of 35.6%. Its genome has 532 predicted protein coding genes and 22 tRNA sequences. Of these predicted protein coding sequences, only 118 were given a putative function. Munch is a relatively novel phage with 159 predicted coding sequences that share no homology to any sequence in the NCBI database. In the genome of Munch, three region containing repeated sequences were found via tool Dotmatcher on CPT Galaxy bioinformatic platform (Figure 15) [161]. Protein sequences from these three region were further compared using BLASTp [159]. Genes from the first repeat region located in the first 20 kb of the genome did not display detectable similarity in protein sequences, suggesting that if these proteins are the result of gene duplication, this event would have occurred in the distant past. The second repeat region was located within the tail fiber protein gene (see Figure 14B), although tandem repeats was detected in DNA sequencing level, there is no obvious protein repeated motif identified in the gene product. Interestingly, the last repeat region with spend the right most 20 kb of the genome contains 13 tandem repeats of a gene encoding a predicted DNA condensation protein. A 21296bp of direct terminal repeat were identified via tool PhageTerm [167]. The most related phage to Munch found in the database is phage 121Q (KM507819.1) that is 55.1% identical in DNA sequence according to Emboss Stretcher [168].

Phage Sw2 is closely related to the well-studied lytic *Siphoviridae*, T5 [81]. Phage Sw2 has a genome of 114,274 bp in size with a GC-ratio of 40.2%. A 8123 bp-long terminal repeat sequence was determined by the tool PhageTerm [167]. The annotation algorithm predicted 197

A.

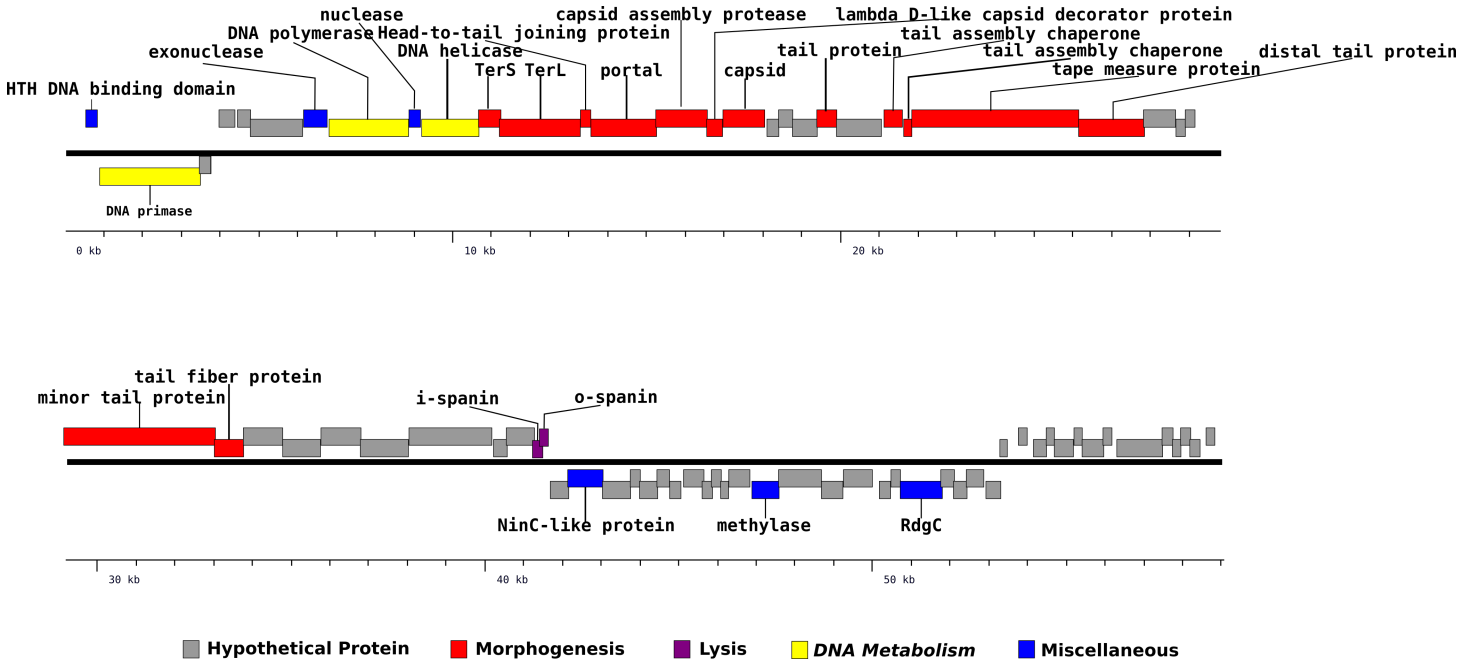

Figure 14. Genome maps of Season12, Munch and Sw2. Genome maps of Season12, Munch and Sw2were shown in figure 14A, 14B and 14C, respectively**.** Maps of phage genomes were obtained by the tool Genome Mapper via CPT Galaxy [161] and WebApollo [162] interfaces (cpt.tamu.edu).

B.

