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CHARACTERIZATION AND USE OF PATHOGEN SPECIFIC BACTERIOPHAGES TO REDUCE THE VIABILITY OF Escherichia coli O157:H7 CONTAMINATION ON FRESH PRODUCE

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CHARACTERIZATION AND USE OF PATHOGEN SPECIFIC BACTERIOPHAGES TO REDUCE THE VIABILITY OF *Escherichia coli* O157:H7 CONTAMINATION ON FRESH PRODUCE

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Agriculture, Food and Environment
at the University of Kentucky

By

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2020

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ABSTRACT OF DISSERTATION

CHARACTERIZATION AND USE OF PATHOGEN SPECIFIC BACTERIOPHAGES TO REDUCE THE VIABILITY OF *Escherichia coli* O157:H7 CONTAMINATION ON FRESH PRODUCE

Fresh produce is one of the most common sources of food-borne outbreaks, involving various pathogenic microorganisms such as *Escherichia coli*. Recent outbreaks have clearly shown that post-harvest washing has limited effectiveness on decontaminating produce and may contribute to cross-contamination of produce due to various limitations. Excessive use of sanitizers and antibiotics has also led to the development of many antibiotic-resistant strains of bacteria that have made the food industry more vulnerable.

Bacteriophages are a bacterial viruses that can selectively infect and replicate within bacteria leading to cell lyse and death. Bacteriophages have become widely recognized due to their ability to selectively eliminate bacteria. Furthermore, their effectiveness in infecting and successfully eradicating various multi-drug resistant strains of bacteria has shown promise in a time of antibiotic resistance. It is for these reasons that bacteriophages are being proposed as an alternative to antibiotics for treating infections in humans, animal production, and as a biocontrol in food for bio-preservation and safety.

Four bacteriophages (C14s, V9, L1, and LL15) of bovine origin were used against *E. coli* O157:H7 to study their efficacy against the pathogen under a controlled and complex environment. A microplate study was used to demonstrate this effectiveness under numerous conditions. A significant reduction ($P<0.01$) in the pathogen was observed. The subsequent study challenged the phage cocktail with 100-ppm bleach and 100-ppm SaniDate 5.0 respectively for three hours to study the ability of phages to tolerate the commercially used sanitizers. The bacteriophages survived the sanitizer concentration and significantly reduced ($P<0.05$) the population of the pathogen. A temperature study was conducted to analyze the ability of bacteriophage to withstand varying temperatures as a component of produce washes with mild heat treatments. Bacteriophages were subjected to 35, 45, and 55°C and were spot tested for effectiveness. The results indicated their ability to tolerate an increase in temperature and effectively produce plaques compared to the control.

The success in demonstrating the phage's ability to reduce pathogens in a controlled environment led to the development of challenging them in a more complex environment, namely a produce wash. Fresh spinach leaves were washed with *E. coli* O157:H7 and bacteriophage cocktail in organic-rich and sterile water. The results indicated that there was a significant reduction ($P<0.01$) in the pathogen under both conditions. The successive study tested the same conditions in the presence of both sanitizers (100-ppm) and bacteriophage cocktail in sterile and high organic load produce wash. The sanitizer made in sterile wash water significantly ($P<0.01$) reduced the pathogen in the presence or absence of a bacteriophage cocktail. However, in the presence of an organic load, the data demonstrated that compared to the control, the phage cocktail significantly reduced ($P<0.01$) the contamination of the pathogen on the

spinach leaves. These results demonstrate the ability of bacteriophages to be used in a produce wash system during post-harvest sanitation to act as a biocontrol in reducing pathogen contamination on fresh produce.

KEYWORDS: *E. coli* O157:H7, Produce-wash, Bleach, SaniDate 5.0, Sanitation, and Dunk wash

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CHAPTER 1. INTRODUCTION

Fresh fruits and vegetables are considered a good source of vitamins, minerals, and other nutrients and are highly recommended by nutritionists and health professionals around the globe (Fan et al., 2009). However, fresh produce remains one of the leading causes of foodborne outbreaks in comparison to other food products such as meat, seafood, and dairy that are considered carriers of pathogens (Center for Disease Control and Prevention, 2020a). More than 400 cases of produce-related foodborne outbreaks have been recorded since 1990 (Murray et al., 2017). Fresh produce such as tomatoes, leafy greens, cantaloupe, and other soft fruits and vegetables are among the top produce that is frequently associated with outbreaks along with sprouted seeds such as clover, mung beans, and alfalfa (Murray et al., 2017). Since fresh produce are usually grown in open fields, the risk associated with exposing the harvestable portion of the crop to enteric pathogens from workers, soil, irrigation water, post-harvest water, wildlife, manure, and other sources are generally elevated (Fan et al., 2009). Additionally, fresh produce are usually consumed raw which in turn increases the risk associated with the consumption of fresh fruits and vegetables (Fan et al., 2009). Table 1.1 summarized from CDC (2020) lists the various outbreaks that were associated with fresh produce in the United States from 2011 to 2019 (Center for Disease Control and Prevention, 2020a).

Table 1.1 Overview of foodborne illness outbreaks associated with fresh produce in the United States from 2011 – 2019 (Center for Disease Control and Prevention, 2020a)

Year	Product	Pathogen	No. of cases
2011	Papaya	*Salmonella enterica* Agona	106
2011	Cantaloupe	*S. enterica* Panama	20
2011	Romaine Lettuce	*Escherichia coli* O157:H7	58
2011	Cantaloupe	*Listeria monocytogenes*	147
2012	Mango	*S. enterica* Braenderup	127
2012	Cantaloupe	*S. enterica* Typhimurium and Newport	261
2012	Romaine lettuce	*E. coli* O157:H7	24
2012	Organic spinach/spring mix blend	*E. coli* O157:H7	33
2013	Cucumbers	*S. enterica* Saint paul	84
2013	Ready to eat salad	*E. coli* O157:H7	33
2014	Cucumbers	*Salmonella* Newport	275
2014	Caramel Apples	*L. monocytogenes*	35
2014	Bean Sprouts	*Salmonella* Enteritidis	115
2014	Bean Sprouts	*L. monocytogenes*	-
2014	Raw Clover sprout	*E. coli* O121	19
2015	Cucumbers	*Salmonella* Poona	907
2016	Alfalfa sprouts	*Salmonella* Abony	36
2016	Frozen vegetables	*L. monocytogenes*	9

Table 1.1 (Continued).

