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Application of Bacteriophage Cocktail in Leafy Green Wash Water to Control Salmonella Enterica

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APPLICATION OF BACTERIOPHAGE COCKTAIL IN LEAFY GREEN WASH WATER TO CONTROL *SALMONELLA ENTERICA*

A Thesis Presented

by

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ABSTRACT

APPLICATION OF BACTERIOPHAGE COCKTAIL IN LEAFY GREEN WASH WATER TO CONTROL *SALMONELLA ENTERICA*

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Produce is responsible for 46% of all foodborne illnesses in the USA. *Salmonella enterica* causes 19,000 hospitalizations each year, and has been associated with produce. Presently, chlorine based sanitizers are most often used, however organic matter reduces its antimicrobial activity. Bacteriophage treatments are an all-natural, alternative method for pathogen inactivation. The objective of this study was to determine the efficacy of a five-strain bacteriophage treatment against a *S. enterica* cocktail in simulated wash waters at different temperatures. Bacteriophage and *S. enterica* were enumerated in simulated wash water solutions. One set of experiments studied bacteriophage and *S. enterica* growth in TSB+vegetable solutions. Bacteriophage behavior was not statistically different ($p < 0.05$) in spinach, romaine, or iceberg lettuce across different concentrations of organic matter. *S. enterica* reduction was approximately 2 log over 135 minutes for vegetable solutions and for the TSB control. *S. enterica* reduction was only 0.5 log in water solutions. The next set of experiments studied bacteriophage and *S. enterica* growth in vegetable solutions. Spinach wash water and tryptone soy broth solutions (TSB) at 20

°C and 37 °C. *S. enterica* was not reduced in spinach solution studies at 20 °C and 37 °C or at broth solutions at 20 °C. However, *S. enterica* was effectively reduced 4 log in broth solutions at 37 °C up to 7.5 hours, but grew to high levels after 24 hours. These results indicate that bacteriophage could not effectively control bacteria levels in produce wash water, and may need to be optimized.

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LIST OF ABBREVIATIONS

CDC-Center of Disease Control

TSB-Tryptone Soy Broth

FSMA-Food Safety Modernization Act

GRAS-Generally Recognized As Safe

PFU-Plaque Forming Units

CFU-Colony Forming Units

GAP-Good Agricultural Practices

EPA-Environmental Protection Agency

USDA-United States Department of Agriculture

CHAPTER 1

INTRODUCTION

Each year, *Salmonella* spp. causes approximately 1.2 million illnesses, and 19,000 hospitalizations *(1)*. It is the second leading cause of foodborne illness in the US *(19)*. Although the Center Disease of Control (CDC) tracks outbreak cases, it is likely that the real incidence is higher, due to undocumented salmonellosis cases. Produce is responsible for 46% of foodborne illness outbreaks *(30)*. As ready to eat produce is minimally processed from farm to store, pathogen control steps must be continually improved to reduce outbreaks. As the CDC has improved its methods in tracking and identifying outbreaks, stricter regulations have been implemented. Most recently, the Food Safety Modernization Act (FSMA) is a key piece of legislation that impacts the food industry. Among other things, it requires is that food handlers have preventative measures against pathogen presence, and requires compliance with FDA inspections *(2, 3)*. Although this affects food stakeholders across the board, farmers are particularly affected by these measures.

In farms, wash water is a primary means for pathogen contamination *(17)*. After harvest, produce is submerged in multiple tubs of water, some with sanitizer. Soil, produce particulate, and bacteria are shed into each tub, and can contaminate subsequent produce. Sanitizers limit the spread of bacteria, and primarily prevent cross-contamination. Typical sanitizers include chlorine derivatives (sodium hypochlorite, calcium hypochlorite), peroxyacetic acid, hydrogen peroxide, and trisodium phosphate.

Chlorine is most commonly used sanitizer, but poses health and environmental risks, and has variable behavior *(17)*. It is well known that chlorine efficacy decreases as the organic load increases *(17, 29)*. Its effectiveness and toxicity vary with pH and temperature. Consumers are also concerned about health and environmental risks from chlorine byproducts *(17)*. Chlorine reacts with organic matter yielding trihalomethanes and other toxic byproducts. Due to variable chlorine behavior, there is a need for a robust disinfectant that can function even in the presence of organic matter.

Bacteriophage treatments have been an appealing alternative, as it is considered all-natural and only targets specific bacteria groups. Bacteriophage are also a clean and effective treatment which can minimize bacteria resistance. They attack pathogens, but not humans or the environment. Currently some bacteriophage treatments have been Generally Recognized As Safe (GRAS) approved by in meat applications, and interest has been to apply the same treatment to produce. The goal of this project was to assess how bacteriophage can be a control strategy in preventing pathogen spread in farm processing steps.

1.1 Objectives

1. Study the growth rates of *S. enterica* in simulated produce wash water.
2. Investigate the impact of organic load on the efficacy of bacteriophage in simulated wash water where organic matter is derived from vegetable juices.
3. Study the impact of organic matter (vegetable juices) on bacteriophage growth and *S. enterica* reduction.

CHAPTER 2

BACTERIOPHAGE APPLICATIONS IN FOOD SYSTEMS: OPPORTUNITIES AND OBSTACLES

2.1. Introduction

Bacteriophage are viruses that exist in nature, and are the world's most ubiquitous microorganism, with 10^{30}-10^{32} entities in the world *(8, 26)*. They are classified into four main families tailed, polyhedral, filamentous, and pleomorphic. Each bacteriophage is between 24-200 nm in size, and consists of a head containing DNA, a sheath, core, and tail fibers (if applicable). Bacteriophage are completely parasitic, and depend on a host to reproduce. They target specific groups of bacteria, but can also infect a broad host range, and closely related bacteria.

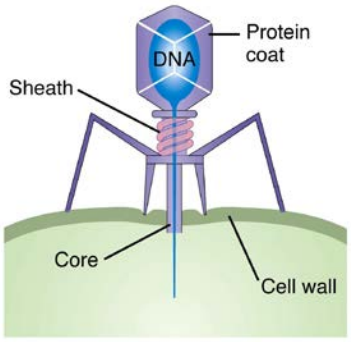

Figure 1: Diagram of a bacteriophage. (Source: Carr, M. 2014)

2.2 Bacteriophage infection cycle

Bacteriophage do not contain any cellular machinery for navigation, but depend on environmental factors for movement. Bacteriophage are inert when out of contact with its target. Once the bacteriophage collides with a bacterium, the infection cycle begins.

The process is classified into four stages: phage adsorption onto bacteria surface, insertion of the phage nucleic acid into the cell, intracellular synthesis of bacteriophage, and propagation.

Bacteriophage attachment is dependent on a host of factors, including the bacteria growth rate, the cell size, and availability of nutrients *(14, 21, 26)*. Some bacteriophage require certain cofactors or molecules such as Ca^{2+} or Mg^{2+} for bacteriophage attachment. The bacteriophage becomes activated when its tail fibers bind to a protein, oligosaccharide, or polysaccharide on the bacteria surface *(26)*. The bacteria cell surface is lined with many proteins and receptors and the inert bacteriophage irreversibly attaches when it comes across a cell. Next, the phage DNA is injected directly into the cytoplasm of the cell. At this point, the phage DNA enters either the lytic phase or the lysogenic phase (Figure 2). In the lysogenic phase, the DNA is incorporated into the bacterial chromosomal DNA, but is not immediately expressed. After certain external factors or conditions, the cell becomes activated and enters the lytic cycle. The bacteria cell becomes a factory for bacteriophage production. The phage DNA is translated and the bacteria cell machinery is overridden so phage progeny are produced. After the phage progeny are mature, the bacteria cell is lysed and the bacteriophage progeny are free to infect other bacteria. Bacteriophage that are lytic skip the lysogenic phase and begin bacteriophage propagation.

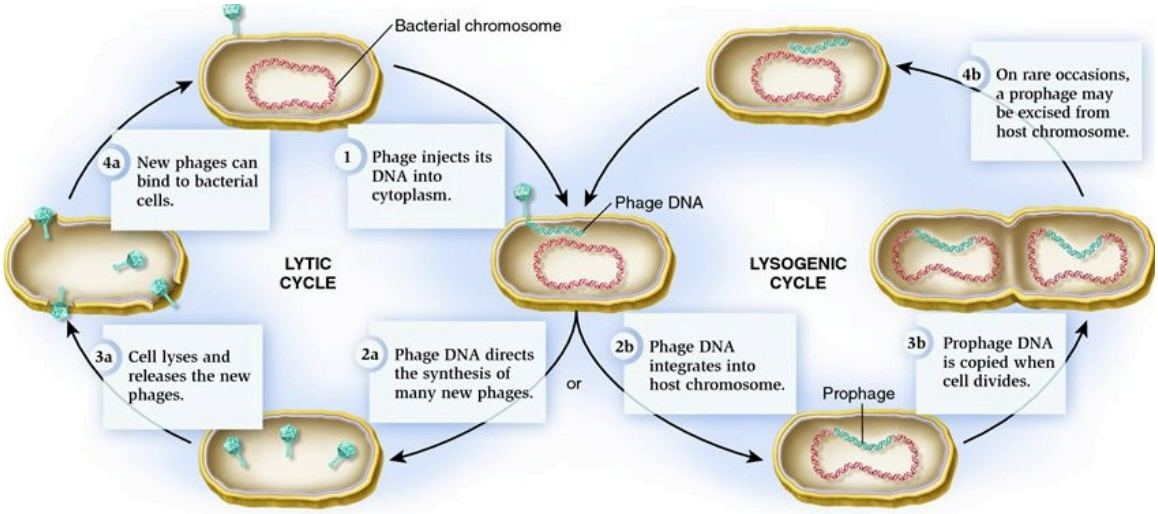

Figure 2: Lytic and lysogenic cycle of bacteriophage. (Source: Biology Forums Gallery 2011)

2.3 History of bacteriophage in medical applications

Frederick W. Twort and Frederick d'Herelle were the two clear frontrunners in bacteriophage studies in the early 1900s. Twort explored how bacteriophage propagated and mistakenly hypothesized that it could be grown in dung, grass, soil, and artificial media. He noticed that when he grew smallpox vaccine on agar, no vaccinia virus grew, but a bacteria micrococcus grew. The micrococcus was watery looking, and over time, became transparent. The transparent colonies could not be subcultured, and when a transparent colony touched another micrococcus, it too became transparent. Further observation under a microscope showed no presence of bacteria. Twort had observed bacteriophage attack on bacteria on agar media.

Around the same time, D'Herelle first noticed that some samples of dysentery grew differently than others among his dysentery patients. He observed clearings on

seeded bacteria, and called them ultraviruses. His investigations found that an invisible agent, later understood to be a bacteriophage, was capable of killing bacteria and propagating. He noticed that phage multiplied in steps and had predictable cycles of infection, multiplication, release, and reinfection. He also found that phage were most likely responsible for patient recovery from bacterial infections. When observing phage titers in stool samples, he noticed that phage titers increased as his patients recovered from dysentery and typhoid. To examine this behavior, he tested chickens against avian typhosis and found that flocks with the oral phage treatment survived the illness better than flocks without *(25)*. He also tested the safety of Shiga-bacteriophage for safety by ingesting his samples himself first, then testing on family and coworkers. None of these trials exhibited any negative side effects.