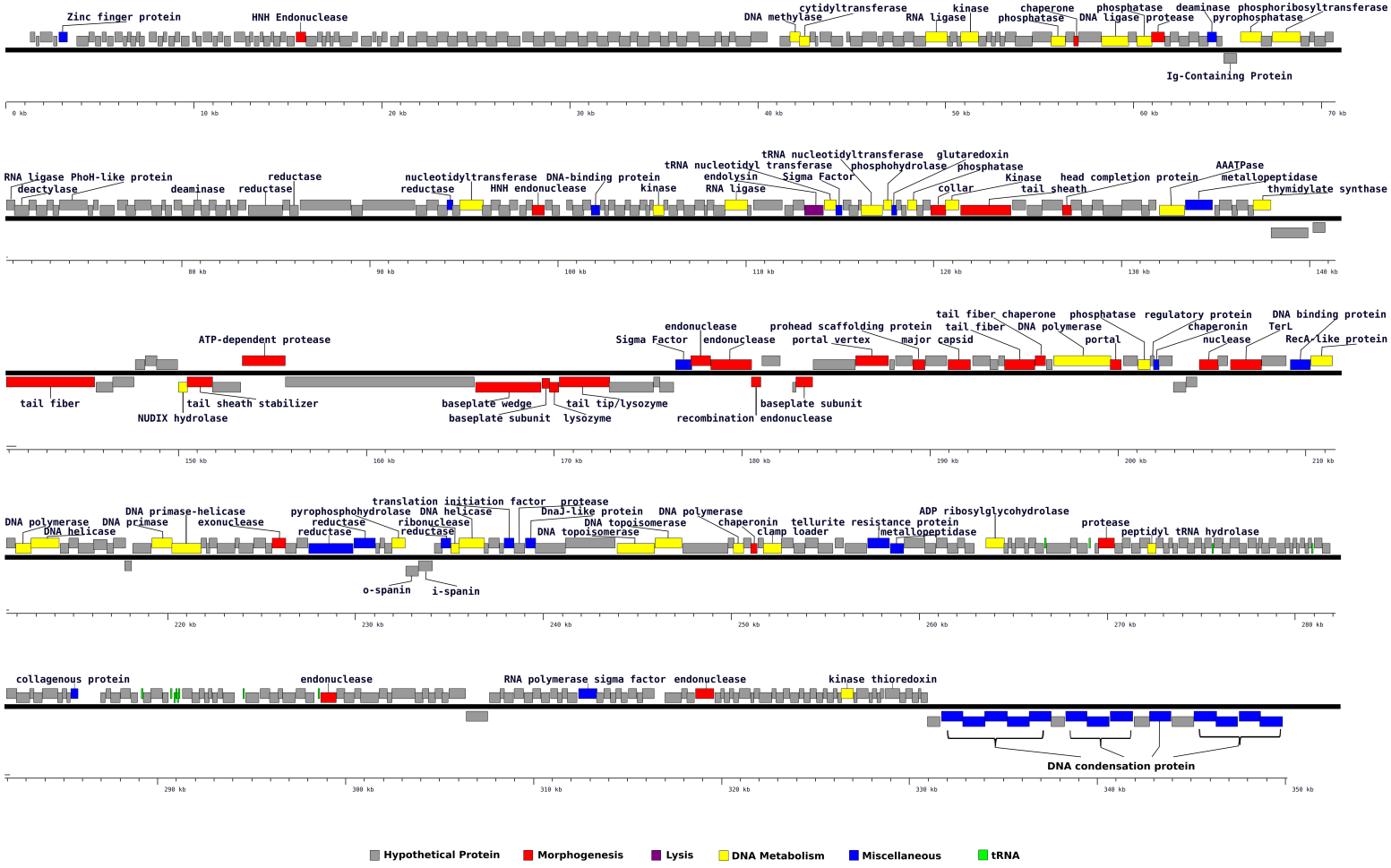

Figure 14, Continued.

C.

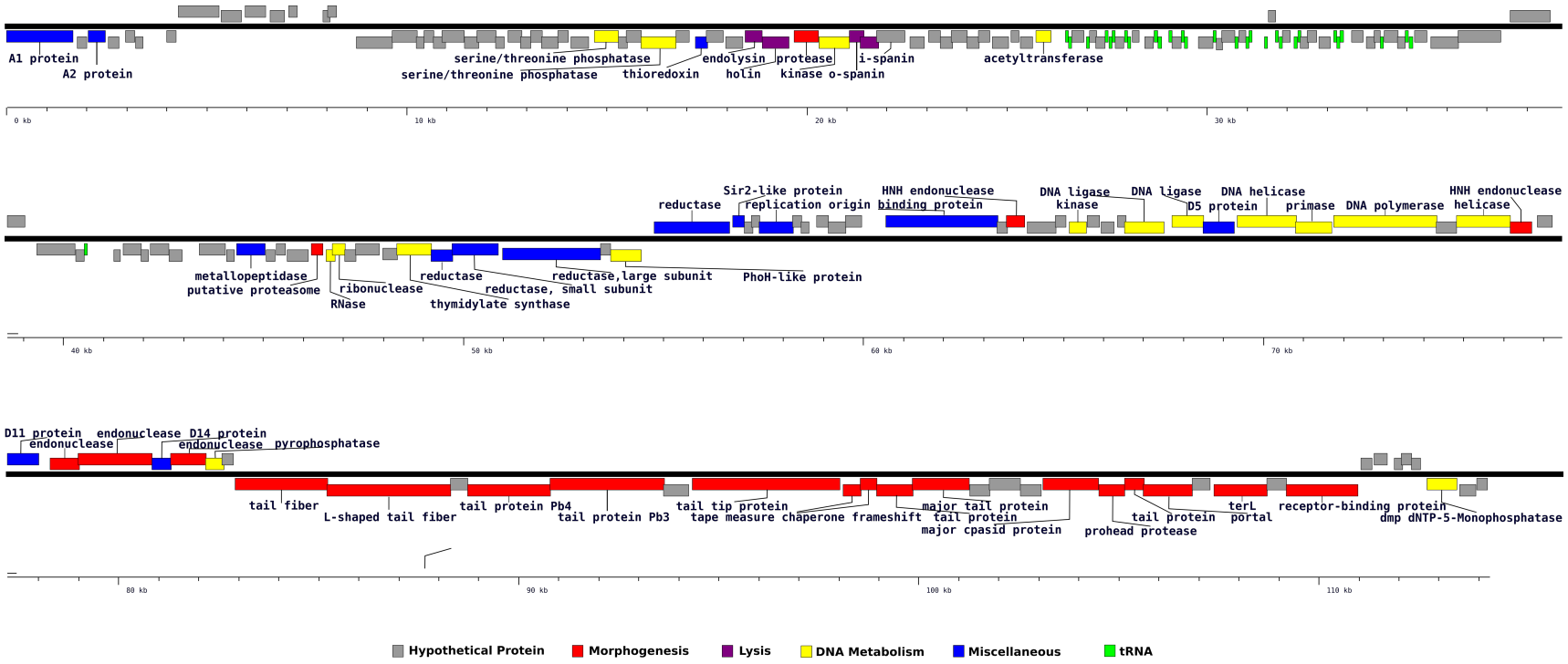

Figure 14, Continued.

protein coding genes, in which 82 were given a putative function. There are 29 tRNA sequences annotated in the genome of Sw2.

3. Growth Kinetics

Adsorption rate, latent period and burst size were obtained by protocols described in Materials and Methods and result of these growth kinetic parameters are summarized in Table 4. The greatest standard deviation of the phage absorption rates observed is 9.58×10^{-10} and the R^2 of the regression line obtained to calculate the slope needed for the calculation for *K* ranged from 0.9443 to 0.9990. The greatest standard deviation measured in burst size across all phages is 4.84.

Table 4. Growth kinetics of phages.