Year	Product	Pathogen	No. of cases
2016	Alfalfa sprouts	*E. coli* O157	11
2016	Alfalfa sprouts	*Salmonella* Muenchen and *Salmonella* Kentucky	26
2016	Packaged salad	*L. monocytogenes*	19
2017	Leafy greens	*E. coli* O157:H7	5
2017	Papaya	*Salmonella* infection	2202
2018	Romaine Lettuce	*E. coli* O157:H7	62
2018	Pre-cut melons	*Salmonella* Adelaide	77
2018	Romaine Lettuce	*E. coli* O157:H7	2101
2018	Raw Sprout	*Salmonella* Montevideo	10
2019	Cut-Fruit	*Salmonella* Javiana	96
2019	Romaine Lettuce	*E. coli* O157:H7	167
2019	Papaya	*Salmonella* Uganda	81
2019	Pre-cut Melon	*Salmonella* Carrau	137

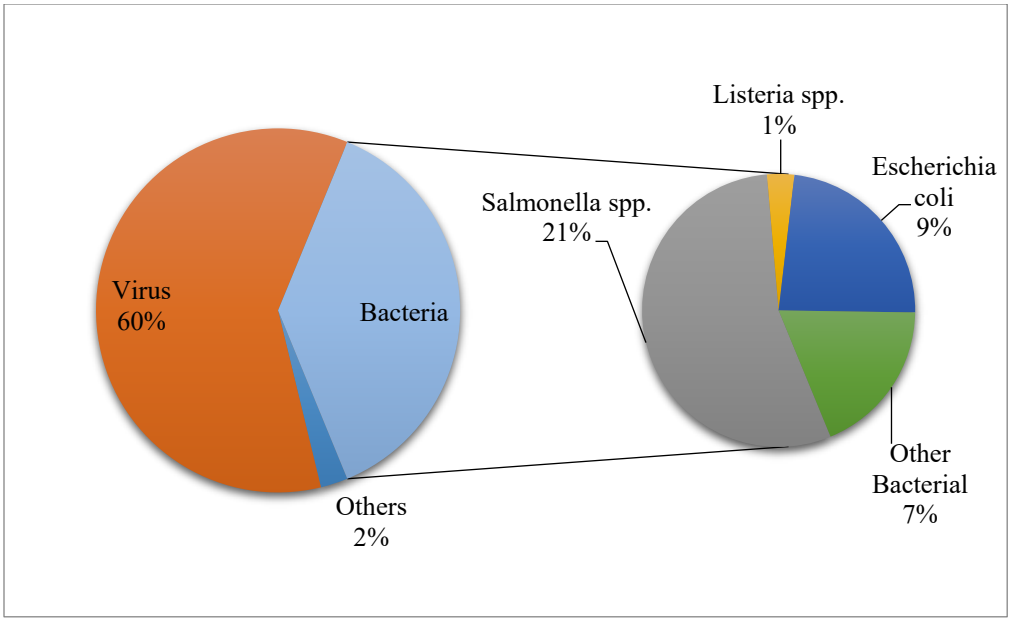

Figure 1.1 Outbreak percentage on fresh produce from 2000-2015 (Center for Disease Control and Prevention, 2018)

Figure 1.1 summarizes the data obtained from the Center for Disease Control and Prevention (2018) which shows the percentage of pathogen contamination associated with fresh produce from 2000 - 2015 (Center for Disease Control and Prevention, 2018). These outbreaks have signified that commercial techniques that are employed for disinfecting produce are not to be relayed on and other novel interventions and strategies are highly necessary for further minimizing the risk of pathogen contamination on fresh produce.

CHAPTER 2. LITERATURE REVIEW

The Need for Prevention-based Food Safety Programs for Fresh Produce

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2.1 Overview

The Food Safety Modernization Act (FSMA) - Produce Safety Rule (PSR), the first set of mandatory federal standards in the United States for growing, harvesting, packaging, and handling fruits and vegetables (Bihn E., 2017), was first published in the Federal Register on November 27, 2015. The primary objective of the rule was to strengthen the current produce food safety system through a prevention-based approach by implementing minimum science-based best practices (U.S. Food and Drug Administration, 2015). Fruit and vegetable growers in the various categories of the PSR must abide by the rules and regulations of FSMA-PSR to fulfill federal regulations. Based on the data obtained from the Center for Disease Control and Prevention (CDC), between 2000 and 2016, 17,338 illness outbreaks were reported, of which 558 were related to produce. These outbreaks led to 15,482 recorded illness, 816 hospitalization, and 20 deaths (Center for Disease Control and Prevention, 2018).

Data obtained from the CDC (Table 2.1) clearly shows how outbreaks have been decreasing over the past few years, perhaps because of increased food safety awareness, buyer requirements (third-party audits), and Good Agricultural Practices (GAP) employed by growers. Although the U.S. food safety regulations have made great strides with respect to produce safety, various developments, such as challenges in the U.S. regulatory bodies, foodborne outbreaks due to new forms of contamination, and increasing costs associated with foodborne illnesses, have led to changes in food safety laws and regulations (Belden and Orden, 2011).