D'Herelle marked the beginning of phage therapy as an alternative option to medicine. However, interest in phage therapy waned in light of the movement for antibiotics. Today, antibiotic treatments are complicated and more costly. Methicillin-resistant *Staphylococcus aureus* and other antibiotic-resistant bacteria make treatments more difficult. Interest in alternative therapies, such as phage therapy are gaining popularity. When comparing phage therapy and antibiotic therapy, some studies show that phage therapy is more effective than antibiotics *(11, 24, 33)*. One study showed an 82% survival with phage therapy as opposed to a 64% recovery rate with antibiotics treatments against disease in lung and pleura *(28)*. So far, phage therapy studies show clear advantages over antibiotics. Phage therapy has no documented side effects, has effective and localized phage response, and prevents against reinfection *(11)*. In

medicine, phage therapy has showed promising advantages, and interest has turned to phage therapy as a potential antimicrobial method in food applications.

2.4 Bacteriophage applications on surfaces

In the studies described below, bacteriophage are an effective means for pathogen reduction on hard surfaces, and could be a replacement for harsh chemical sanitizers. Most facilities use chemical sanitizers such as chlorine, quaternary ammonium compounds, and iodine. These can be corrosive and can pose a health risk to workers. Keeping food contact surfaces clean is very important in product safety, and is especially important in foods that have no heat processing step. Without the processing step, it becomes much easier for pathogens to grow and affect consumers. In post-processing procedures, it is also especially important that food contact surfaces are clean. After the processing step, the food product is essentially ready for market sales and consumer consumption. Surfaces need to be routinely and properly sanitized to prevent contamination. Although chemical sanitizers are mostly used, bacteriophage can be an effective and safer alternative.

According to one study, bacteriophage have been able to reduce *E. coli* O157:H7 levels on glass surfaces and gypsum boards (drywall) *(5).* When bacteriophage was applied on glass surfaces at a 10^8, 10^9, 10^{10} PFU/ml concentration for 5 minutes, *E. coli* O157:H7 was reduced 99.99%, 98%, and 94% respectively. On gypsum surfaces, *E. coli* O157:H7 was reduced 100%, 95%, and 85%. This study shows that highly concentrated bacteriophage are most effective in eliminating *E. coli* O157:H7 and remain effective even in the presence of organic matter (dried skim milk on surface).

Another study focused on glass and stainless steel surfaces. They found that a 5 minute treatment of SalmoFresh was effective in reducing a *S. enterica* levels >99% (2.1-4.3 log CFU) *(39)*. The study demonstrated that the bacteriophage cocktail had a significant effect on *S. enterica* levels, and could partially replace the use of chemical sanitizers in food facilities.

One study focused on *Yersinia pestis* on glass, gypsum boards, and stainless steel surfaces and found that a 5 minute treatment of a bacteriophage cocktail at 10^8, 10^7 PFU/ml 100% reduced *Y. pestis (32)*. A 10^6 PFU/ml treatment yielded a 99.97% reduction.

Studies on bacteriophage efficacy against biofilms have been effective. One study shows that a 6 hr contact time of bacteriophage against *L. monocytogenes* on stainless steel yielded a 3 log reduction *(22)*. This treatment was comparable to a lactic acid treatment under same conditions.

Another study found that single bacteriophage strains could act against *Campylobacter jejuni* biofilms. It reduced 1-3 log CFU/cm^2 within 24 hours. The study also documented transmission electron microscopy images of the *C. jejuni* biofilms with bacteriophage propagation.

Overall, bacteriophage have been effective on food facility surfaces, and are able to reduce pathogen presence over an extended period of time. Biofilms, which are a pervasive problem, can be reduced with bacteriophage. In conjunction with sanitizers, bacteriophage are a viable method to reduce sanitizer use while maintaining antimicrobial strategies.

2.5 Bacteriophage applications in meats and dairy products

Bacteriophage applications were first Generally Recognized As Safe (GRAS) approved in meat and dairy applications. Intralytix™, Micreos, and Omnilytics have developed bacteriophage cocktails that target harmful pathogens in foods. Studies show that they have been an effective strategy in reducing pathogen levels in foods.

In 2006, Listex™ by Micreos was approved with a GRAS status. Bacteriophage were isolated from the environment, that had no presence of endotoxin genes. A 9 log PFU/g food dosage was proposed in cheese production to control *L. monocytogenes* presence *(35)*.

In 2013, Intralytix™'s SalmoFresh™, a six strain lytic bacteriophage cocktail, was GRAS-approved for meat applications against *S. enterica*. The purpose was to prevent cross-contamination after the processing step when applied on food surfaces, namely meats, at 7 log PFU/g food. Intralytix™ isolated and sequenced the bacteriophages from the environment, and chose bacteriophages that did not contain any undesirable genes. Undesirable genes included expression of bacterial toxins or evidence of transduction such as the 16S RNA genes. According to preliminary and unpublished studies by Intralytix™ in the GRAS application, SalmoFresh™ has exhibited a range of results *(36)*. Studies show a 90% reduction of *Salmonella* levels in RTE deli meats, a 98% reduction in oven-roasted chicken, and a 65-90% reduction on raw turkey breast after 24-120 hours in cold storage. Additional studies explore its use in other meats, and the application indicates that SalmoFresh™ is an effective antimicrobial against *Salmonella* in both raw and cooked meats at refrigerated and room temperatures.

Studies show that bacteriophage treatments have been successful against a variety of pathogens. One study tested bacteriophage MOI 4 treatment against *L. monocytogenes* in RTE meats. After 6 days at 6 °C, a 3 log CFU/g reduction was observed in hot dogs, a 1.5 log CFU/g log reduction in sliced turkey meats, and a 1 log reduction in smoked salmon *(20)*. The bacteriophage levels were consistent even after 6 days.

In raw chicken skin, bacteriophage with an MOI of 1 reduced *S. enterica* Enteritidis populations 1 log CFU/cm and bacteriophage levels remained near initial population levels over 48 hours *(18)*. With a MOI between 100-1,000 a 99.7% bacteria reduction was observed. Higher concentrations of bacteriophage yielded a larger pathogen reduction.

In another study, bacteriophage were isolated from the environment against *E. coli* O157:H7. On 7 out of 9 meat surfaces, a 2 log CFU/surface of *E. coli* O157:H7 was 100% reduced. 2 out of the 9 meat surfaces had <10 CFU/ml after enrichment.

The studies show that meat applications have significant control on pathogen levels. They indicate that bacteriophage control is maintained during storage at refrigerated temperatures. Bacteriophage has been successfully applied in meats, and its applications in produce are also significant.

2.6 Bacteriophage applications in produce safety

2.6.1 Background information of produce safety

Produce safety is a growing concern, as produce consumption has increased by over 20% from 1970 to 2000 *(4)*. Produce has become an interstate commodity over the last few decades, and foodborne illness outbreaks have been more easily identified and

tracked by the CDC. Forty-six percent of foodborne illnesses are linked to the consumption of fresh produce *(30)*. Most commonly, *Escherichia coli O157:H7, Listeria monocytogenes,* and *Salmonella spp*. have been associated with produce such as sprouts, seeds, lettuce varieties, carrots. Ready to eat (RTE) foods are especially high risk because of minimal processing. In 2011, Jensen Farms was responsible for a *L. monocytogenes* outbreak through their farmed cantaloupes, which affected 146 people, 30 of whom died. The investigation report found many violations of food safety guidelines, especially the lack of sanitizer in wash solutions. Consequently, the wash water was a conducive environment for *L. monocytogenes* to spread.

The government has taken measures to keep the food system safe. FSMA provides a guideline for preventative controls, inspection and compliance, response, import regulations, and strengthens the partnership between the Federal Drug Association (FDA) and food distributors. Produce safety guidelines mandate farms to identify hazards, and use scientifically proven methods to control microbial levels and physical hazards in all stages of produce growth. This has forced farmers to take an active stance in processing steps and presents a need for more research in produce safety. There are a number of entry points for pathogens in the farming system—workers, animals in the field, poor hygiene, contaminated equipment, and the processing step.

Contamination risk is high in produce during pre-harvest and post-harvest stages. In pre-harvest, soil, irrigation water, manure, wild animals, and human handlers are all potential sources of pathogens. During post-harvest, handlers, harvesting equipment, wash water, improper storage and temperature can also contaminate and introduce pathogens. Preventative measures such as the Good Agricultural Practices (GAP)

certification program provides training for farm workers and educates them on hygiene, water management, animals, and packaging. It gives an important platform for employee education. While there are many preventative measures that can be taken, produce wash water sanitation is a key control point. Produce is minimally processed and typically leave the facility raw. Most farms employ a wash step to remove field heat from their crops. Most produce are submerged in water, where soil debris, produce runoff, and bacteria are shed. At this point, bacteria may contaminate subsequent lots of produce washed in the same water. If sanitizer is not present, or at ineffective levels bacteria may contaminate produce and lead to foodborne illnesses. The FDA and Environmental Protection Agency (EPA) offer very limited recommendations on chemical sanitizers, wash water monitoring, and wash water disposal. Many farmers are unsure how to use and monitor sanitizer in their wash water.

The main purpose of sanitizer is to control the microbial load in wash water and prevent cross contamination. Research has shown that inoculated lettuce dunked in wash water was able to contaminate subsequent uninoculated lettuce lots *(7, 27).* Chemical sanitizers such as chlorine and hydrogen peroxide are used as antimicrobials. Chlorine, the more affordable choice, is typically used in the following forms: chlorine gas, calcium hypochlorite, sodium hypochlorite, and chlorine dioxide. Chlorine gas is the cheapest, but also the most dangerous. Chlorine gas is toxic, and must be used with proper machinery and monitoring to protect workers. Calcium hypochlorite is the most commonly used source of chlorine in disinfection, and is more stable than sodium hypochlorite. Sodium hypochlorite is used more often on small-scale farm operations. With an oxidation potential 2.5 times more powerful than chlorine gas, chlorine dioxide

is more effective, but must be strictly monitored. Although the oxidizing power of chlorine derivatives differ, the antimicrobial effect of chlorine rapidly decreases in the presence of organic matter. Soil and vegetable runoff quickly pollute the water, leading to a variable efficacy of chlorine. Depending on the produce type, wash water chlorine levels are recommended between 50-400 ppm within pH of 6-8 *(37)*. Studies show that chlorine reduces microbial populations 1-3 log/g, but in the presence of organic matter, its antimicrobial activity is no better than water *(29)*.

Finding an effective sanitizer is one challenge, but convincing farmers to use any disinfectant is another problem altogether. Although research clearly advocates farms to use sanitizer in their wash water, a survey shows that out of 300 farms, 91% do not use any sanitizer in their wash water *(12)*. FSMA requires that farmers practice methods to reduce foodborne illness outbreak risks. Using sanitizer is a key component in produce safety, but with the variability of chlorine-based sanitizers, there is a need to find a more robust treatment that can withstand the presence of organic matter that also meets consumer acceptance.

2.6.2 Bacteriophage applications in produce

Most bacteriophage studies show a significant antimicrobial effect, indicating their potential in sanitizing applications. Bacteriophages also remain effective during storage at refrigeration temperatures. Overall, research shows a range of reduction which depends on the produce type.

One study inoculated the surfaces of tomato and spinach with three strains of *E. coli* O157:H7 (700 CFU/g, 14,000 CFU/g respectively), and found a 94% reduction in

tomatoes within 120 hours, 100% reduction in spinach within 24 hours *(6)*. Another study showed a 4 log CFU/g reduction over 7 days in cantaloupes, and a 2 log CFU/g reduction over 7 days in lettuce *(34)*. The range of reduction is dependent on the matrix—pH, temperature, and other physical factors.