Parameters / Bacteriophages	Adsorption Rate (Phage^{-1}cell^{-1}mL^{-1}min^{-1})	Latent Period (Min)	Burst Size (PFU/Infected Cell)
Sergei	1.47 x 10^{-9}	46	48
Season12	2.71 x 10^{-9}	52	39
Munch	4.70 x 10^{-9}	63	16
Sw2	1.07 x 10^{-9}	62	21

4. Characterization of Phage-Resistant Mutations

The efficiency of plating (EOP) obtained by testing each of four phages against the wild type *S.* Anatum FC1033C3 and four phage-resistant mutations of tested strains were shown in Table 5. The EOP of each phage against the wild type strain FC1033C3 was standardized as 1,

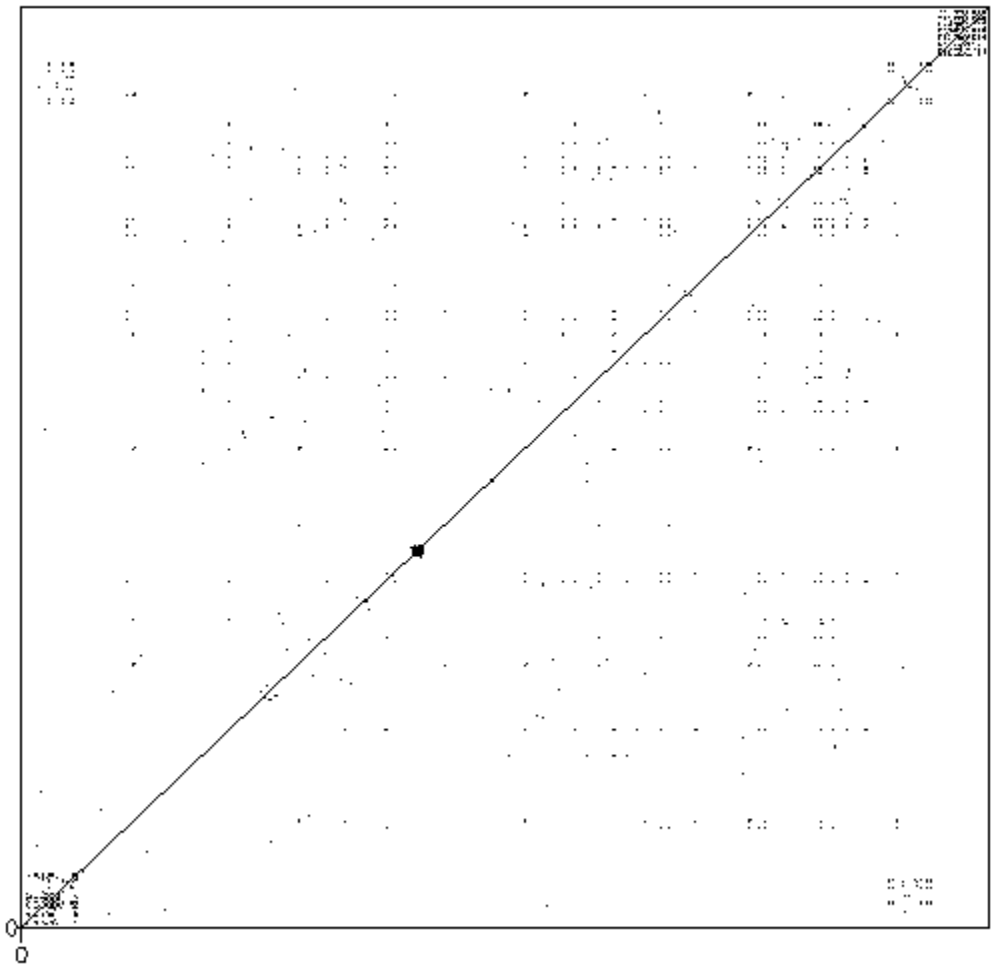

Figure 15. DNA Dot plot obtained by mapping genomic sequence of Munch. Result obtained by plotting the genomic sequencing or Munch again itself via tool dotmatcher on CPT Galaxy bioinformatic platform [161].

and the EOP of phages tested against phage-resistant mutants are shown as the ratio of EOP against wild type. According to the results shown in Table 5, two different types of phage resistant independences are observed. Phage Sergei and Munch were able to infect the phage resistant mutants of each other with EOPs close to 1, implying that Sergei and Season12 are genetically independent for phage resistance. In contrast, phage Munch was able infect the Season12-resistant mutant while phage Season12 was unable infect the Munch-resistant mutant, implying that Season12 and Munch are only partially independent for this phenotype.

5. Efficacy Testing on Single and Mixed Phages Against *S.* Anatum FC 1033C3 in a Microtiter Plate Liquid Assay

With the observation from Table 5, using the combination of two phages that are fully independent for phage resistance was hypothesized to be capable of improving antimicrobial efficacy in a microtiter plate liquid assay compared to testing single phages against the wild type strain. In contrast, using a combination of phages that are partially independent was hypothesized to be incapable of improving antimicrobial efficacy compared to using single phages. To test this hypothesis, two phage combinations, phage Sergei and Munch that are fully phage resistance independent, as well as Munch and Season12 that are partial phage resistance independent, along with Sergei Munch and Season12 alone were tested against the wild type strain according to the Materials and Methods. Results obtained from this experiment are shown in Figure 16. By testing phage Sergei, Munch and Season12 alone against the wild type *Salmonella* strain, regrowth of bacterial culture was observed starting at 7, 6 and 5 hours, respectively. Observation of bacterial regrowth is consistent with the rise of phage-insensitive mutants in the culture. By using the combination of two phages that are fully resistance independent (Sergei and Munch),

no regrowth was observed during the 12-hour experiment, demonstrating a significant improvement of antimicrobial efficacy against tested *Salmonella* strain. In contrast, by using the combination of two phages that are partial resistance independent (Season12 and Munch), no improvement of antimicrobial efficacy was observed.

Table 5. Characterization of phage cross-resistance

Bacteria / Bacteriophages	WT[1]	MutSergei[2]	MutSeason12	MutMunch	MutSw2
Sergei	1.00[3]	$<10^{-7}$	0.98	0.75	0.70
Season12	1.00	1.10	$<10^{-7}$	$<10^{-7}$	1.15
Munch	1.00	0.02	0.02	$<10^{-7}$	0.02
Sw2	1.00	1.17	1.42	1.42	$<10^{-7}$

[1.] WT stands for wild type strain of *S.* Anatum FC1033C3.
[2.] MutSergei stands for Sergei-resistant mutant of *S.* Anatum FC1033C3.
[3.] Numbers displayed indicates EOP = number of plaques formed on each phage mutation/number of plaques formed on wild type *S.* Anatum FC1033C3.