Table 2.1 Produce-related outbreaks in the United States (2000–2016) (Center for Disease Control and Prevention, 2018)

Year	2000-2005	2006-2010	2011-2016
Outbreaks	220	179	159
Illness	6,305	5,470	3,707
Hospitalization	169	374	273
Deaths	3	7	10

2.2 Summary

It is important to understand that, FSMA – PSR, in general, includes minimum science-based standards for growing, harvesting, packing, and holding fruits and vegetables intended for human consumption. In addition, it is essential to understand where fruits and vegetables come from, including routes of contamination and the microbiology not only of fruits and vegetables but also the environment in which they are grown and the various resources used to produce them. Although many different routes of pathogen entry into fruits and vegetables are possible, soil and water have been the top two routes of contamination. Numerous studies have been conducted to understand the way in which contamination occurs when produce is exposed to contaminated water, soil, or manure during production, harvesting, packing, and storage (Brandl and Mandrell, 2002; Harris et al., 2003; Islam et al., 2004; Penteado et al., 2004; Johannessen et al., 2005; Barker-Reid et al., 2009; Mootian et al., 2009; Oliveira et al., 2011).

Foodborne outbreaks in fresh produce have been identified in many parts of the world (Lynch et al., 2009). In 2015, the CDC estimated that approximately 48 million new

cases of foodborne illness are reported every year, resulting in 128,000 hospitalizations and 3,000 deaths (Center for Disease Control and Prevention, 2018). It was also estimated that the average national cost of foodborne illness was around $55.5 billion (Scharff, 2015). The proportion of outbreaks linked to fresh produce in the U.S. has been increasing significantly, from < 1 % to almost 6 % from 1970 to the 1990s, with 54% of the outbreaks linked to known pathogens (Sivapalasingam et al., 2004). Consumption of fruits and vegetables has significantly increased in the United States, because of its association with a healthy lifestyle (Callejón et al., 2015). Significant amounts of produce are consumed raw, and outbreaks associated with these products are growing correspondingly (Buck et al., 2003). The complex cycle of bacterial contamination and persistence on plants by adhesion of pathogens to the surfaces restricts the usefulness of conventional processing and chemical sanitizing methods to prevent the transmission of organisms in produce (Lynch et al., 2009). Outbreak investigations conducted over the years have led researchers to analyze different opportunities for contamination at the farm level in the farm-to-fork network (Lynch et al., 2009). Future achievements in preventing produce-related outbreaks depend on understanding the various factors influencing potential contamination, as well as maintenance of best practices to reduce and eliminate contamination (Kozak et al., 2013). Therefore, creating awareness and understanding of pathogen-produce interactions are vital for controlling the growth of unwanted microorganisms on fresh produce and delivering safe food to the community.

2.3 Pathogens contaminating fresh produce

Various pathogenic microorganisms are associated with the contamination of fresh produce (Table 2.2). These include *Campylobacter* spp., *Clostridium botulinum*,

Clostridium perfringens, enterotoxigenic *Bacillus cereus*, *Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli* (STEC), *Listeria monocytogenes*, *Salmonella* spp., *Shigella* spp., enterotoxigenic *Staphylococcus aureus*, *Vibrio cholerae*, *Yersinia enterocolitica*, certain viruses, and protozoa (Steele and Odumeru, 2004). The likelihood of fruits and vegetables from a field or orchard becoming contaminated with pathogenic microorganisms during harvesting, post-harvesting, processing, or distribution was analyzed by Beuchat in 1996 (Beuchat, 1996). Beuchat discussed the ability of pathogenic microorganisms to cause human diseases and to survive and be present in the water which is used for irrigation or in the soil used for growing produce.

Table 2.2 Sources of pathogenic microorganisms on fresh produce (Beuchat, 1996)

Harvest	Source
Pre-harvest	• Feces • Soil • Irrigation water • Green or inadequately composted manure • Air (dust) • Wild and domestic animals, and • Human handling
Post-harvest	• Feces • Human handling (workers, consumers) • Harvesting equipment • Transport containers (field to packing shed)

Table 2.2 (Continued).

Harvest	Source
	• Wild and domestic animals • Air (dust) • Wash and rinse water • Processing equipment (sorting, packing, and cutting) • Ice • Transport vehicles • Improper storage (temperature, physical environment) • Improper packaging • Cross-contamination (other foods in storage, preparation, and display areas) • Improper display temperature • Improper handling after wholesale or retail purchase

Numerous outbreaks linked to contaminated fruits and vegetables have been recorded in recent years (Hussain and Gooneratne, 2017). These outbreaks have called attention to the effect of consumption of contaminated produce on human health, particularly when produce is consumed raw (Steele and Odumeru, 2004). *L. monocytogenes* outbreaks and prevalence in fresh produce was reviewed in 2017 by Zhu et al. (Zhu et al., 2017) who focused on fresh produce-related listeriosis outbreaks, the organism's corresponding prevalence in the environment, contamination levels of fresh produce, and challenges associated with fresh produce safety. The author concluded that

L. monocytogenes is typically present in most fresh produce and ascribed this finding to the crop growing environment, post-harvest processing methods, and the retail setting. Measures to enhance produce safety in order to reduce the presence of these pathogenic microorganisms on fresh produce, including prevention of biofilm formation through effective sanitation methods (Zhu et al., 2017), were highly recommended.

Another major pathogen contaminating fresh produce is Shiga-toxin producing *Escherichia coli* (*E. coli*), specifically serotype O157:H7, which has been identified as a causative agent in many foodborne outbreaks of gastroenteritis. Even though infections with STEC have been associated largely with consuming undercooked beef, several outbreaks linked to this pathogen have been traced back to consumption of contaminated produce, such as radishes, sprouts, and pre-packaged spinach (Berger et al., 2010). It has been demonstrated that these pathogens have the ability to adhere to the leaves of fresh produce, such as salad leaves, through alternative mechanisms involving the filamentous type III secretion system (Shaw et al., 2008) or through flagella-mediated attachment (Shaw et al., 2011).

Fruits and vegetables have a high potential to act as vehicles for disease transmission. Fresh produce can be contaminated with pathogens by coming in contact with improperly treated manure, contaminated water or soil, poorly implemented washing/sanitizing operations, or food handlers who are infected and who handle produce improperly (Steele and Odumeru, 2004). Table 2.3, from Harris et al. (2003), details some characteristics of pathogens and their associated contamination sources (Harris et al., 2003). It is obviously important to review good agricultural and food safety practices

periodically to keep up with newly identified microbial problems in order to improve food safety standards.