Another study compared bacteriophage control in atmospheric and modified atmospheric (MA) conditions in leafy greens against *E. coli* O157:H7. Bacteriophage was least effective in spinach, but more effective on romaine lettuce. Under atmospheric conditions at 4 °C, 1.19 log decrease was observed, while under MA conditions it was slightly more effective with a 2.18 log decrease CFU/cm^2 in spinach. At 10 °C, atmospheric conditions yielded a 1.99 log decrease while under MA conditions, a 3.08 log CFU/cm^2 decrease was observed. Bacteriophage control was more effective in MA conditions and at the abusive temperature (10 °C). At 4 °C a 3.25 log CFU/cm^2 reduction was observed in atmospheric and MA conditions in romaine lettuce. However, at 10 °C, a 3.99 log CFU/cm^2 reduction, and a 4.34 log CFU/cm^2 reduction was observed in atmospheric and MA conditions respectively. Although the reduction variability in leafy vegetables was not explained, bacteriophage were effective in controlling pathogen levels in leafy greens. This effect was more pronounced under MA conditions. MA conditions help slow the growth of spoilage microorganisms and also control pathogen growth. Bacteriophage can be used in these applications to control pathogen levels post-processing.

In another study, bacteriophage and *Enterobacter asburiae* were applied to mung beans. *E. asburiae* is an antagonistic bacteria that acts against *S. enterica*. When bacteriophage and *E. asburiae* were used alone on mung beans, a 3.41 log CFU/g and a

5.56 log CFU/g reduction on *S. enterica* alone respectively. However, when used together *S. enterica* was only detectable upon enrichment.

Bacteriophage was also able to reduce *S. enterica* levels on melons, but not apples. At 5 °C and 10 °C, a 3.5 log CFU/slice was observed on melon. Even at 20 °C, a 2.5 log CFU/slice reduction was observed. However, no significant reduction was observed on apple slices at any of the three temperatures. Upon further investigation, the pH levels of the fruits played a major role in bacteriophage activity. Bacteriophage levels remained constant on melon slices (pH 5.8), however bacteriophage levels decreased to undetectable levels on apple slices (pH 4.2).

Studies show that bacteriophage are effective on produce, but can be used in conjunction with other treatments for a greater bacteria reduction. The produce type is also important in bacteriophage efficacy. A low pH may inactivate some types of bacteriophage. In response, a pH tolerant bacteriophage can be isolated from nature. A genetically modified bacteriophage may also be used.

2.6.3 Recommendations on optimum bacteriophage applications in produce

Studies thus far show that bacteriophage are capable of controlling pathogen levels, but have not achieved the gold standard of a 5 log reduction. However, bacteriophage application has yet to be optimized to maximize its antimicrobial activity. When using bacteriophage, it is important to use specific bacteriophages in a system where the bacteriophage are most efficient.

Bacteriophage, a living microorganism, is susceptible to the same physical factors as bacteria. pH and UV light play a role in bacteriophage activity *(26)*. UV light can kill

bacteriophage, and extreme pH can inactivate them as well. When produce is treated with chemical sanitizers, the bacteriophage is optimal when applied after the treatment. Depending on how much residual sanitizer is left, a water rinse may be recommended before bacteriophage application. Bacteriophage are an effective control presence against pathogens on the surface and can prevent cross-contamination post-washing.

Using bacteriophage in hurdle strategies may be effective in further reducing any pathogen presence. A chemical sanitizer or physical disinfectant strategy may reduce any pathogen presence. A bacteriophage application may further reduce the remaining population. Because bacteriophage are susceptible to pH and other physical factors, application after a chemical sanitizer or irradiation is ideal.

Bacteriophage application in a solution is recommended because they may collide with their target. Brownian motion or an external agitation may facilitate the collision between bacteria and the bacteriophage. High concentrations of bacteriophage (10^9 or 10^{10} PFU/ml) increase the chance of colliding with and adhering to target bacteria. However, environmental conditions of the solution play a role in bacteriophage adhesion. Divalent cations play an important role in positioning bacteriophage for attachment *(26)*. Mg^{2+} and Ca^{2+} ions may need to be present to increase the bacteriophage efficacy.

The physiological state of the host may also play a role in bacteriophage attack. The bacteria surface changes as the bacteria growth phase changes. In one study, the surface area of *E. coli* B/r changed depending on the growth medium and temperature, which influenced the rate of bacteriophage adsorption. One study showed that bacteriophage reduction of *S. epidermis* was slower in biofilms and stationary phase when compared to in exponential phase *(10)*. Most bacteria exist in the biofilm form and

may be less susceptible to bacteriophage. It is therefore important to make the other environment factors as conducive for adhesion as possible.

A spray application of bacteriophage is an alternative to a dunking application. Intralytix recommends that bacteriophage be sprayed onto produce to avoid cross contamination within the solution. A high concentration of bacteriophage is recommended so that any bacteria on its surface is immediately infected with the bacteriophage. However, the produce surface plays an important role in bacteriophage contact with bacteria. It is well known that a leaf surface may facilitate bacterial attachment and colonization *(13, 38).* The crevices and ridges in the leaf may hide bacteria from bacteriophage spray. The application may not effective in bacteriophage coverage. Once the bacteriophage is applied, it is only effective on the portions of the leaf it is in contact with. At a high enough concentration, the coverage may be effective enough to infect all areas with bacteria presence, and may remain viable during storage time. They present an advantage because it has continued antimicrobial activity after its application. During storage times, it is able to reduce bacteria, and can potentially reduce cross-contamination. Even during a low temperature, the contact between the bacteria and bacteriophage, once establish provides a reduction.

Multiple bacteriophage strains to target one group bacteria is also recommended. Bacteriophage may have attack a wide range of bacteria serovars. One bacteria strain may be resistant to one strain of bacteriophage, but is likely to be susceptible to another strain. Bacteriophage to target different groups of bacteria (i.e. *Salmonella, Listeria, E. coli* species) should also be used together. Because there may be a risk for multiple pathogen contamination, bacteriophage application should target multiple groups. A cocktail of

different bacteriophage strains to target different groups of and multiple species within a group of bacteria is most effective.

Using bacteriophage in a controlled environment helps it work better. In a sealed MA environment, the bacteria population on the produce is confined. The bacteriophage can focus on attacking and reducing levels in the package. If the package is open, bacteria may float onto the produce and bacteriophage reduction is limited. As bacteriophage cannot move on its own, it must come into contact. Bacteriophage works best by being used at a high MOI to immediately eliminate pathogen presence.

Bacteriophage-resistant bacteria is a concern. However, bacteriophage, can be found in all environments, which gives a large pool of alternative bacteriophage strains. A new bacteriophage can be isolated from the environment to use against pathogens. To minimize bacteriophage resistant, it is important to eliminate pathogens immediately. Using bacteriophage in a cocktail (multiple strains) is recommended.

2.7 Conclusion

Bacteriophage have been effective in meat applications and also in produce applications. Optimizing in each application is needed to make bacteriophage most effective. More study is needed to understand how bacteriophage can be best applied. Treatments must be validated, and treatment methods can be optimized.

CHAPTER 3

S. ENTERICA GROWTH IN WASH WATER SOLUTIONS

3.1 Experimental Background

The growth of *S. enterica* in produce wash water solutions is not well studied. On farms, bacteria are shed from produce into the wash water, and may grow if not killed by sanitizer. Farm wash water is changed infrequently, and bacteria may grow to high numbers if given enough time. The goal of this study was to understand how *S. enterica* grows in different wash water solutions. Experiments were conducted in two wash water systems—one with TSB and one without. This study helps better understand the viability of *S. enterica* in wash water solutions and the importance in sanitizer .

3.2 Materials and Methods

3.2.1 *S. enterica* strain preparation

Five *S. enterica* strains were obtained from ATCC (ATCC Manassas, VA 13311, 14028, 9712, 51962), and grown to 50 μg/ml nalidixic acid resistance. A single colony of each strain was grown in tryptone soy broth (*abbreviated* TSB, ThermoScientific™, Waltham, MA), and then amended incrementally with 25 μg/ml nalidixic acid (Fisher BioReagents, Fair Lawn, NJ) to a final concentration to 50 μg /ml nalidixic acid in TSB at 37 °C. After 24 hr, the cultures were pelleted and washed twice. Strains were stored in glycerol and TSB at -80 °C. Strains were resurrected in TSB + 50 μg /ml nalidixic acid for 16 hr at 37 °C for use.

3.2.2 Simulated wash water solution preparation

Vegetables (spinach, iceberg lettuce, and romaine lettuce) were purchased from local grocery stores and stored at 4 °C before use. Within 5 days of purchase, each vegetable was blended with distilled water with an immersion blender and diluted to turbidity concentrations of 25, 50, and 100 NTU (Hach 2100Q Portable Turbidimeter, Ames, IA) as necessary. Solutions were autoclaved before use, and turbidities and pH were measured after autoclaving.

3.2.3 *S. enterica* growth curve studies in TSB + 100 NTU vegetable solutions

S. enterica was grown overnight in TSB + 50 μg/ml nalidixic acid. Solutions of equal parts TSB and 100 NTU vegetables were warmed to 37 °C before *S. enterica* was added. 10^4 CFU *S. enterica* was added to solutions to yield a final concentration of 10^2 CFU/ml. Bacteria counts were measured at regular increments for up to 12 hours. This experiment was repeated in duplicate.

3.2.4 *S. enterica* growth curve studies in 100 NTU vegetable solutions

S. enterica was grown overnight in TSB + 50 μg/ml nalidixic acid. Solutions of 99 ml 100 NTU of spinach, romaine, and iceberg solutions were warmed to 37 °C before *S. enterica* was added. 10^4 CFU was added to 99 ml of wash water solutions yielding a final concentration of 10^2 CFU/mL. Bacteria counts were measured at regular increments for up to 24 hours. This experiment was repeated in triplicate.

3.2.5 Statistical Analysis

Data was analyzed through two-way analysis of variance followed by a Tukey's test with significance where $p \leq 0.05$.