6. Abilities of Phages on Reducing *Salmonella* Population in Two Testing Models

Treatments with single and mixed phages were tested against *S.* Anatum FC1033C3 in a cattle hide model and a soil model. Preliminary study of soil model was performed by testing Sergei and combination of Sergei and Munch at concentration 10^9 PFU/mL against *S.* Anatum

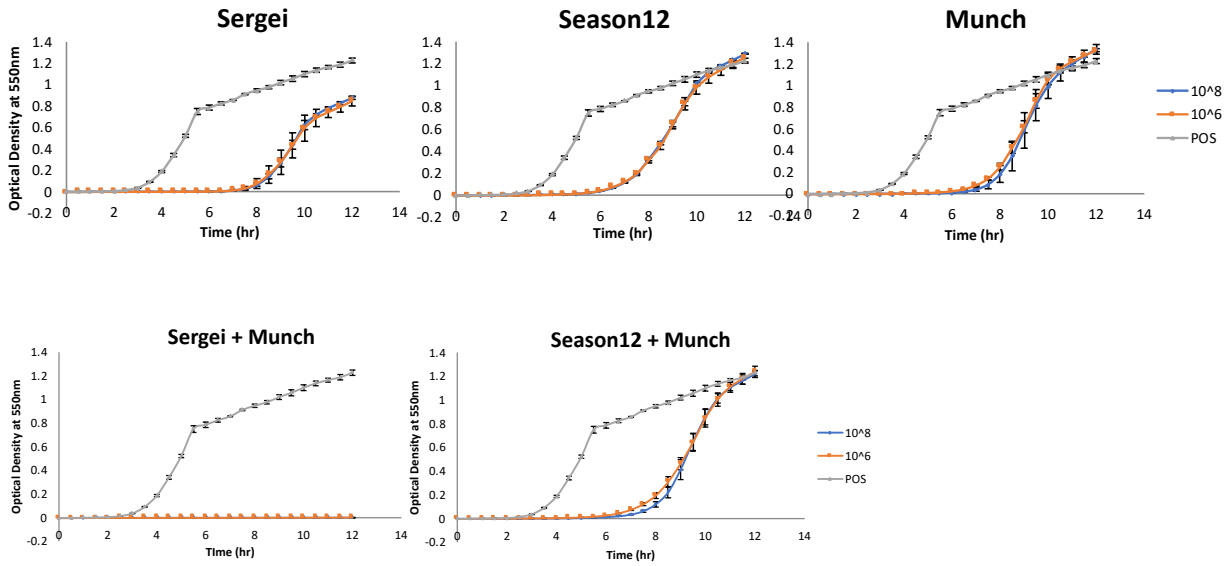

Figure 16. Efficacies of phages alone and in combination against *S.* Anatum FC1033C3 in a microtiter-plate based assay. Bacterial inoculum at ~10^5 CFU/mL was placed in 96-well microtiter plates and challenged separately with single phages or phage mixtures at concentration 10^8 and 10^6 PFU/mL. The plates were incubated at 37 °C with double orbital shaking in a Tecan Spark 10 M plate reader and growth was monitored by measuring OD_{550nm} at 30-min intervals for 12 h. Growth curves were achieved by plotting OD after baseline adjustment against time. Three biological replicates were performed in this assay. Blue, orange and gray curves represent growth profiles of phage treatments at 10^8 PFU/mL, phage treatments at 10^9 PFU/mL and positive control.

FC1033C3 and results were shown in Figure 17. Phage treatment duration greater than 24h didn't exhibit difference in bacterial reduction (see Figure 17), thus, soil experiment with 1h and 24h treatment duration were selected.

Results showing the bacterial survival of controls and phage treatments in cattle hide and soil models are shown in Figure 18 and Figure 19, respectively. Results from additional sand model are presented in Figure 19.

In the hide model, except for phage Season12 in both treatment concentrations, phages alone or in combination in either treatment concentration were able to significantly reduce *Salmonella* populations on cattle hides compared to the positive control at 5.74 $\log_{10}$ CFU/cm^2. A reduction of 1.75 $\log_{10}$ CFU/cm^2 was obtained by using phage treatment of Sw2 alone at 10^9 PFU/mL, which was the highest bacterial reduction among all treatments performed. Dosage effects were observed across bacterial survival of phage treatments, with statistically significant differences observed between two treatment concentrations in phage Sergei and Sw2.

In the soil model with 1 hr treatment duration, statistically significant reductions were observed only in treatment concentrations at 10^9 CFU/mL of phage Munch, Sergei and Sw2 alone and combinations of Sergei + Sw2 and Sergei + Munch. Sergei was able to reduce bacterial concentration from 6.33 $\log_{10}$ CFU/g (control) to 4.95 CFU/g, and this 1.38 CFU/g reduction was the greatest reduction observed across all phage treatments. Phage Sergei alone and its combination with Munch showed statistically significant dosage effect ($p<0.05$) (Figure 19). In the 24 hr treatment experiment, phage Sw2 and its combination with Sergei was able to significantly reduce the bacterial population in soil compared to the control treatment at either treatment concentration, and Sergei alone was only able to significantly reduce bacterial load

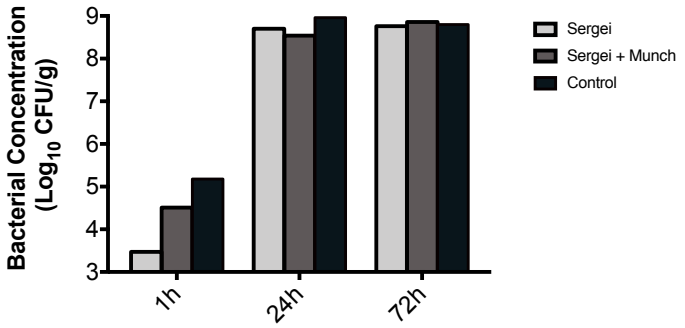

Figure 17. Bacteriophage survival after phage treatments in soil preliminary experiment. Bars in this figure indicate bacterial survivals of 1h, 24h and 72h phage treatments in soil preliminary experiment. The colors of bars indicate phage treatments with Sergei alone, Sergei and Munch combined and positive control. Detection limit of this experiment is 2 $\log_{10}$ CFU/g.