Table 2.3 Characteristics of some microbial pathogens that have been linked to outbreaks of produce-associated illnesses (Harris et al., 2003)

Microorganism	Incubation period	Infectious Dose	Source
Clostridium botulinum	12 to 36 hours	Toxin production in food	River, lakes, decaying vegetation,
Escherichia coli O157:H7	2 to 5 days	10 to 1000	Animal feces, especially cattle, deer, and human: cross-contamination from raw meat, produce
Salmonella spp.	18 to 72 hours	10 to 100,000	Raw meat, poultry, or eggs
Shigella spp.	1 to 3 days	About 10	Human Feces
Listeria monocytogenes	1 day to 5 or more weeks	Unknown, dependent upon the health of an individual	Food processing environments
Hepatitis A	25 to 30 days	10 to 50	human feces and urine

2.4 Agricultural water

According to the FDA, any water used in covered activities, i.e., where water is intended for use on fresh produce or on surface in contact with it, is called agricultural water. Agricultural water can be classified into pre- or post-harvest water, depending on its application and intended use during production, harvesting, and packaging (Bihn E., 2017).

2.4.1 Pre-harvest water

In recent years, many pathogens have been isolated with increasing frequency from fresh produce. Wastewater is increasingly employed as a source of irrigation to supplement scarce water supplies and to provide nutrients to crops. Improperly treated irrigation water can contain high levels of foodborne pathogens, which could adversely impact the quality and safety of fruits and vegetables produced using that water. Poor water quality has long been associated with fruit and vegetable contamination by various pathogenic microorganisms (Solomon et al., 2003). Irrigation water as a potential pre-harvest source of bacterial contamination on vegetables was studied by Ikabadeniyi et al. in 2002 (Ijabadeniyi et al., 2011), who studied the effect of the water source used for irrigation on the bacterial load in the water and the subsequent levels of bacterial contamination found on fresh produce during a 12-month sampling period. They used logistic regression analysis to predict the potential bacterial load of *Salmonella spp*., *L. monocytogenes*, and intestinal *Enterococcus* in irrigation water and vegetables. Analysis of variance ($P \leq 0.05$) was employed to determine whether there were significant differences between the levels of turbidity, oxygen demand, aerobic plate count, aerobic spore former counts, and anaerobic spore-former counts in 36 water samples. Results indicated that logistic

regression of the aerobic colony counts and *S. aureus* counts were statistically dependable in predicting the presence of *L. monocytogenes* on vegetables. Similarly, a significant difference was observed between the aerobic plate counts and the anaerobic spore-former counts (Ijabadeniyi et al., 2011). These findings were used to predict the potential presence of intestinal *Enterococcus* and *Salmonella,* respectively. The data indicated that the water used for irrigation was a likely source of contamination in fresh produce. Treatment of pre-harvest irrigation water was highly recommended, along with good agricultural practices, especially in producing ready-to-eat vegetables (Ijabadeniyi et al., 2011).

In 2009, Braker-Reid et al. (Barker-Reid et al., 2009) studied the persistence of *E. coli* on injured iceberg lettuce in a field irrigated with contaminated water. The research team conducted assays to evaluate the persistence of *E. coli* on injured lettuce plants irrigated with water applied via overhead irrigation and inoculated with nonpathogenic *E. coli*. Specifically, physically damaged plants were treated on day 0 by applying 1 liter of inoculum (7 $\log_{10}$ CFU/ml) to each plant head, using a watering can. *E. coli* was subsequently detected on all lettuce head samples, and data analysis demonstrated that injury to the leaf prior to *E. coli* inoculation and harvest ($P = 0.00067$) significantly increased the persistence of the pathogen on lettuce samples, thus significant persistence of *E. coli* was seen on plants that had very recent injuries, and it was concluded that growers should avoid using contaminated water for irrigating lettuce crops for a minimum of 2 days before harvesting (Barker-Reid et al., 2009), a recommendation that should minimize food safety risk, since damage from farm management practices or environmental effects may cause pathogen retention on fresh produce. Growers were also advised to consider

chlorination or ozonation of water prior to its use, in order to provide safe irrigation water for crops (Barker-Reid et al., 2009).

Mootian et al. (2009) analyzed (Mootian et al., 2009) the transfer of *E. coli* O157:H7 from the soil, water, and manure to lettuce plants. The main aim of the study was to determine whether exposure to low levels of the pathogen in the rhizosphere (near root portion) and phyllosphere (above ground portion) of lettuce plants would result in detectable levels of pathogen in the phyllosphere. Plants were exposed to different concentrations of the pathogen through contaminated soil and manure or through surface irrigation with contaminated water. It was observed that 21% of the plants tested positive for *E. coli* O157:H7. Surface sterilization did not result in complete elimination of the pathogen, as the bacteria were protected in crevices of lettuce tissue. Contamination of produce often increases close to harvest and can increase the risk of pathogens being present in the produce at the time of harvest (Mootian et al., 2009). It was concluded that future efforts are necessary to avoid human pathogen contamination of produce, rather than focusing solely on disinfecting technologies (Mootian et al., 2009).

Recovery of *Salmonella enterica* subsp. *Newport,* introduced through irrigation water, from tomato fruits, stems, and leaves, was studied by Hintz et al. in 2010 (Hintz et al., 2010). The objective of the study was to determine whether tomato plants irrigated with the target pathogen had the potential to uptake the organisms. The study involved using irrigation water containing 7 $\log_{10}$ CFU/ml of *S. Newport* on commercially-produced 7-week-old tomato plants. Leaves, roots, stems, and fruits were sampled at different stages during development, homogenized, and then enumerated on XLT-4 agar for *S. Newport*. The results indicated that 35 of the 92 obtained samples (65% roots, 40% stems, 10%

leaves, and 6% fruits) were positive for *S. Newport*. Significant differences were observed for the presence of *S. Newport* according to the tissue type sampled, but no association was observed between the growth stages and contamination levels (Hintz et al., 2010).