3.3 Discussion

3.3.1 Growth curves of *S. enterica* in simulated wash water solutions with TSB

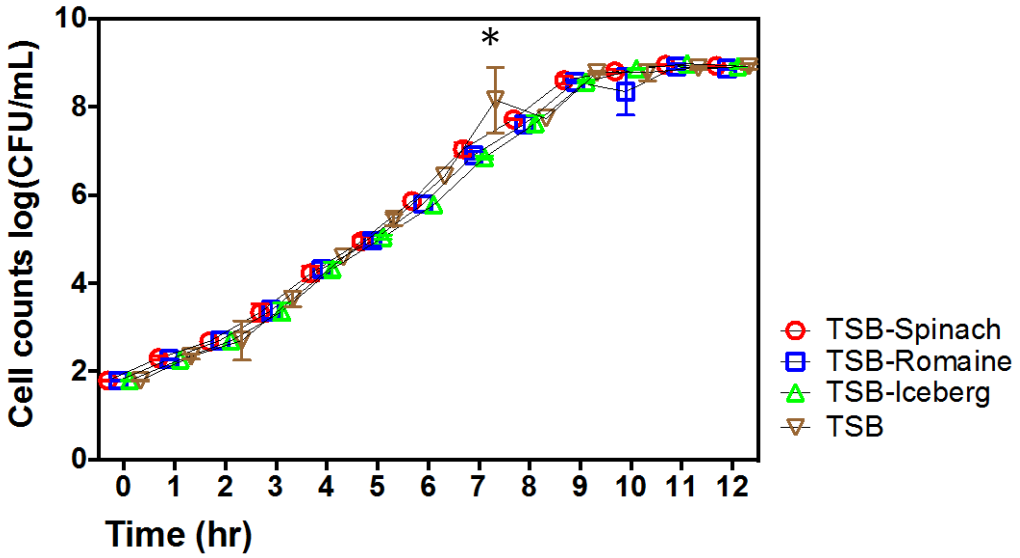

Figure 3: Growth curves of *S. enterica* in wash water solutions. Wash water solutions contained equal parts of TSB and 100 NTU spinach, romaine lettuce, or iceberg lettuce. Experiment was conducted at 37 °C over 12 hours.
* Indicates a statistical difference ($p < 0.05$)

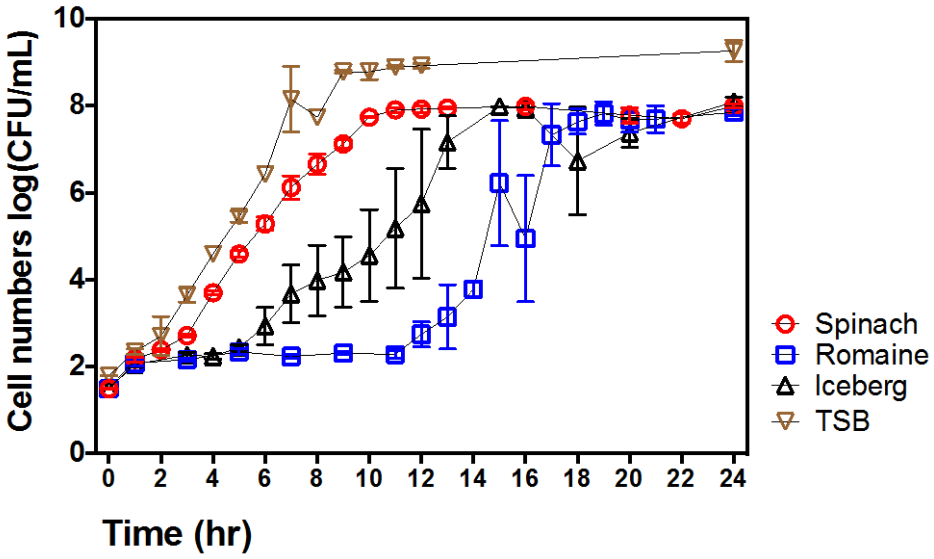

Figure 4: Growth curves of *S. enterica* in 100 NTU spinach, romaine, and iceberg lettuce solutions at 37 °C. No statistical analysis was conducted.

S. enterica growth was compared between two wash water models. In **Figure 3**, where wash water solutions contained TSB, growth among all solutions (with vegetables and without) were not statistically different except at hour 7 ($p < 0.05$, **Figure 3**). In the ideal growth medium TSB, the lag phase was 1.5 hours. In vegetable solutions, the lag phase was 2 hours for spinach and iceberg lettuce, and 2.5 hours for romaine lettuce. Stationary phase was reached in 9 hours for all solutions. Although *S. enterica* levels at hour 7 was slightly higher in TSB solutions, its growth similar to other solutions ($p > 0.05$). Given the large standard error of mean, if the experiment was replicated again, the *S. enterica* levels may not be statistically different. The overall trend of *S. enterica* was similar in solutions with and without organic matter. If a real life wash water solution was nutrient-rich (containing amino acids, sugars, and salts) along with organic matter, *S. enterica* is capable of growing to high levels within hours. Given that the infectious dose

of *S. enterica* is 100 cells, it is important that wash water contain sanitizer to prohibit bacterial growth.

3.3.2 Growth curves of *S. enterica* in simulated wash water solutions

The second model of wash solutions contained organic matter from vegetables only. In this model, *S. enterica* growth was drastically different across the 100 NTU vegetable solutions (**Figure 4**). Unlike the previous growth measurements (**Figure 3**), *S. enterica* responded very differently in spinach, romaine lettuce, and iceberg lettuce solutions compared to TSB solutions ($p < 0.05$).

The lag phase in spinach was shorter than in romaine or iceberg lettuce. *S. enterica* entered the exponential phase after approximately two hours, and reached stationary phase at hour 10. Growth was also the most consistent in spinach, as noted by the small standard deviations. By hour 5, *S. enterica* levels were significantly different in romaine lettuce and iceberg lettuce solutions ($p < 0.05$) but were not significant different in spinach solutions ($p > 0.05$). This suggests that the nutritional content in romaine and iceberg lettuce was significantly less than spinach and TSB, making a less favorable growing environment for *S. enterica*.

S. enterica growth in romaine and iceberg lettuce varied greatly in each replicate experiment. *S. enterica* did not grow consistently, as lag phase took anywhere from 11 hours to 17 hours. The large error bars in the exponential phase indicate that *S. enterica* was not consistently at the exponential phase during those hours.

Iceberg lettuce solutions also displayed similar activity. Exponential phase ranged between hour 6 or hour 10. This variable growth is not well understood, but may be

attributed to the differences in nutritional values in each vegetable. Each replicate used a different vegetable batch, and the batches may have different nutritive substances that *S. enterica* needed. The faster they grew, the more available nutrients *S. enterica* was able to use. Spinach was most nutrient dense, as indicated by the shortest lag phase. When spinach was compared to romaine and iceberg lettuce, the most notable differences were in minerals (**Appendix, Table 1**). Spinach contained more iron, calcium, potassium, sodium, and Vitamin C than romaine and iceberg lettuce. These micronutrients may have played an integral role in *S. enterica* growth. Alternatively, the nutritional content from spinach may have been more robust. All solutions were autoclaved, and proteins and other nutrients may have unfolded in the process. Content from spinach may have better withstood autoclave conditions and been available for *S. enterica* use. Although this behavior was not further explored, this hypothesis could be tested by adding micronutrients to romaine and iceberg lettuce solutions and testing if *S. enterica* growth is accelerated.

In comparison to the previous set of growth curves with the TSB addition, it is also clear that *S. enterica* was largely dependent on the nutrients from the TSB to grow, not the vegetable nutrients. Growth was slower using vegetable nutrients alone, taking a longer time to reach exponential phase for romaine and iceberg lettuce. As a result, it was concluded that TSB would impact the bacteriophage and *S. enterica* activity greatly.

In the next steps, the bacteriophage studies were only conducted in spinach solutions because spinach solutions yielded the most consistent *S. enterica* growth. A preliminary study of bacteriophage and *S. enterica* activity was conducted (**Appendix Figure 1** and **Figure 2**), and responses were identical across spinach, romaine lettuce,

and iceberg lettuce. Bacteriophage behavior in spinach may be applied to romaine lettuce, and iceberg lettuce.

3.3.3 Conclusion

S. enterica behavior varies in the wash water environment. In a nutrient-rich environment with TSB, *S. enterica* grows quickly and reaches exponential phase after hour 2. This demonstrates the viability of *S. enterica* and the possibility it may spread quickly as levels increase. However, *S. enterica* growth was significantly different in spinach, romaine lettuce, and iceberg lettuce solutions. *S. enterica* grew to significantly different levels by hour 5 when romaine and iceberg lettuce solutions were compared to TSB. Spinach solutions yielded the most similar growth to TSB, and suggest that the nutrient profile of the vegetable plays a big role in *S. enterica* behavior and spread.

CHAPTER 4

BACTERIOPHAGE AND *S. ENTERICA* BEHAVIOR IN SIMULATED WASH WATER SOLUTIONS WITH TRYPTONE SOY BROTH AND VEGETABLES

4.1 Experimental Background

There is a lack of literature studying bacteriophage activity with organic matter from vegetables. On farms, wash water is an important point in the processing step to remove field heat from produce. Consequently, the water often contains organic matter from soil debris and produce, which may influence bacteriophage behavior. Organic matter influence on bacteriophage was elucidated. Bacteriophage were first incubated with different concentrations of vegetable organic matter and tested for lytic activity using plaque assays. These solutions contained organic matter from vegetables, and were mixed with TSB. At the same time, bacteriophage activity and *S. enterica* populations were studied in 100 NTU solutions of organic matter. This approach helps determine if organic matter inhibits bacteriophage from reducing *S. enterica* in a rich environment.

4.2 Materials and Methods

4.2.1 Simulated wash water solution preparation

Vegetables (spinach, iceberg lettuce, and romaine lettuce) were purchased from local grocery stores and stored at 4 °C before use. Within 5 days of purchase, each vegetable was blended with distilled water with an immersion blender and diluted to turbidity concentrations of 25, 50, and 100 NTU (Hach 2100Q Portable Turbidimeter,

Ames, IA) as necessary. Solutions were autoclaved before use, and turbidities and pH were measured after autoclaving.

4.2.2 Bacteriophage activity in wash water solution studies

Bacteriophages were diluted in bacteriophage broth (24 g/L tryptone, 5 g/L NaCl, 150 mg/L $CaCl_2$, 200 mg/L $MgSO_4$, 50mg/L $MnSO_4$, 18 g/L sodium acetate trihydrate, and 6 g/L glucose), and 100 μL was added to 0.5 mL of vegetable juice. The mixture was incubated at 25 °C for 30 and 60 minutes before titration. A control was tested with the same bacteriophage dilution portion, and a titer was immediately taken.

4.2.3 Challenge studies simulated wash water with broth and S. enterica studies

44.5 mL of vegetable juice and 44.5 mL of TSB were warmed to 37 °C before use. 10^8 CFU *S. enterica* (overnight cultures of ATCC 13311, 14028, 9712, 6958, 51962) and 10^8 PFU of Intralytix™ SalmoFresh™ (Baltimore, MD) bacteriophage cocktail was added. The final MOI was 1. The system was incubated at 37° C at 200 RPM. Bacteriophage and bacteria levels were measured at 45, 90, and 135 minutes.

4.2.4 Statistical analysis

Data from was analyzed through two-tailed t-tests, with significance where $p < 0.05$. Challenge studies data were analyzed through two-way analysis of variance with a Tukey's test where necessary with a significance where $p < 0.05$.

4.3 Discussion of Results

4.3.1 Incubation studies of bacteriophage and organic matter

Incubation studies of bacteriophage with vegetable juices showed that bacteriophage responded in similar ways (**Figure 5**). There was no significant inhibitory effect of organic matter on bacteriophage ($p > 0.05$).