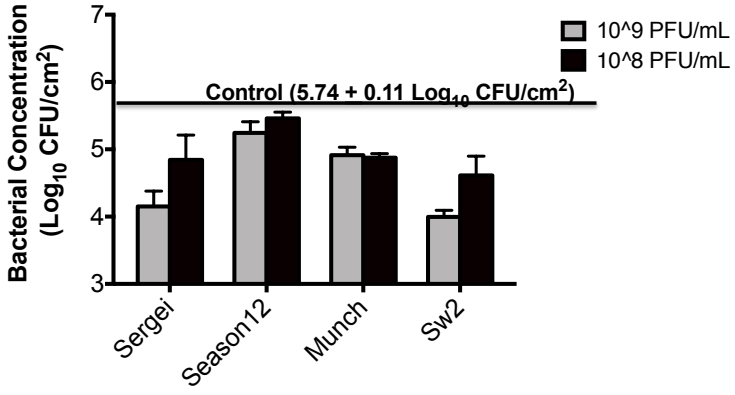

Figure 18. Bacterial survival after phage treatments in a cattle hide model. Bars in this figure indicate bacterial survivals of 1h phage treatments in cattle hide model. The colors of bars indicate two phage treatment concentrations at 10^8 and 10^9 PFU/mL. Solid line indicates positive control of testing model. Detection limit of this experiment is 1.15 $\log_{10}$ CFU/cm^2.

A. B.

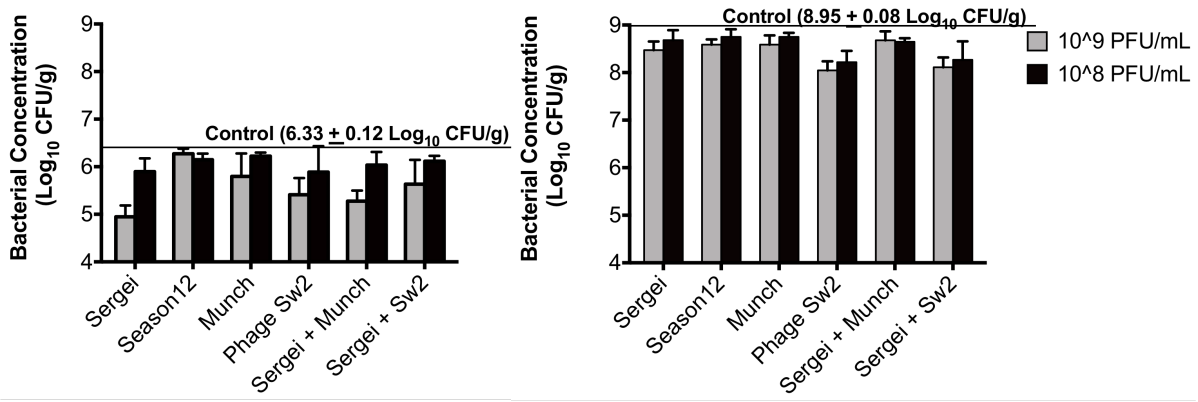

Figure 19. Bacterial survival after phage treatments in a soil model. Figure 19A and 19B indicate bacterial survivals of phage treatments in soil model with treatment duration of 1h and 24h, respectively. Bars in this figure indicate bacterial survivals of 1h phage treatments in a soil model. The colors of bars indicate two phage treatment concentrations at 10^8 and 10^9 PFU/mL. Solid lines indicate positive control of testing models. Detection limit of this experiment is 2 $\log_{10}$ CFU/g.

when applied at 10^9 PFU/mL. Phage treatment with Sw2 at concentration 10^9 PFU/mL resulted in the greatest reduction of $\log_{10}$ 0.82 CFU/g.

Comparing with the bacterial survival results between two models, treatments with phages alone or in combination seemed to demonstrate better efficacies in the hide model versus the soil model. This phenomenon led to two hypotheses of why phages displayed poor antimicrobial efficacies in soil: 1) due to potential inactivation by components in soil; 2) due to high surface area of soil needed to be covered by treatments. To further examine these hypotheses, Sw2 at concentration of 10^9 PFU/mL was selected as a representative against the tested *Salmonella* strain in a sand model with the same experimental protocol described in soil model. The sand model was designed to mimic the physical property of soil while providing an inert condition where minimal interaction between the sand and phage occurs. Results from the sand model are displayed in Figure 20. Sw2 was able to significantly reduce the *Salmonella* population by 0.8 $\log_{1o}$ CFU/g in 1 hr treatment duration and continued to suppress bacterial growth by 0.64 $\log_{10}$ CFU/g in 24 hr. The inert condition provided by sand did not demonstrate an increased ability of the phage treatment to reduce bacterial population.

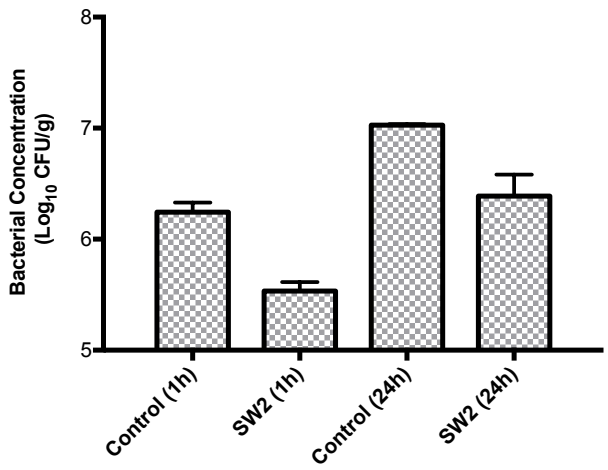

Figure 20. Bacterial survival after treatment with Sw2 at 10^9 PFU/mL in a sand model. Bars in this figure indicate bacterial survivals of 1hr and 24 hr phage treatments in a sand model. Detection limit of this experiment is 2 $\log_{10}$ CFU/g.

Discussion

1. Morphological, Genomic and Cross-Resistant Characterizations of Phages

Before adopting bacteriophage treatment as a means of controlling *Salmonella* in cattle feedlot environments, it is important to understand the fundamental biology of phages, including the dynamics of phage infection, development of phage resistance in bacterial hosts, and ultimately the information encoded in their genomes responsible for these phenotypes.

Except for Munch, genomic DNA of phages sequenced in this study showed a high level of similarity to well-known groups of phages. Conserved functional and structural proteins found in these three phages provided useful information to understand dynamics of phage infection. For instance, finding conserved tail fiber proteins in phage genomes is essential to preliminary prediction of phage receptors on bacterial surface and helpful for interpretation of results from the phage cross-resistance experiment.