These studies clearly point out the risks of using contaminated water to irrigate crops, especially for fresh produce that may be consumed raw. Recently, the diverse opportunities for plants to become exposed to and contaminated with a huge array of human pathogens have been the focus of much discussion and research. It was previously believed that pathogens exposed to crops during cultivation would not persist through the different stages of harvest, post-harvest storage, handling, and transport (Solomon et al., 2003). The ability of *Salmonella* spp. to survive on the edible portion of cilantro leaves was studied by Brandl and Mandrell in 2002 (Brandl and Mandrell, 2002). Researchers demonstrated the ability of *S.* Thompson to survive on the cilantro plants, despite low water availability and dry conditions, for an extended period of time (Brandl and Mandrell, 2002). This study provides evidence that outbreaks of foodborne illness can result from pre-harvest contamination of fresh produce.

In addition to pathogens remaining on the surface of the edible portions of plants, potential internalization and persistent survival inside the plant creates additional produce food safety challenges that are yet to be fully investigated. Hence, efforts to reduce microbial contamination during pre-harvest, along with proper post-harvest inactivation or removal of microorganisms, are likely necessary to reduce the microbial load on fresh produce and thereby minimize the incidence of associated foodborne illness outbreaks.

2.4.2 Post-harvest water

Many outbreaks of human illness related to the consumption of washed produce have been reported in the United States. Changes in agronomy, harvesting, distribution, processing, and consumption patterns have contributed significantly to an increase in foodborne illness (Beuchat and Ryu, 1997). Various pathogens, such as *Listeria spp., Clostridium spp., Bacillus spp., Escherichia spp.*, parasites, and viruses, are likely to contaminate fresh produce, not only through infected manure, irrigation water, or soil, but also through contaminated wash water employed during post-harvest washing (Beuchat and Ryu, 1997). Fresh cut produce processors usually rely on wash water, along with sanitizers, to reduce the risk of microbial contamination of their products. Employing wash water with sanitizers is used specifically to prevent cross-contamination and to improve the hygiene of produce by eliminating soil particles and debris (Gil et al., 2009). Despite the use of sanitizers with wash water for reduction of microorganisms during washing, epiphytic organisms are capable of growing rapidly during storage. The main problems encountered with using wash water are the type and concentration of sanitizers employed. Treatment with chlorinated water, one of the most common post-processing methods for washing fresh produce, reduces the population of pathogenic and other microorganisms but cannot eliminate them completely. It is clear that current concentrations of chlorine employed by the industry to wash produce cannot be relied upon to eliminate all pathogens (Beuchat and Ryu, 1997). The multitude of alternative methods and sanitizers now available for produce washing highlight the problems encountered in using chlorine and suggests that many industries may benefit from supplementing, if not replacing, the traditionally used disinfectant. In addition, many European countries are now using potable

water instead of chemical disinfecting agents for washing fresh-cut vegetables and fruits (Gil et al., 2009).

Evidence of Salmonella internalization into fresh mangos during a simulated post-harvest procedure was analyzed by Penteado et al. in 2004 (Penteado et al., 2004). The research team investigated a nationwide recall on mangos in the United States that was due to possible contamination with Salmonella, even though the crop had been disinfected with chlorine. Salmonella enterica S132, which expresses a green fluorescence protein, was used as the target microorganism for the study. Mangos (immature and ripe) were processed according to the post-harvest handling procedure. Enumeration of the microorganism was carried out on processed mangos by sectioning the fruits into stem-end, middle-side, and bottom-end segments. Samples were homogenized, plated on BHI agar and incubated at 37°C for 18–24 hours. Overnight incubated plates were then examined, using UV light to enumerate colonies. Both the immature and ripened mangos tested positive for Salmonella internalization. The degree of ripeness had no significant effect on the frequency of contamination. Internalization was significantly higher ($P < 0.05$) on the stem-end segment (83%) than on the middle (19%) or the blossom end (9%). Salmonella levels inside the pulp varied greatly between treatments, and the pathogen was detected within the pulp after 1 week of incubation at various temperatures. The study concluded that poor-quality wash water that was not properly chlorinated or was contaminated during processing may have served as the contamination route. Employing high-quality water for post-harvesting processing is a necessity to minimize the likelihood of contamination. Additional studies are required to establish the effectiveness of existing

disinfestation procedures on preventing internalization of pathogens during post-harvesting processes (Penteado et al., 2004).

Pathogens have long been observed to have the ability to be transferred from different sources onto the edible portions of plants at any point from harvest to consumption. Employing high-quality wash water free of organic matter, along with an effective sanitizer, is highly recommended to avoid cross-contamination, especially if the water is recycled. The impact of wash water quality on *E. coli* cross-contamination of fresh-cut escarole was studied by Allende et al. in 2008 (Allende et al., 2008), who employed different types of wash water (such as potable, recirculated, and diluted recirculated water) inoculated with microorganisms to study the ability of bacteria to cross-contaminate produce. A significant amount of transmission of *E. coli* from the inoculated to the un-inoculated samples occurred during washing. It was concluded that the contamination level may impact water quality and the efficacy of added sanitizers for reducing the concentration of waterborne pathogens. It was also shown that cross-contamination of fresh-cut produce can occur if even a small amount of contaminant is present during washing, thus demonstrating the need for using good quality wash water with an effective sanitizer to control or prevent contamination (Allende et al., 2008). In 2004, Rodgers et al. compared chemical sanitizers for inactivating *E. coli* O157:H7 and *L. monocytogenes* on apples, lettuce, strawberries, and cantaloupe (Rodgers et al., 2004). They employed ozone (3 ppm), chlorine dioxide (3 and 5 ppm), chlorinated trisodium phosphate (100 and 200 ppm) and peroxyacetic acid (80 ppm) with regard to their effect on reduction of *E. coli* O157:H7 and *L. monocytogenes* in an aqueous system. Pathogens employed for the study were prepared by using three different strains of each organism, resulting in a cocktail