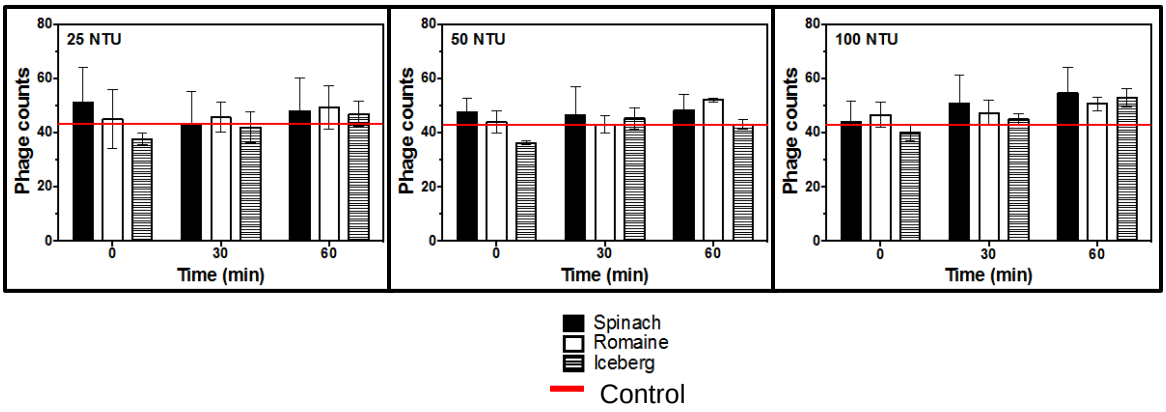

Figure 5: Incubation of bacteriophage and spinach, romaine lettuce, and iceberg lettuce solutions. The red line indicates the control (bacteriophage activity from a standard plaque assay).
* no statistical significance observed in solutions (two-tailed t-test, $p < 0.05$)

Bacteriophage activity was also consistent across different types of organic matter sources (vegetables). Results were within range of the control, which was an immediate measurement of the bacteriophage cocktail. Even across different concentrations of organic matter and time incubation, bacteriophage maintained its lytic ability. Bacteriophage activity was not inhibited or increased in the presence of higher organic matter concentrations. Also, the source of organic matter did not play a significant role. Bacteriophage activity was similar across different vegetables ($p > 0.05$). Overall, bacteriophage retained its ability to lyse on a bacterial lawn. Initial studies indicate that organic matter does not inhibit bacteriophage activity.

4.3.2 Bacteriophage and *S. enterica* studies in simulated wash water solutions with TSB

Bacteriophage growth was observed in TSB-vegetable solutions (**Figure 6**). Over two hours, one burst was observed by the end of 90 min. By the end of two hours, the bacteriophage had amplified 100-fold. A second burst was not observed, but may have been detected if the incubation time was extended.

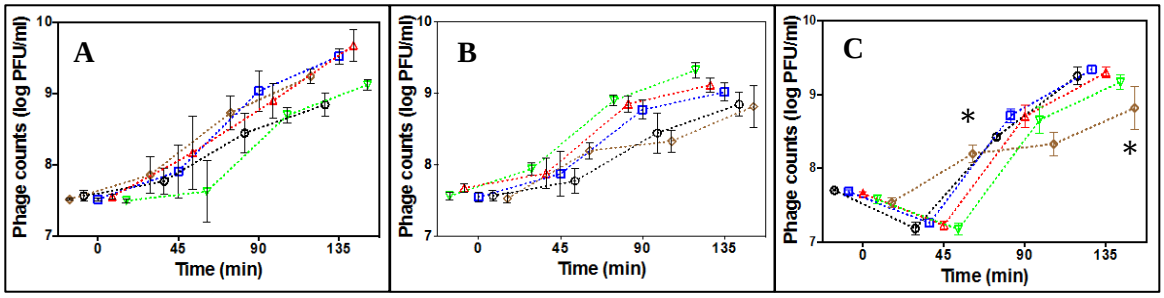

Figure 6: Bacteriophage growth curves over 135 minutes in TSB + vegetable solutions. (**A**. Spinach; **B.** Romaine lettuce; **C.** Iceberg lettuce.) at 37° C 200 RPM.
(Broth; Water; 25 NTU; 50 NTU; 100 NTU.) Experiments were repeated in triplicate. Error bars represent SEM.
* Phage levels in TSB were statistically significant at 45 minutes and 135 minutes (two-way analysis of variance followed by Tukey's test, $p < 0.05$)

Within each vegetable, the concentration of organic matter did not have a significant effect on bacteriophage behavior. In spinach solutions, bacteriophage increased 2 log PFU in 25, 50, and 100 NTU solutions. Bacteriophage levels were not significantly different among solutions ($p < 0.05$).

In romaine lettuce solutions, bacteriophage increased 1.4 log PFU in 25 NTU solutions, 1.5 log PFU in 50 NTU solutions, and 1.8 log PFU in 100 NTU solutions. Again, bacteriophage levels were similar among solutions ($p > 0.05$).

In iceberg lettuce solutions, bacteriophage increased 1.6 log PFU in 25, 50, and 100 NTU solutions. However, after 45 minutes of incubation, bacteriophage levels were significantly higher in TSB than other solutions ($p < 0.05$). The high level of bacteriophage most likely corresponds to a faster burst. *S. enterica* may have been more quickly infected in TSB solutions after 45 minutes than other solutions. Although there was an initial burst in TSB solutions at 45 minutes, bacteriophage levels were slightly lower at the 135 minute time point. This suggests that bacteriophage were infecting other *S. enterica* and undetected by the plaque assay. If time was extended, higher bacteriophage levels may be observed.

Overall, bacteriophage grew the most in spinach (1.8 log), but levels were not statistically different from other solution types ($p < 0.05$). When compared to control solutions, bacteriophage levels in TSB grew 1.8 log PFU, and 1.5 log PFU in water solutions. No significant difference was observed in bacteriophage growth between controls and organic loaded solutions. This suggests that the presence of organic matter had no inhibitory effect on bacteriophage efficacy. Therefore, bacteriophage may potentially be used in wash water solutions with a large load of organic matter.

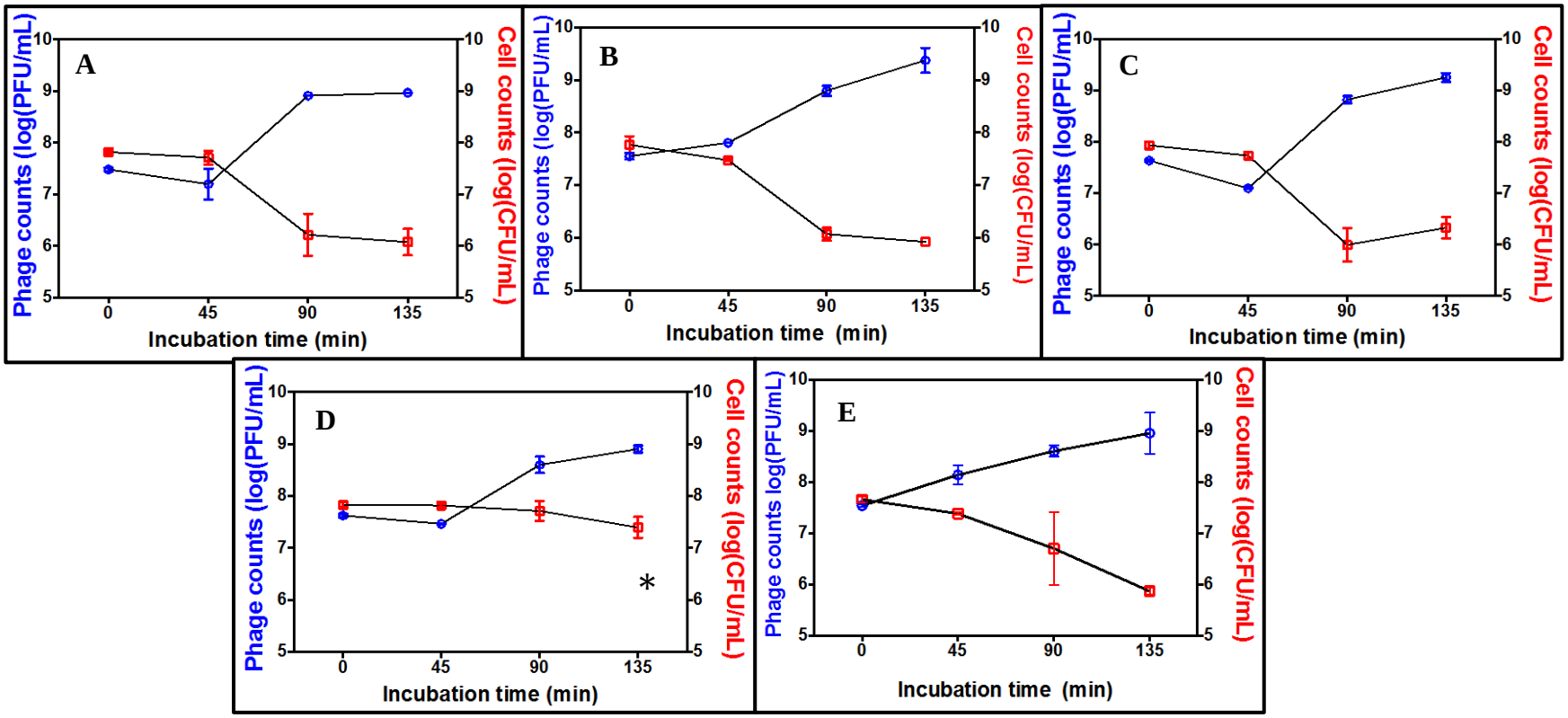

Figure 7: Bacteriophage and *S. enterica* populations in TSB + 100 NTU vegetable solutions **(A**. 100 NTU spinach, **B**. 100 NTU Romaine lettuce, **C**. 100 NTU Iceberg lettuce, **D**. Water, **E**. TSB) during incubation at 37° C and 200 RPM.

(*S. enterica populations;* Bacteriophage populations) Experiments were repeated in duplicate. Error bars represent SEM, * *S. enterica* populations were statistically different in Water solutions compared to all other solutions at 135 minutes ($p < 0.05$).

S. enterica reduction in combination with bacteriophage amplification was also analyzed. In all solutions, except water, *S. enterica* was reduced approximately 1.5 log in 135 minutes and bacteriophage grew approximately 2 log (**Figure 7**). In water, *S. enterica* reduction was only 0.5 log, although bacteriophage amplified 1.5 log. *S. enterica* levels were higher ($p < 0.05$) in water solutions at 135 min when compared to other solutions. In water, bacteriophage and *S. enterica* may have been less active as the solution did not contain any nutrients. Nutrients such as divalent cations play an important role in bacteriophage attachment to bacteria. Its lack may explain the lack of *S. enterica* reduction. Although water solutions had similar levels of bacteriophage as in other vegetable and TSB solutions, it is likely that bacteriophage were less active in water.

In vegetable and TSB solutions, bacteriophage may have infected *S. enterica*, rendering the bacteriophage and the *S. enterica* particles undetected. Once a bacteriophage infects a bacteria cell, it is no longer detectable by a plaque assay. This may explain why *S. enterica* levels were lower in water, while bacteriophage levels were comparable. Bacteriophage levels may actually be higher in vegetable and TSB solutions, and its levels would have been confirmed if incubation times were extended to observe another burst. In general, *S. enterica* was not effectively controlled in water solutions.

When different concentrations of organic matter were compared, 100 NTU solutions generally yielded the highest bacteriophage counts, with levels in 25 and 50 NTU solutions being slightly lower. This suggests that organic matter does not limit bacteriophage activity, but on the contrary, aids it. The highest concentration of vegetable solution contains the most vegetable residue. The nutritional content in the vegetables

may have helped bacteriophage work more effectively. Unlike other chemical sanitizers such as chlorine, bacteriophage efficacy was maintained, and heightened in the presence of organic matter.

Overall, bacteriophage levels in all solutions were comparable and not statistically different ($p < 0.05$). Bacteriophage amplification and *S. enterica* reduction was at equal levels in organic matter solutions and TSB control solutions. Only in water solutions was the ability for bacteriophage to reduce *S. enterica* diminished ($p < 0.05$). These studies indicate that bacteriophage was not hindered in the presence of organic matter, and can effectively reduce *S. enterica* levels.