Sergei is closely related to phage 9NA, the type phage of a proposed new genus of phage *9NAlikevirus* [169]. The structural related proteins of Sergei, including virion portal protein, major capsid proteins and major tail proteins are highly identical within this phage group, with less than average 10% different from one another. The tailspike protein of 9NA is homologous to *Salmonella* phage P22, implying that 9NA binds to the *Salmonella* O antigen, has endorhammosidase activity, and is capable of cleaving the polysaccharide receptor [170]. The tail tip protein of Sergei is 80% identical to predicted tail tip protein 9NA, suggesting a possibility of Sergei attaching to the O-antigen as phage receptor.

Season12 is ~ 90% identical with Chi and a well-studied Chi-like phage iEPS5 in nucleotide sequence [89,171]. Genes that are associated with DNA replication and transcription,

such as DNA primase, DNA polymerase and DNA helicase as well as structural proteins, such as portal protein head-to-tail joining protein, decorator protein, major capsid protein share high degree of identity to Chi. Its major tail fiber protein is > 99% identical to Chi and iEPS5, with only two amino acid variations. Bacteriophage Chi was first isolated in 1936 and known for its ability to infect flagellated *Salmonella* spp. [172]. Adsorption mechanism of Chi-like phages were later studied in a closely related phage, iEPS5 [171]. Choi *et al.* indicated that phage iEPS5 were only able to infect the bacterial host when the flagellum is rotating counterclockwise, suggesting the physical movement of flagellum generates a power that attracted phages moving toward to the bottom of flagellum and attaching to the bacterial surface [171]. Compared to phage Chi (YP_009101117), the nearly identical major tail fiber protein with 2 amino acid changes found in Season12 strongly suggests the flagellum being the receptor of Season12. Three tandem repeats of a hypothetical protein containing 320 – 339 amino acid residues were identified located downstream of the tail fiber protein. Interestingly, the hypothetical protein of the tandem repeats in Season12 was also identified in the genomes of closely related phages such as iEPS5 (KC677662) (9) and SPN19 (JN871591.1), but these phages contain only one copy of this protein.

Sw2 was found to share ~65% sequence identity with phage T5 at the DNA level as determined by Emboss Stretcher. Compared to 16 tRNA genes found in T5, 29 tRNA were annotated in Sw2 [81]. Like other closely related phages in the genus of *T5likevirus*, the genome of Sw2 can be divided into pre-early genes, early and late genes [81]. Proteins encoded in pre-early genes are associated with host shutdown, including 5'-deoxyribonucleotidase, A1, and A2 [81]. The early gene cluster functions related to DNA metabolism, replication, regulation, and

lysis, followed by late gene region consists of virion structure [81]. High similarity of tail fiber protein found in Sw2 indicate its potential utilization of FhuA as its receptor to bacterial host, like T5 [173].

Phage Munch has an usually large genome that is >300kb with 78% of genes annotated with a unknown function. The term “jumbo phage” is given to phages with a genome size larger than 200kb [174]. Jumbo phages isolated previously generally exhibited high level of genetic diversity with little linkage to known clusters based on phylogenetic analysis [174]. As a jumbo phage, morphologically, Munch has notable large virion head. Unlike most of *Salmonella* phages, Munch is AT-rich with a GC content of 35.6%. The majority of jumbo phages (95.6%) were isolated against Gram-negative hosts that are GC-rich, but jumbo phages tend to display an AT-rich genome [174,175]. Structural proteins annotated in Munch, such as major capsid proteins, portal proteins and baseplate proteins, are majorly associated with structural proteins of phage T4. Other genes that are associated with nucleotide metabolism and replication, for instance, DNA and RNA polymerase, were also annotated in the genome of Munch. Interestingly, there is a tandem repeat region located in the last 20kb of the genome, in which 13 repeats of a DNA condensation protein, separated by three hypothetical proteins, were identified. This unit repeat region were not found in any related jumbo phages such as phages 121Q (KM507819.1), vB_Eco_slurp01 (LT603033.1), vB_CsaM_GAP32 (JN882285.1), PBECO 4 (KC295538.1) and vB_KleM-RaK2 (JQ513383.1). Tandem duplication was also identified in other jumbo phages such as 121Q and G [176]. Hua *et al.* suggested the possibility of jumbo phages expanding their genomes though tandem duplication to accommodate large virion capsid [176]. Putative tail proteins annotated in phage Munch shows low level of homology to other

known phage in the NCBI database, making it difficult to predict its putative receptors on the bacterial host.

Phages binding to host receptor is the initial and most essential step of infection, and bacterial hosts develop resistance to phage majorly via modification of phage receptors. This defense strategy targeting phage receptor region includes loss of receptors, production of extracellular matrix or masking proteins [115]. Therefore, in phage therapeutic applications, it is preferable to use phage cocktails not only because of the advantage of expansion of host coverage by multiple phages, but also the necessity of utilizing phages attaching to independent receptors to prevent bacterial phage-resistance and improve infection efficacy [109]. According to the phage cross-resistance characterization performed in this study, Sergei, Season12 and Sw2 demonstrated resistance independence as they were able to infect the phage-resistance mutants from each other. This observation could potentially serve as an evidence of our prediction of phage receptors obtained from bioinformation analysis. Further experiment of testing combinations two phages that are fully phage-resistance independent in the microtiter-plate based method approved the concept of using phage cocktail described by Gill *et al.* [109]. By using Sergei and Munch, capable of infecting resistant mutants of each other, regrowth of bacterial culture was inhibited.

2. Factors Contribute to the Antimicrobial Efficacies of Phage in Two Models

Suggested by Gill *et al*., host range and virulence are two essential components of a successful phage treatment as 1) specificity of phage treatments determines the bacterial host range that phage treatments are able to target; 2) efficacies of phage infection determine how well the treatment is able to lyse bacterial host and prevent resistance development [109].

Host range and virulence were determined in a previous study using a microtiter-plate based assay described by Xie *et al.* [96] and the results of four phages used in this study were extracted and displayed in Figure 21 and 22 (Figure 21. Host range assays were performed using the agar overlay spot method; Figure 22. Host range and virulence assays were performed using microtiter-plate based liquid assay). Phage Munch and Sw2 demonstrated a broader host range, capable of infecting 11 out of 20 *Salmonella* strains tested across multiple serovars observed in the microtiter-plate based assay. On the other hand, phage Sergei was able to infect strains in serovar Anatum, suggesting that Sergei potentially utilizes the outer decoration region of O-antigen that is specific to this serovar instead of the conserved region towards to inner core. Although four phages displayed similar efficacies of plating in the spot assay, according to the liquid scores obtained in this study, phage Sw2 displays greatest suppression of bacterial growth against tested strain used in current study, *S.* Anatum FC1033C3, among all phages, followed by Sergei with second highest liquid score. This observation suggests that phage Sw2 and Sergei are more virulent against the tested strain in this microtiter-plate based assay and are likely to have better antimicrobial efficacy against targeting strain in testing models.