mixture prepared at a concentration of approximately 6 log CFU/ml. Four sanitizers were prepared at the appropriate concentrations, using distilled water (wash water), which was also employed as a control, at 21° and 23°C. Samples were homogenized and plated on various media to quantify mesophilic bacteria, *E. coli* O157:H7, *L. monocytogenes*, yeasts, and molds. Significant reductions in both pathogens occurred, with ozone being the most effective treatment, followed by chlorine dioxide, chlorinated trisodium phosphate, and peroxyacetic acid (in decreasing order of efficacy). Quantification of organisms yielded relatively similar results for all nine days of sampling, although toward the end of the study, mold and yeast populations were significantly higher for samples treated with chlorine dioxide and ozone. It was concluded that chlorine dioxide, chlorinated trisodium phosphate, and ozone all effectively reduced the counts of *E. coli* O157:H7 and *L. monocytogenes* (Rodgers et al., 2004).

Plain water can be used for reducing the probability of contamination during washing, but it also can transfer pathogenic microorganisms (Gil et al., 2009). Washing fresh produce with an effective sanitizer is therefore important to obtaining products free of organic matter and especially to preventing cross-contamination between clean and contaminated products. The aforementioned experiments clearly demonstrate the importance of employing good-quality post-harvest wash water along with a sanitizer to reduce pathogens and spoilage organisms on fresh produce.

2.5 Soil and Manure

Soil has long been known to provide essential nutrients for the growth and development of plants (Bezdicek et al., 1996). Soil and manure have both played major roles in exposing plants to a diverse array of microflora comprised of both beneficial and

harmful microorganisms. Many foodborne outbreaks have been linked to consumption of fruits and vegetables grown in soil contaminated with manure or polluted irrigation water (Oliveira et al., 2011). Contamination of produce with improperly treated or contaminated soil, manure, or compost on the farm can cause pre-harvest contamination of fresh produce (Islam et al., 2005). Although competition from natural soil flora and unexpected environmental conditions may hinder the growth and development of pathogens (Islam et al., 2005), the potential of pathogens to persist and survive has led researchers to study their ability to adapt to extreme environmental conditions. Islam et al. in 2004 studied the fate of *Salmonella enterica* serovar Typhimurium on field-grown carrots and radishes exposed to different types of compost inoculated with the target organism (Islam et al., 2004). The three types of compost employed (poultry manure, dairy cattle manure, and alkaline-pH stabilized dairy cattle manure), along with irrigation water, were inoculated with 10^7 and 10^5 CFU/ml of *Salmonella*. Crops were grown in the contaminated field, and samples were withdrawn to study the persistence of *Salmonella*, which was shown to survive for an extended time and was detectable in the soil for 203 to 231 days (Islam et al., 2004). Similar results were observed in the case of contaminated irrigation water. The team concluded that employing either contaminated manure or irrigation water could play a major role in contaminating the soil, leading to prolonged persistence of the pathogen, which could eventually contaminate produce, especially root vegetables (Islam et al., 2004).

Transfer of *Listeria innocua* from contaminated compost and irrigation water to lettuce leaves was studied by Oliveira et al. in 2011 (Oliveira et al., 2011). The objective was to determine the transfer of the pathogen from contaminated compost and water to the

edible portion of the plants as well as the survival of the pathogen through two seasons, fall and spring. Viable *L. innocua* were retrievable from the field for up to 9 weeks, at a concentration of 10^5 CFU/gdw in fall and 10^3 gdw (gram by weight) in spring (Oliveira et al., 2011). The team was also able to successfully demonstrate the transfer of the pathogen from contaminated soil and water to the edible portion of the plant, especially the outer leaves. It was concluded that the pathogen survived better in fall than in spring, which indicates that temperature and humidity play major roles in regulating growth of the bacteria. In general, employing contaminated compost and irrigation water will contribute to the presence of foodborne pathogens on vegetables (Oliveira et al., 2011).

Johannessen et al. in 2005 studied the potential uptake of *E. coli* O157:H7 from organic manure into crisp head lettuce (Johannessen et al., 2005). Lettuce seedlings were planted in soil which was fertilized with contaminated bovine manure containing 10^4 CFU/g of *E. coli* O157:H7 and grown in a climate-controlled greenhouse for 50 days, after which samples were withdrawn randomly and tested for the presence of the pathogen. The pathogen was not detected on the edible portion, the outer leaves, or the roots of the lettuce harvest, despite the persistence of the pathogen in the soil for almost 8 weeks. It was concluded that the *E. coli* O157:H7 was not transmitted from contaminated manure to lettuce under the test conditions (Johannessen et al., 2005).

Large quantities of animal manure are applied to agricultural lands in the U.S., with an estimated 1.36 billion tons being applied annually, 90% of which consists of cattle manure (US Senate Agriculture Committee, 1998). Although application of manure or compost improves soil fertility, applying improperly treated or contaminated manure and compost, especially of animal origin, which contains various enteric pathogens, could

allow pathogens to enter the food chain (Islam et al., 2005). Pathogens may be introduced into the soil from contaminated manure, compost, irrigation water, and surface runoff water from production operations such as those used for raising cattle, swine, or poultry. On the basis of results of the aforementioned studies, it can be concluded that application of manure to production fields may result in persistence of microorganisms in the environment for extended periods of time, thereby increasing the risk of contamination of the produce

2.6 Conclusion

Increases in production, distribution, and consumption of fresh produce, along with inconsistent agricultural practices and varying production methods, may explain the high incidence of produce-associated foodborne illness outbreaks. In the past decade, food safety has become a major concern, and the frequency of outbreaks has reduced consumer confidence, which has led the food industry to take steps necessary to produce safe food and thus rebuild consumer acceptance. Various environmental factors during pre- and post-harvest may contribute significantly to contamination of fresh produce by spoilage organisms and potential pathogens. It is clear that microorganisms, including human pathogens, have the ability to survive in water, soil, and manure, and on fresh produce, for prolonged periods of time because of their ability to adapt to extreme conditions.