4.4 Conclusion

Studies show that bacteriophage activity was not hindered by the presence of organic matter. Bacteriophage were able to amplify over 135 minutes in the presence of different sources and concentrations of organic matter. Bacteriophage growth was also correlated to *S. enterica* reduction. In 100 NTU solutions, *S. enterica* was effectively reduced approximately 2 log CFU/ml in all solutions except water within 135 minutes. *S. enterica* levels in water solutions were not effectively reduced and suggest that bacteriophage are more effective in solutions containing organic matter.

CHAPTER 5

BACTERIOPHAGE AND *S. ENTERICA* BEHAVIOR IN SIMULATED WASH WATER SYSTEMS IN ORGANIC MATTER DERIVED FROM VEGETABLES

5.1 Experimental Background

From the previous study, bacteriophage activity was not hindered by the presence of organic matter. The next stage studied bacteriophage in a more realistic system—organic matter from vegetables only. From growth curve studies, *S. enterica* growth was most consistent in spinach solutions. Subsequent experiments studied bacteriophage and *S. enterica* growth trends in spinach solutions alone. Temperature and bacteriophage concentrations were varied. The purpose was to study the efficacy of bacteriophage-mediate lysis of *S. enterica* cells at differing temperatures and initial concentrations. This study evaluates the efficacy of the bacteriophage cocktail in reducing *S. enterica* levels in a more realistic wash water system.

5.2 Materials and Methods

5.2.1 Simulated wash water solution preparation

Vegetables (spinach, iceberg lettuce, and romaine lettuce) were purchased from local grocery stores and stored at 4 °C before use. Within 5 days of purchase, each vegetable was blended with distilled water with an immersion blender and diluted to turbidity concentration 100 NTU (Hach 2100Q Portable Turbidimeter, Ames, IA) as necessary. Solutions were autoclaved before use, and turbidities and pH were measured after autoclaving.

5.2.2 Challenge studies in simulated wash water and *S. enterica* studies

Bacteria strains were grown in TSB at 37 °C for 18 hr. 99 mL of vegetable were warmed to 20 °C or 37 °C before use. A final concentration of 10^4 CFU/mL of five strains of *S. enterica* (ATCC 13311, 14028, 9712, 6958, 51962) were added 10^3 or 10^6 PFU/mL of Intralytix™ SalmoFresh (Baltimore, MD) bacteriophage cocktail was added (final MOI of 0.1 or 10). Bacteriophage and bacteria concentrations were measured every 90 minutes for 7.5 hr. A final measurement at 24 hr was also taken.

5.3 Discussion of Results

5.3.1 Bacteriophage and *S. enterica* trends in simulated wash water solutions

Studies showed that temperature and solution content had an important influence on bacteriophage activity. Bacteriophage were effective in controlling *S. enterica* levels within TSB solutions at 37 °C, but not at 20 °C. In general, bacteriophage were also ineffective within 100 NTU spinach solutions.

At 20 °C, bacteriophage was ineffective in reducing *S. enterica.* Bacteriophage and *S. enterica* levels did not change up to 7.5 hours, but at 24 hours they reached high levels. Bacteriophage was unable to infect *S. enterica* as illustrated by the plateau in bacteriophage levels and *S. enterica* levels (**Figures 8, 9**). However, bacteriophage may have exhibited some antimicrobial effect between 7.5 and 24 hours. Most likely, *S. enterica* and bacteriophage needed at least 7.5 hours to adapt to the environment before becoming active. Bacteriophage control in 20 °C was not observed, but may have been observed if testing continued between 7.5 and 24 hours. Temperature plays an important role in bacteriophage efficacy. D'Herelle demonstrates temperature-dependent

bacteriophage activity in his studies *(16)*. Bacteriophage were able to infect bacteria strains within its optimum range, but at higher temperatures, the bacteriophage grew more slowly and did not have as pronounced of an effect against bacteria. Given the temperature effects on bacteriophage, the bacteriophage strains may have been less effective at 20 °C as it may have been out of its range. However, at 37 °C, bacteriophage were effective in controlling *S. enterica* in TSB solutions only. A 4 log CFU reduction was observed at MOI 0.1 and 10 at 7.5 hr when compared to the bacteria control. However, there was no complete kill in solution. By 24 hours, *S. enterica* grew to high levels (> 9 log CFU/ml), which was comparable to levels in bacteria control solutions. Although further experiments were not conducted to understand why *S. enterica* was not successfully eliminated, bacteriophage-resistant *S. enterica* populations are a likely cause. Five different strains of *S. enterica* were used, all which were susceptible to the bacteriophage cocktail. One or more of the *S. enterica* strains may have developed a resistance to the bacteriophage and dominated the system. The bacteriophage virulence in each *S. enterica* strain was not studied in depth, but bacteriophage lytic activity was observed in individual *S. enterica* strains in plaque assays (results not shown). To better understand the bacteriophage-resistance trends, the experiments may be repeated with individual *S. enterica* strains. However, some studies suggest that antimicrobial activity may differ between studies with individual and multiple strains of bacteria *(23)*. Studying bacteriophage effects with individual *S. enterica* strains may show different results, but may help elucidate the bacteriophage resistant trends of *S. enterica.*

The SalmoFresh™ bacteriophage cocktail also contained five different strains of bacteriophage. Although multiple bacteriophage decreased the chance of bacteriophage-

resistance, it may still occur. Individual bacteriophage strains may not be isolated from the commercially available bacteriophage cocktail. Instead, experiments with single strains of *S. enterica* may help reveal which strains are becoming resistant.

The physiological state of *S. enterica* may also have played an important role in bacteriophage activity. In spinach solutions, no *S. enterica* reduction was observed (**Figures 8, 9**). Bacteriophage was able to amplify over the 7.5 hours, but its growth did not yield any decrease of *S. enterica* populations. *S. enterica* grew to similar levels as did the bacteria control. Bacteriophage were able to grow as observed in **Figures 8, 9** but were not able to eliminate it to slow or stunt *S. enterica.*

As mentioned earlier, certain strains of *S. enterica* may have been less susceptible to the bacteriophage than others. In an environment with less nutrients and sugars than TSB, *S. enterica* may have been in a stressed state, which may affect its cell physiology. Some studies show that bacteria may undergo a physiological change to develop bacteriophage-resistance *(15, 21, 31).* Cellular receptors and proteins on its surface change with cell metabolism. In a more stressed state, the *S. enterica* strains may not have been displaying the target receptors, making it less vulnerable to bacteriophage attack. This gives rise to bacteriophage-resistant strains which allow for certain strains to dominate. Bacteriophage is no longer effective, and the risk of *S. enterica* contamination increases. As discussed in other studies, Delbrock et al found that the host physiology had an effect on phage adsorption. They measured a sixty-times difference between optimal and poor conditions of bacteria *(14).* Similar activity may be observed within these experiments.

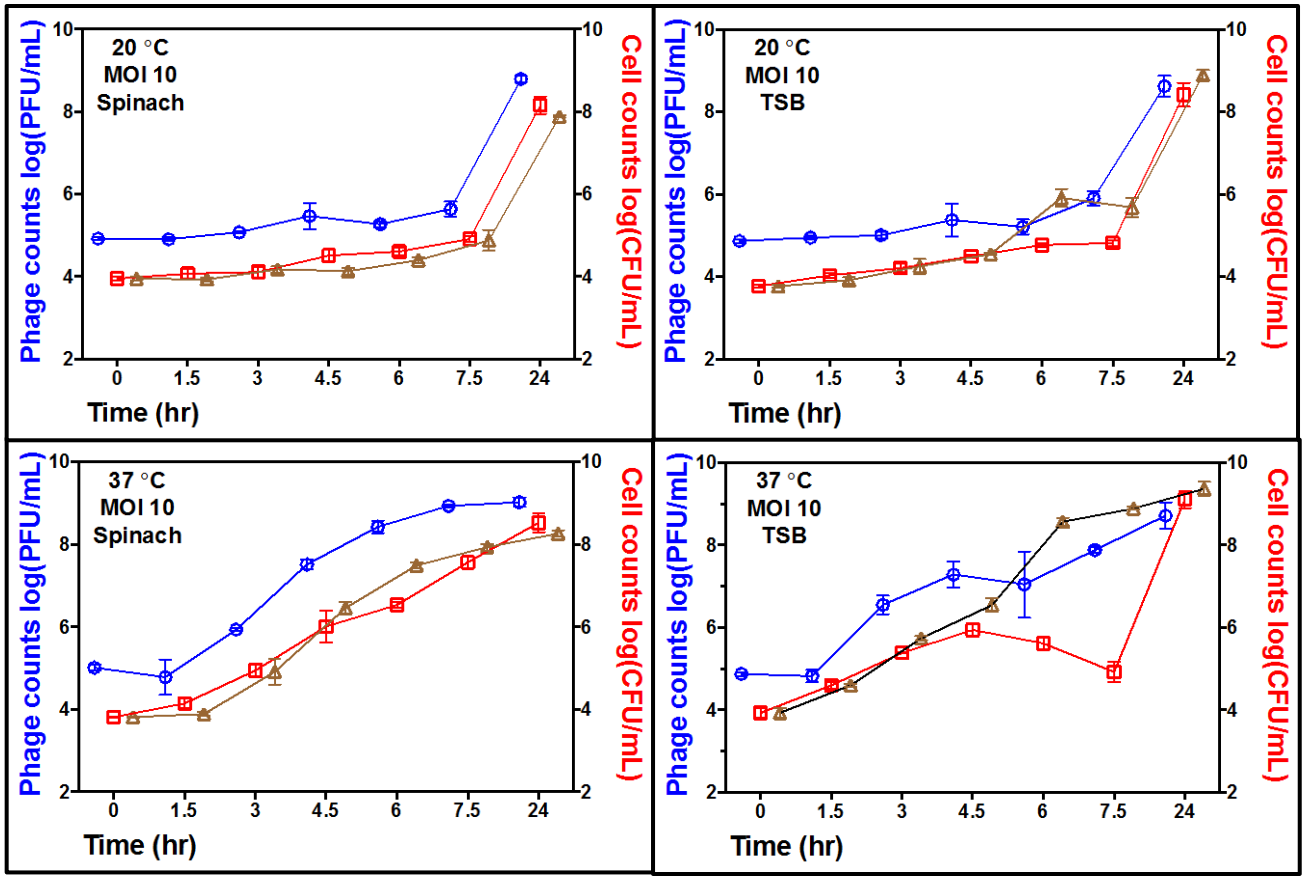

Figure 8: Bacteriophage MOI 10 and *S. enterica* levels in spinach and TSB solutions at 20 °C or 37 °C. (*S. enterica populations* with bacteriophage; Bacteriophage populations; *S. enterica* populations without bacteriophage) Experiments were repeated in triplicate. Error bars represent SEM,