Virulence of phage, or efficacy of phages to infect and lyse bacterial host is an integrated result of its growth kinetics, including adsorption rate, latent period and burst size [137]. Theoretically, phages with high adsorption rate constants, short latent periods and large burst sizes would be expected to produce new virions efficiently during the infection cycle [138]. Others suggested that a high adsorption rate is associated with low plaque size and productivity, which could further lead to slower growth rate [177]. In the current study, by looking into adsorption rate alone, Sergei and Sw2 with lower adsorption rate produced a high liquid score

when measuring virulence in the microtiter-plate assay against *S.* Anatum FC1033C3, which correlated with a model suggested by Gellet *et al* [177]. Gellet *el al*. suggested that phages with low adsorption rate advanced in plaque size and productivity, which allows phage to reproduce efficiently for bacterial elimination [177]. Lindberg *et al.* proposed in their model study that phages with high adsorption rates but longer lysis times grow slower than ones with lower adsorption rate but shorter lysis time, this theory did not seem to correlate with the kinetics parameter measured in this study [178]. Lindberg *et al.* also indicated in their study that the lack of correlation between growth kinetics and integrated growth rate is due to the fact that individual phage traits are poor surrogates for phage growth rate, even though the phage growth rate is determined as a function of individual phage traits [178].

To better demonstrate the correlation between two host range assays and phage antimicrobial efficacies in *ex vivo* models, experimental results obtained in previous and current studies are summarized in Table 6 [97]. Sergei and Sw2 that were capable of producing a strong signal in the microtiter plate liquid assay were also capable of achieving a higher reduction in both *ex-vivo* models, implying that antimicrobial efficacies of phages in testing models are correlated with their virulence in the liquid assay. The tendency of spot assays to overestimate phage virulence was also observed by Henry *et al.*, in which phage Season12 and Munch that are capable of efficiently making plaques does not exhibit strong bacterial suppression in either microtiter-plate assay and two *ex-vivo* models [112]. This phenomenon demonstrated a similar concept to the observations of Lindberg *et al.* [113], where phage growth rate in liquid culture was a strong predictor of phage *in vivo* efficacy in an insect model.

Strains	USDA4 Anatum	FC1033C3 Anatum	S2029C2 Anatum	S2028C1 Anatum	FD 1001A1 Muenchen	H2006-1 Cerro	08-022 Dublin	SGSC 2475 Enteritidis	3115 Enteritidis	SGSC 2480 Heidelberg	3117 Heidelberg	USDA3 Montevideo	H1042-3 Montevideo	USDA2 Newport	10-014 Newport	330-1 Reading	USDA1 Typhimurium	3116 Typhimurium	LT2 Typhimurium	USDA5 Kentucky
Sergei	4	4	4	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Season12	0	4	0	0	0	2	0	2.7	0	0	0	0	0	4	4	0	2	0	4	0
Munch	4	4	4	3.3	0.3	0	4	4	4	3.7	4	4	1.7	2	4	2	2	0	0	0
Sw2	4	3.3	4	4	0	0	4	3	2	1.5	4	0	0	0	0	3.3	0	0.6	3	0

Figure 21. Host range from agar overlay spot method. Phage host range as measured by spotting on soft agar overlays. Adapted from Xie *et al.,* 2018 [96]. Phages were plated at the routine test dilution (RTD, determined as the first tenfold serial dilution of phage that formed countable plaques lawns of its own host) and 100 X the RTD. Phage were tested against a panel of twenty *Salmonella* strains and scored on the following criteria: phage forming > 50% of the number of plaques formed on its host strain at its RTD = 4; phage forming 5% to 50% of the number of plaques formed on its host strain at its RTD = 3; phage forming a zone of confluent lysis but no individual plaques at 100 × RTD = 2; phage forming individual plaques at 100 × RTD = 1; no plaque or clearing formation at either dilution = 0. The scores from three replicate experiments were averaged and color intensity indicates greater score [96].

Phage	Initial phage titer (PFU/ml)	*Salmonella* strains																			
		USDA4 Anatum	FC1033C3 Anatum	S2029C2 Anatum	S2028C1 Anatum	FD 1001A1 Muenchen	H2006-1 Cerro	08-022 Dublin	SGSC 2475 Enteritidis	3115 Enteritidis	SGSC 2480 Heidelberg	3117 Heidelberg	USDA3 Montevideo	H1042-3 Montevideo	USDA2 Newport	10-014 Newport	330-1 Reading	USDA1 Typhimurium	3116 Typhimurium	LT2 Typhimurium	USDA1 Kentucky
Sergei	10^8	65	55	57	63	0	2	6	0	0	0	0	0	0	0	0	0	0	1	0	0
	10^6	58	51	52	56	0	2	4	0	0	0	0	0	0	0	0	0	0	0	0	0
Season12	10^8	13	10	0	0	25	80	1	49	7	13	11	4	1	79	79	2	12	12	25	3
	10^6	2	32	0	0	3	46	0	34	0	0	1	0	1	76	79	0	8	2	21	0
Munch	10^8	9	27	9	9	19	0	17	60	27	29	0	48	45	56	17	7	31	0	0	0
	10^6	6	0	8	7	0	0	21	45	7	0	0	47	39	23	17	9	47	0	0	0
Sw2	10^8	77	86	77	78	0	2	21	21	0	13	27	0	4	0	0	21	8	35	19	0
	10^6	73	79	68	72	1	0	21	22	0	15	21	0	1	0	0	21	0	0	21	0

Figure 22. Host range from microtiter plate liquid assay. Adapted from Xie *et al.,* 2018 [96]. Values indicate average liquid assay scores are described by Xie *et al.* To aid the reader, cells are shaded with stronger color intensity indicating a greater score. Assay score represents the differences in area under the bacterial growth curve with and without phage. Since the largest standard deviation observed in any individual assay was 10.89, an assay score of greater than 10.9 was used as a cutoff to distinguish a legitimate signal from noise. Larger numbers indicate a greater suppression of bacterial growth in the presence of phage. Boxed cells indicate the initial isolation and propagation host of the phage [96].