Illnesses associated with produce are sporadic. Although numerous studies have demonstrated the ability of pathogens to contaminate fresh produce, experimental studies do not mimic real farm environments, and their implications are “one size fits all;” prescriptive and reactive approaches have not, to date, provided adequate solutions. Microbial contamination is difficult to remove and can easily become internalized through

natural features such as stem scars or leaf injury. Employing effective sanitation plays a major role in eliminating pathogens; however, it is evident that the current options employed for sanitizing produce are insufficient to combat the sporadic contaminations that may occur in a produce growing and handling environment. Emphasis must be placed on employing multi-level sanitation processes that use hurdle technology to make produce safer for human consumption. Because of the numerous routes and weak links in production, storage, and distribution of fresh produce, complete elimination of pathogens is difficult, since contamination can occur at any point along the chain. To prevent produce-related contamination, we need to look at the entire food chain from field to consumption with an eye to identifying major control points and establishing essential risk-based prevention steps. Prevention of produce related outbreaks also requires a collaborative effort from industry, government, health agencies, and academia (Howard and Gonzalez, 2001).

The majority of produce-related outbreaks in the past were associated with leafy greens (25%), sprouts (25%), and melons (10%) (Bihn E., 2017), leading many people to think that the focus of food safety programs should be only such high-risk commodities. However, restricting food safety practices to these high-risk commodities does not meet the overall purpose of producing safe food for human consumption, because every crop produced in the field has a chance to become contaminated with human pathogens. Thus, employing proactive and prevention-based food safety programs such as those described in GAP/GHP and the FSMA Produce Safety Rule should be most effective in reducing food safety risks.

CHAPTER 3. BACTERIOPHAGE

3.1 Introduction

Bacteriophages are bacterial viruses that can infect and replicate within the host bacterium, leading to cell lysis and death. In 1896, Ernest Hanbury Hankin discovered a bactericidal action from an unknown entity within the waters of the Ganges and Jumna rivers in India (Abedon et al., 2011). Years later, in 1971, the term “bacteriophage” was coined by microbiologist Felix d’Herelle upon successful isolation of this unknown virus from human stool samples (O'Sullivan et al., 2019). Bacteriophages (phage) are considered one of the most widely distributed entities, with an estimated global population of more than 10^{31} particles (Hendrix, 2003). Phages are considered an obligate intracellular parasite and require a living host for growth and propagation (O'Sullivan et al., 2019). Although phages are ubiquitous, they are usually found in places where their corresponding host bacteria thrive. Some phages are considered a persistent threat to specific food industries, especially the fermentation and dairy industries, as they can infect and inhibit the growth of starter cultures (O'Sullivan et al., 2019). Alternatively, other phages are used to control spoilage and eliminate pathogenic bacteria from contaminating food; thus, reducing food waste and foodborne illnesses (O'Sullivan et al., 2019).

3.2 Morphology

A wide range of morphological characteristics is observed in bacteriophages that are isolated from environmental samples. Typically, bacteriophages have a defined protein coat enclosing their genetic material, which is either RNA or DNA (Clark and March, 2006). Most phages have a head which is polyhedral in structure, except for particular filamentous phage (Ackermann, 1998). The head of the phage is attached to a connector,

with or without fibers, that is referred to as the tail or collar. The tail typically carries specific receptors, used for host identification and attachment (Haq et al., 2012).

Based on the nucleic acid composition, bacteriophages are divided into four families: Caudovirales, Microviridiae, Leviviridae, and Cystoviridae (Dias et al., 2013). Caudovirales carry double-stranded DNA (ds DNA) and are commonly characterized by the presence of a tail (Dias et al., 2013). The characteristics of the tail can further divide these phages into three sub-categories: Siphoviridae (long flexible tail), Myoviridae (contractile tail), and Podoviridae (short tail) (Dias et al., 2013). Caudovirales represent almost 96% of the total phages identified to date (Dias et al., 2013). In contrast, Microviridiae typically contains single-stranded DNA (ss DNA), Leviviridae contains single-stranded RNA (ss RNA), and Cystoviridae contains double-stranded RNA (ds RNA) (Dias et al., 2013).

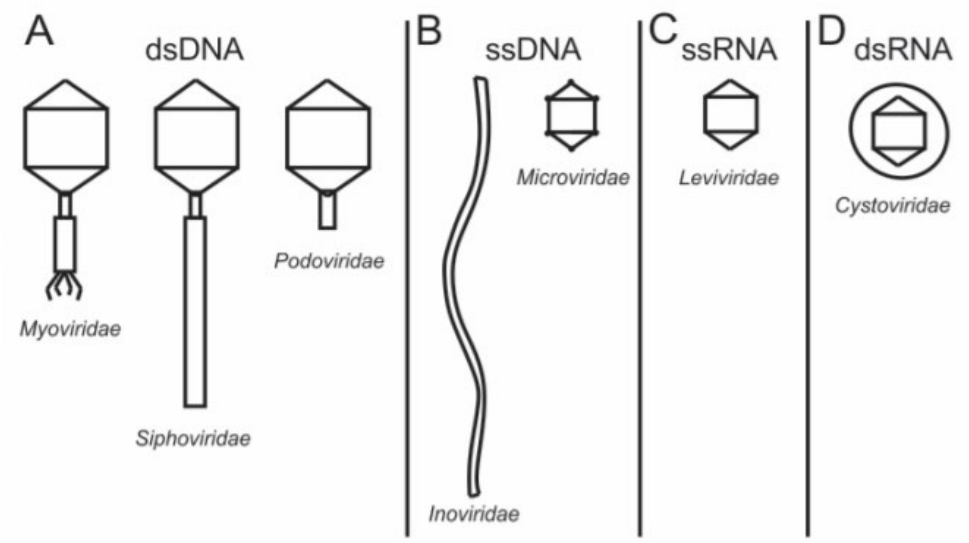


Figure 3.1 A - Caudovirales (dsDNA), B - Microviridiae (ssDNA), C - Leviviridae (ssRNA), and D - Cystoviridae (dsRNA) (Dias et al., 2013).