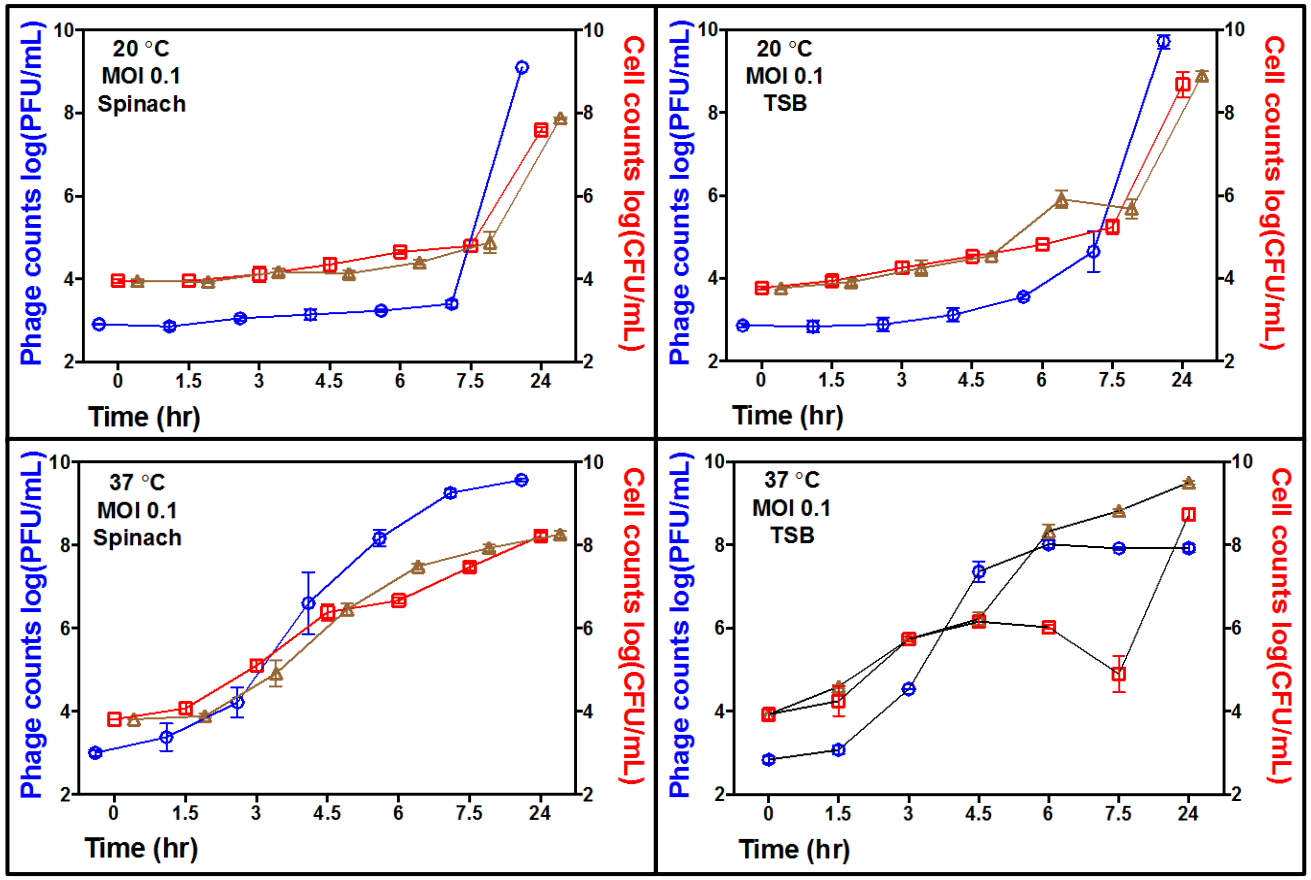

Figure 9: Bacteriophage MOI 0.1 with *S. enterica* levels in spinach and TSB solutions at 20 °C or 37 °C. (*S. enterica populations* with bacteriophage*;* Bacteriophage populations; *S. enterica* populations without bacteriophage) Experiments were repeated in triplicate. Error bars represent SEM,

Bacteriophage application strategy plays an important role in bacteria reduction. Some studies show that bacteriophage was able to reduce bacteria within 24 hours in a spray or spot application at refrigerated temperatures *(5, 9, 32)*. In these conditions, bacteriophage were directly applied to areas containing bacteria. Studies showed a significant reduction in bacteria, even at storage temperatures. Direct contact between bacteriophage and bacteria help facilitate bacterial reduction. However, within a solution, bacteriophage contact with bacteria was randomized, even with agitation. Because contact between was not constant, which may explain bacteriophage inability to reduce *S. enterica*. This inability may be further exacerbated as temperature and medium played an important role in *S. enterica* reduction. Within a solution-based application, bacteriophage efficacy may be optimized with a more temperature-adapted bacteriophage, and a more dramatic MOI. Although MOI 0.1 and 10 did not show a significant difference in bacteriophage levels and *S. enterica* levels, a MOI of 100 or 1000 may display an effect.

5.3.2 Conclusion

Bacteriophage efficacy was independent of MOI, but dependent on temperature and solution type. Although it was able to amplify over 24 hours within spinach solutions, it was ineffective in controlling *S. enterica* levels in spinach solutions. However, bacteriophage effectively controlled *S. enterica* at 37 °C within TSB solutions, but not at 20 °C. This demonstrates that bacteriophage were not successful in controlling *S. enterica* populations in a real life model with organic matter derived from spinach, but were effective in a nutrient rich environment such as TSB.

CHAPTER 6

SUMMARY AND CONCLUSION

Bacteriophage applications in wash water studies did not effectively reduce *S. enterica* levels. Although studies with a TSB component showed that organic matter had no inhibitory effect on bacteriophage, bacteriophage was ineffective without TSB. *S. enterica* levels continued to grow in the presence of bacteriophage. However bacteriophage levels also grew, indicating that a partial kill occurred. However, a complete kill of *S. enterica* was not attained, indicating that it was ineffective in organic matter solutions from spinach. TSB solutions, an ideal environment for *S. enterica* and bacteriophage, showed a 4 log decrease of *S. enterica.* Although *S. enterica* control was observed, TSB would not be present in farm wash water applications. Bacteriophage applications in wash water may need to be optimized to achieve a high kill.

Bacteriophage activity at a low temperature (20 °C) was very limited up to 7.5 hours and displayed no antimicrobial effect on *S. enterica.* The likelihood of cross-contamination of bacteriophage in a dip application is high. However, at warm temperature (37 °C), bacteriophage was able to grow in spinach solutions and also reduce *S. enterica* up to 7.5 hours. However, over an extended period of time, bacteriophage could not completely eliminate *S. enterica*, and bacteria levels grew to ~9 log. Wash water solutions are ideally cold (4 °C) to remove field heat from crops, but bacteriophage were unable to control bacteria at 20 °C. They only displayed a controlling effect at 37 °C which is unrealistic for real life applications. Also, it is unlikely that a wash water solution be high in sugars and salts, unless artificially added. Even so, bacteriophage

required at least 4.5 hours before it had a significant effect on *S. enterica* levels. Bacteriophage treatments were not instanteously effective against *S. enterica* and the risk of cross-contamination within solution remains high.

Although bacteriophage activity was not practical in wash water studies, bacteriophage remain a promising option for bacterial control. They have been tested with hurdle techniques and have effectively reduced bacteria levels on produce. Future work may investigate bacteriophage application in the post-wash water stage, during refrigerated storage.

APPENDIX

SUPPLEMENTARY TABLES AND GRAPHS

Table 1: Nutritional comparison of 100 g of iceberg lettuce, romaine lettuce, and spinach.

Source: USDA National Nutrient Database for Standard Reference 27 Software v.2.2.4

		Spinach	**Romaine Lettuce**	**Iceberg Lettuce**	**TSB**
Nutrient	**Unit**	**1Value per 100 g**	**1Value per 100 g**	**1Value per 100 g**	**1Value per 100g**
Proximates					
Water	g	91.4	94.61	95.64	97
Protein	g	2.86	1.23	0.9	2
Total lipid (fat)	g	0.39	0.3	0.14	
Carbohydrate, by difference	g	3.63	3.29	2.97	
Fiber, total dietary	g	2.2	2.1	1.2	
Sugars, total	g	0.42	1.19	1.97	0.25
Minerals					
Calcium, Ca	**mg**	**99**	**33**	**18**	
Iron, Fe	mg	2.71	0.97	0.41	
Magnesium, Mg	mg	79	14	7	
Phosphorus, P	mg	49	30	20	
Potassium, K	**mg**	**558**	**247**	**141**	
Sodium, Na	**mg**	**79**	**8**	**10**	**500**
Zinc, Zn	mg	0.53	0.23	0.15	
Vitamins					
Vitamin C, total ascorbic acid	**mg**	**28.1**	**4**	**2.8**	
Thiamin	mg	0.078	0.072	0.041	
Riboflavin	mg	0.189	0.067	0.025	
Niacin	mg	0.724	0.313	0.123	
Vitamin B-6	mg	0.195	0.074	0.042	
Folate, DFE	µg	194	136	29	
Vitamin A, RAE	µg	469	436	25	
Vitamin A, IU	IU	9377	8710	502	

Vitamin E (alpha-tocopherol)	mg	2.03	0.13	0.18	
Vitamin K (phylloquinone)	µg	482.9	102.5	24.1	

Table 2: pH of TSB-vegetable solutions in growth curves

Solution	**pH**
TSB-Spinach	7.25 ± 0.03
TSB-Romaine lettuce	7.27 ± 0.08
TSB-Iceberg lettuce	7.28 ± 0.05
TSB	7.30 ± 0.05

Table 3: pH of 100 NTU vegetable solutions

100 NTU Solution	**pH**
Spinach	6.68 ± 0.06
Romaine lettuce	6.51 ± 0.15
Iceberg lettuce	6.21 ± 0.10

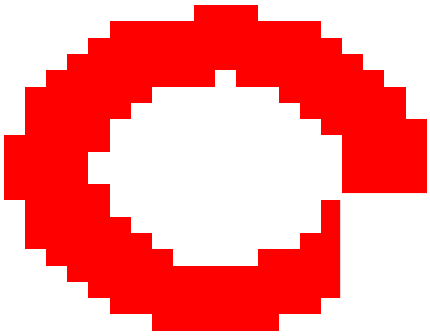

Figure 10: Bacteriophage growth over 5 hours in ○ 100 NTU spinach, ○ 100 NTU romaine lettuce, and ○ 100 NTU iceberg lettuce, and ○ Water at 37 °C, 200 RPM. No statistical analysis conducted.