In addition to their virulence predicted in liquid culture, bioinformatic analysis could also provide additional information explaining phage efficacies in *ex-vivo* models. High level of homology in the tail region of Season12 suggested that it likely attaches bacterial flagellum as its reception during the infection [89,179,180]. Conditions involved with the *ex-vivo* models performed in this study are sub-optimal to bacterial growth and survival, including nutrient starvation, desiccation and pH shift, resulting in potential stress response of *Salmonella* [181]. Downregulation of genes responsible of protein synthesis was often observed during the stress response in *Salmonella*, including several genes involved in flagellar biosynthesis (e.g *flgA, flgB, flgH, flgBA*), resulting in decreased motility of bacteria cells [182]. The fact that *Chilikevirus* were only capable of infecting motile *Salmonella* cells explained its poor antimicrobial efficacies in *ex-vivo* models due to potential non-motile stage of *Salmonella* in stressed condition.

A reduced antimicrobial efficiency of phage treatments was observed in the soil model compared to the hide model, suggesting that either phages were inactivated by chemical compounds or physical structure in soil, or phage treatment was incapable of covering a high amount of surface in fine particles like soil. The additional inert model by using sand provided important information that explained the reduced phage efficacies in soil model. First, bacterial growth between 1and 24 hr treatments in the soil and sand model are 2.63 $\log_{10}$ CFU/g and 0.78 $\log_{10}$ CFU/g, suggesting that the lack of nutrition supporting bacterial growth in the sand model resulted in only a minor increase of bacterial population compared to the soil model. In addition, using an inert sand model for efficacy testing of phage did not result in a greater reduction of bacteria load, implying that the reduced phage efficacies observed in the soil model is unlikely due to phage inactivation by soil. Using a real-time coevolution model, Gomez *et al.* suggested

Table 6. Comparison of two host range method in predicting phage efficacies in two models

Experiments / Phages	Host Range Scores		Reductions in Model Testing Phage Concentration at 10^9 PFU/ml		
	Agar Overlay Spot	Microtiter Plate Liquid (10^8 PFU/ml)	Cattle Hide (Log_{10} CFU/cm^2)	Soil (Log_{10} CFU/g)	
				1 h	24 h
Sergei	4	55	1.60*	1.38*	0.45*
Season12	4	10	0.50	0.22	0.33
Munch	4	27	0.83*	0.53*	0.33
Sw2	3.3	86	1.75*	0.77*	0.88*

* Star symbols indicate that the bacterial reductions are significantly compared to controls

that the increased resources in soil available for bacterial growth led to an increase of bacterial resistance to phages [183]. In our current study, by using a sand model with reduced nutrient resources, phage efficacy did not improve with reduced bacterial growth, indicating that phage-resistance developed during the 24 hr treatment may not contribute to the reduced efficiency in phage treatment. This phenomenon may also explain why treatments with phage combinations did not exhibit a synergy effect compared to single phage treatments. Sergei and Sw2, which displayed stronger bacterial inhibition in the cattle hide model, also achieve greater reduction in the soil model with 2 treatment durations, implying that treatment efficacies achieved in testing models are phage-dependent instead of subject-dependent. In our current model, bacterial reduction achieved by phage treatments seemed to be relevant to virulence of phage itself and the

availability of physical contact between the treatment object and phages, with little association with inactivation of phages by treatment object or development of phage resistance.

CHAPTER IV

CONCLUSION

Asymptomatic *Salmonella* carriage in beef cattle is a food safety concern and the beef feedlot environment and cattle hide are reservoirs of this pathogen. Previous studies on mechanisms of *Salmonella* transmission and colonization of lymph nodes suggested that entry route of *Salmonella* could be through oral uptake via contaminated food and drinking water, or transdermal transmission via skin abrasion and insect bites. Previous study identified the feedlot environment as reservoirs of bacteriophages that may prove useful as a means of controlling *Salmonella* in the beef cattle feedlot environment and cattle hide. In recent decades, attention for new antimicrobials to control bacterial pathogens and treat infectious diseases were increased with growing threat of antibiotic resistance. Bacteriophages have re-emerged as an attractive alternative to combat antibiotic resistant bacteria. The capacity of bacteriophages to efficiently infect and lyse the target bacterial strain is essential for successful phage therapy [109-111]. Prediction models used to determine treatment efficacies of phages *in vitro* yield various accuracies depending on methods used.

In the current study, a liquid culture-based assay was developed in a 96-well microtiter plate format to measure the phage host range and virulence for a collection of 15 *Salmonella* phages against a panel of 20 *Salmonella* strains representing 11 serovars. The majority of the host range results from two methods were in agreement including in cases where a bacterial strain was insensitive to the phage. Each method produced a false-negative result in 19/300 (6%) of the measured phage-host combinations when compared to the other method. The spot method

tended to indicate greater phage sensitivity than the microtiter assay even though direct comparisons of the response magnitude between the two methods is difficult since they operate on different mechanisms.

In the two *ex-vivo* models used to determine antimicrobial efficacy of phage treatments, phage Sergei and Sw2, which displayed greater virulence in the microtiter plate based assay, were able to achieve greater bacterial reduction of *Salmonella.* The ability of phages on suppressing bacterial growth in *ex-vivo* models showed high degree of correlation with their virulence measured by the liquid assay but little association with the spot assay, suggesting that spot assay tends to overestimate antimicrobial efficiency of phages. This tendency was also observed by Henry *et al.* [112] in which phage PhiKZ displaying a EOP of 1.2 performed poorly in liquid culture and in an in vivo model of phage therapy, which suggests that measures of phage virulence may be more useful than measures of plating efficiency when selecting phages for use in antibacterial applications. The antimicrobial efficacies of phage treatments are also found to be phage-specific but not model-dependent, implying that a phage capable of achieving great bacterial reduction in one model is likely to perform well in another.

The current study also explored reasons behind the reduced treatment efficacies of phage in soil model by using a sand model. Result obtained in this study suggested a potential common difficulty of antimicrobial treatments in fine particle model due to the high surface area needed to be covered by treatments. In addition, treatments with phage combinations did not display an increased bacterial reduction in both models, suggesting that bacterial resistance to phage may not be critical during a short term treatment period.

To better validate the microtiter-plate based liquid assay in predicting antimicrobial efficacies of phages, future studies can be expanded to other bacterial-phage combinations as well as other *ex-vivo* or *in-vivo* models. Phages demonstrating ability of significant bacterial reduction could be used in challenge studies in *in-vivo* cattle hide models and in feedlot environment to explore practical application of phages as an intervention to prevent *Salmonella* transmission and improve overall food safety of beef.

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