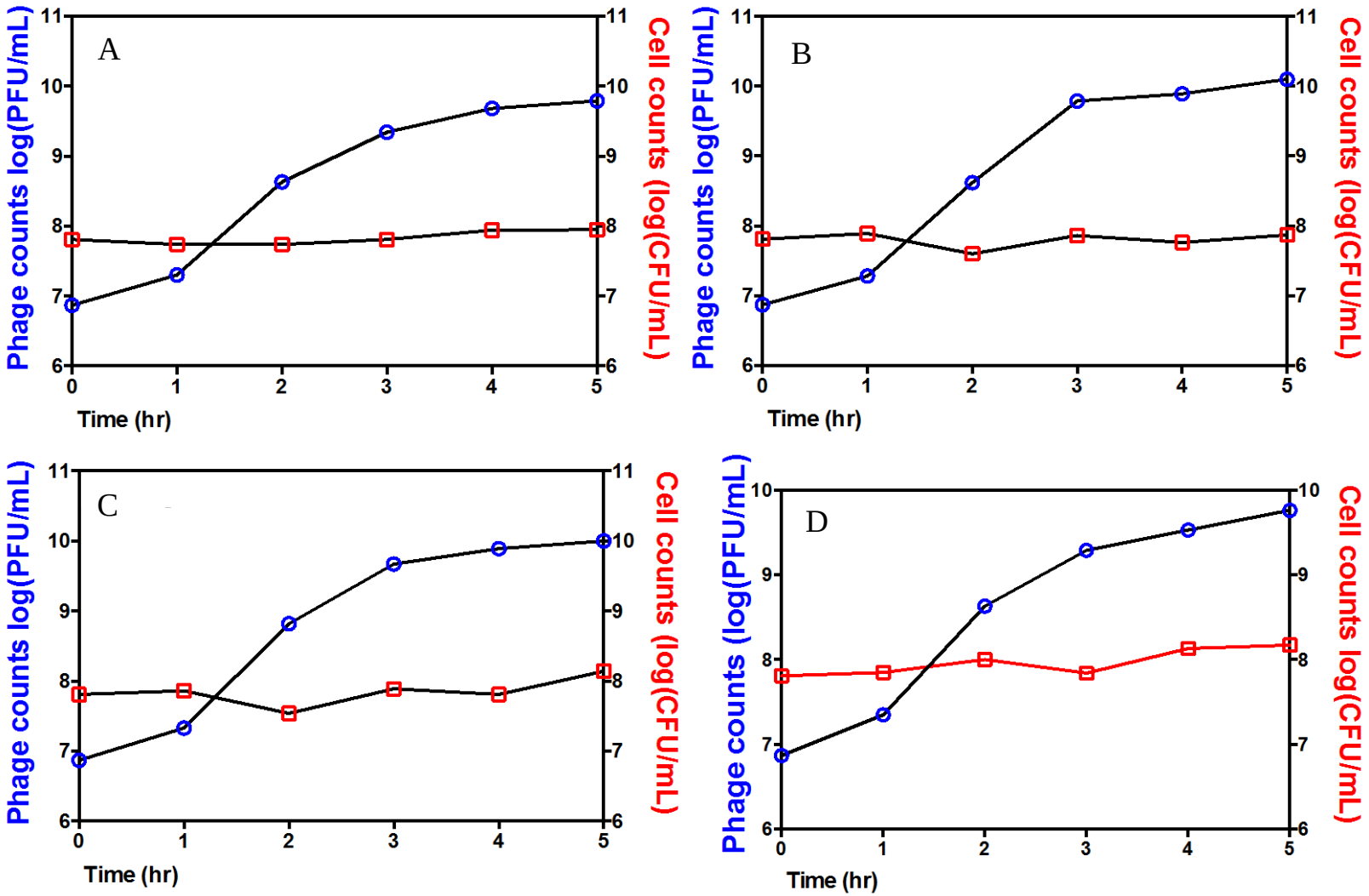

Figure 11: Bacteriophage growth and *S. enterica* levels during a 5 hour incubation at 37 °C, 200 RPM in A. 100 NTU spinach, B. 100 NTU romaine lettuce, C. 100 NTU iceberg lettuce, D. Water solutions. (Bacteriophage, *S. enterica* levels)

REFERENCES

1. Anonymous. 2012. Pathogens causing US foodborne illnesses hospitalizations, and deaths, 2000–2008. .

2. Anonymous. 2015. FSMA Facts. Available at: http://www.fda.gov/downloads/Food/GuidanceRegulation/UCM263773.pdf. 2015. .

3. Anonymous. 2011. FDA Food Safety Modernization Act. 111-353.

4. Anonymous. 2003. Agricultural Fact Book 2001-2002. .

5. Abuladze, T., M. Li, M. Y. Menetrez, T. Dean, A. Senecal, and A. Sulakvelidze. 2008. Bacteriophage reduce experimental contamination of hard surfaces, tomato, spinach, broccoli, and ground beef by Escherichia coli O157:H7. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY* 74:6230-6238.

6. Abuladze, T., M. Li, M. Y. Menetrez, T. Dean, A. Senecal, and A. Sulakvelidze. 2008. Bacteriophages Reduce Experimental Contamination of Hard Surfaces, Tomato, Spinach, Broccoli, and Ground Beef by Escherichia coli O157:H7
 . *APPLIED AND ENVIRONMENTAL MICROBIOLOGY* 74:6230-6238.

7. Allende, A., M. V. Selma, F. López-Gálvez, R. Villaescusa, and M. I. Gil. 2008. Impact of Wash Water Quality on Sensory and Microbial Quality Including Escherichia coli Cross-Contamination of Fresh-Cut Escarole. *Journal of Food Protection* 71:2514-2518.

8. Brussow, H., R. W. Hendrix. 2002. Phage Genomics: Small is Beautiful. *Cell* 108:13-16.

9. Carter, C., Parks, A., Abuladze, A., Li, M., Woolston, J., Magnone, J., Senecal, A., Kropinski, AM., Sulakvelidze, A. 2012. **Bacteriophage cocktail significantly reduces Escherichia coli O157** H7 contamination of lettuce and beef, but does not protect against recontamination. *Bacteriophage* 2:178.

10. Cerca, N., R. Oliveira, and J. Azaredo. 2007. Susceptibility of *Staphylococcus epidermis* planktonic cells and biofilms to the lytic action of staphylococcus bacteriophage K. *Letters in Applied Microbiology* 45:313-317.

11. Chanishvili, N., T. Chanishvili, M. Tediashvili, and P. A. Barrow. 2001. **Phages and their application against drug-resistant bacteria**. *Journal of Chemical Technology & Biotechnology* 76:689-699.

12. Cohen, N., C. Hollinsworth, R. Brennan Olson, M. Laus, and W. Coli. 2005. Farm Food Safety Practices: A Survey of New England Growers. *Food Protection Trends* 25:363-370.

13. Critzer, F. J., M. Doyle. 2010. Microbial ecology of foodborne pathogens associated with produce. *Current Opinion in Biotechnology* 21:125-130.

14. DELBROCK, M. 1940. ADSORPTION OF BACTERIOPHAGE UNDER VARIOUS PHYSIOLOGICAL CONDITIONS OF THE HOST. *Journal of General Physiology* 20:631-642.

15. Demerec, M., U. Fano. 1945. Bacteriophage-resistant mutants in Escherichia coli. *Genetics* 30:119-136.

16. D'Herelle, F. 1926. The Bacteriophage and its Behavior. The Williams & Wilkins Company, Baltimore, MD.

17. Gil, M. I., M. V. Selma, F. Lopez-Galvez, and A. Allende. 2009. Fresh-cut product sanitation and wash water disinfections: Problems and solutions. *International Journal of Food Microbiology* 134:37-45.

18. Goode, D., V. M. Allen, and P. A. Barrow. 2003. Reduction of Experimental Salmonella and Campylobacter Contamination of Chicken Skin by Application of Lytic Bacteriophages. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY* 69:5032-5036.

19. Gould, L. H., K. A. Walsh, A. R. Vieira, K. Herman, I. T. Williams, A. J. Hall, and D. Cole. 2013. **Surveillance for Foodborne Disease Outbreaks — United States, 1998–2008**. .

20. Guenther, S., D. Huwyler, S. Richard, and M. J. Loessner. 2009. Virulent Bacteriophage for Efficient Biocontrol of Listeria monocytogenes in Ready-To-Eat Foods. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY* 75:93-100.

21. Hadas, H., M. Einav, I. Fishov, and A. Zaritsky. 1997. Bacteriophage T4 development depends on the physiology of its host Escherichia coli. *Microbiology* 143:179-185.

22. Hibma, A., S. A. A. Jassim, and M. W. Griffiths. 1997. Infection and removal of L-forms of Listeria monocytogenes with bred bacteriophage. *International Food Journal of Microbiology* 34:197-207.

23. Jydegaard, A. M., A. Gravesen, and S. Knochel. 2000. Growth condition-related response of Listeria monocytogenes 412 to bacteriocin inactivation. *Letters in Applied Microbiology* 31:68-72.

24. Kochetkova, V., A. Mamontov, R. Moskovtseva, E. Erastova, E. Trofimov, M. Popov, and S. Dzhubalieva. 1989. **Phagotherapy of postoperative suppurative-inflammatory complications in patients with neoplasms**. *Sov Med* 23-26.

25. Kutter, E. 2009. Phage Host and Efficiency of Plating. *Bacteriophages* 501:141-149.

26. Kutter, E., A. Sulakvelidze (eds). 2005. Bacteriophages: Biology and Applications. CRC Press, Florida.

27. López-Gálvez, F., A. Allende, M. V. Selma, and M. Gil. 2009. **Prevention of *Escherichia coli* cross-contamination by different commercial sanitizers during washing of fresh-cut lettuce**. *International Journal of Food Microbiology* 133:167-171.

28. Meladze, G. D., M. G. Mebuke, N. S. Chkhetia, N. I. Kiknadze, G. G. Koguashvili, I. I. Timoshuk, N. G. Larionova, and G. K. Vasadze. 1982. The efficacy of staphylococcal bacteriophage in treatment of purulent diseases of lungs and pleura. *Grudn. Khir* 53-56.

29. Olaimat, A. N., R. A. Holley. 2012. Factors influencing the microbial safety of fresh produce: A review. *Food Microbiology* 32:1-19.

30. Painter, J. A., R. M. Hoekstra, T. Ayers, R. V. Tauxe, C. R. Braden, F. J. Angulo, and P. M. Griffin. 2013. Attribution of Foodborne Illnesses, Hospitalizations, and Deaths to Food Commodities by using Outbreak Data, United States, 1998–2008. *Emerging Infectious Diseases* 19:407-415.

31. Rakhuba, D., E. Kolomiets, S. Szwajcer Dey, and G. Novik. 2010. Bacteriophage receptors, mechanisms of phage adsorption and penetration into host cell. *Polish Journal of Microbiology* 145-155.

32. Rashid, M. H., T. Revazishvili, T. Dean, A. Butani, K. Verratti, K. A. Bishop-Lilly, S. Sozhamannan, A. Sulakvelidze, and C. Rajanna. 2012. A Yersinia pestis-specific, lytic phage preparation significantly reduces viable Y. pestis on various hard surfaces experimentally contaminated with the bacterium. *Bacteriophage* 168-177.

33. Sakandelidze, V. M. 1991. The combined use of specific phages and antibiotics in different infectious allergoses. *Vrachebnoe delo* 60-63.

34. Sharma, M., J. R. Patel, W. S. Conway, S. Ferguson, and A. Sulakvelidze. 2009. Effectiveness of Bacteriophages in Reducing Escherichia coli O157:H7 on Fresh-Cut Cantaloupes and Lettuce. *Journal of Food Protection* 72:1481-1485.

35. Steele, E. 2006. Submission of GRAS Notification of Bacteriophage P 100. .

36. Sulakvelidze, A. 2012. Intralytix GRAS notification for SalmoFresh. .

37. Suslow, T. **Chlorination in the production and postharvest handling of fresh fruits and vegetables**. .

38. Teplitski, M., K. Warriner, J. Bartz, and K. R. Schneider. 2011. Untangling metabolic and communication networks: interactions of enterics with phytobacteria and their implications in produce safety. *Trends in Microbiology* 19:121-127.

39. Woolston, J., A. Parks, T. Abuladze, B. Anderson, M. Li, C. Carter, L. F. Hanna, S. Heyse, D. Charbonneau, and A. Sulakvelidze. 2013. Bacteriophages lytic for Salmonella rapidly reduce Salmonella contamination on glass and stainless steel surfaces. *Bacteriophage* 3:.