3.3 Mechanisms of the Infection Cycle

Like all viruses, phages go through several steps during the infection cycle, including absorption, injection, expression, and replication of the viral genome. Following the entry into the cytoplasm of the host cell, bacteriophages can follow the lytic or lysogenic pathways (Dias et al., 2013; O'Sullivan et al., 2019). If the viral genome integrates itself with the host chromosome or remains as a non-expressed plasmid in the host cytoplasm, the pathway is referred to as a lysogenic cycle (Figure 3.2). During this phase, the genetic material is passed on to the progeny of the host cell. On the other hand, if the genome, after integration with the host chromosome, results in active replication of the phage particle, then the pathway is referred to as the lytic cycle (Figure 3.2) (Dias et al., 2013). Apart from the two main pathways, bacteriophages can also perform other infection cycles such as pseudolysogenic or chronic. In the pseudolysogenic cycle, only a certain fraction of the phage multiplies within the host while the rest act as a strict carrier of the plasmid (Dias et al., 2013). In the chronic cycle, the progeny of the phage is constantly released from the host through the process of budding or extrusion (Dias et al., 2013).

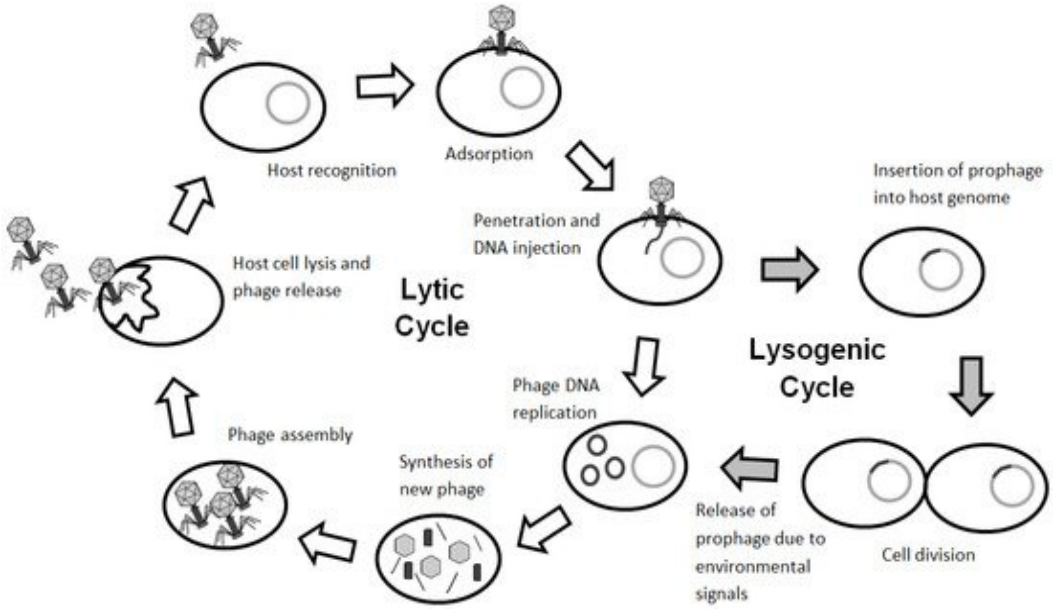


Figure 3.2 The phage life cycle (Doss et al., 2017).

3.4 Phage therapy

Before the discovery of antibiotics, bacteriophage was used to successfully treat infections (Hanlon, 2007). Phage therapy was short-lived due to a lack of understanding about the basic phage biology and the rapid development of new antibiotics (Hanlon, 2007). Decades of using antibiotics and various synthetic antimicrobials have led to the development of multiple-drug resistant bacteria, which results in a serious issue in controlling infections with the use of commercially available antibiotics and other antimicrobials (Dias et al., 2013). Current research and funding agencies are now focusing on finding alternative resources that are cheap, easy, safe, and effective to employ (Dias et al., 2013).

Phage therapy has several advantages over conventional antibiotic therapy (Doss et al., 2017). The isolation of phage is considered comparatively simple, fast, and inexpensive (Parasion et al., 2014). Phages tend to be infective under extreme conditions and have a

tendency to replicate until the host bacterial population has been significantly reduced (Schmelcher and Loessner, 2014). Bacteria also tend to develop resistance to phage 10 times slower than developing resistance to an antibiotic (Parasion et al., 2014). Most phages have shown a high specificity to their host bacteria. This eliminates the possibility of them infecting humans since phages do not display an affinity for eukaryotic cells (Parasion et al., 2014).

Phages have successfully been used in animal models to treat infections (O'Sullivan et al., 2019). Pathogenic *E. coli* strains are considered common causes of colibacillosis in avian species. This infection can lead to a decrease in egg production, carcass rejection at slaughter, and even pre-mature mortality (Guabiraba and Schouler, 2015). Huff et al. (2003) successfully demonstrated the ability of phages to decrease the *E. coli* infection in broiler chickens when administered either through aerosol or intramuscular injection (Huff et al., 2003). The study indicated that the aerosol spray administration and intramuscular injection resulted in a significant reduction in the mortality rate from 50% to 20% and 53% to 17% respectively (Huff et al., 2003). The results demonstrated the ability to utilize bacteriophages as an alternative to traditional antibiotics in order to control bacterial infections in animal production (Huff et al., 2003). Bovine mastitis, caused by *Staphylococcus aureus*, is a leading cause of decreased milk yield and quality in the dairy industry (Breyne et al., 2017). Overuse of antibiotics is considered one of the major problems faced by the industry, due to occasional non-curative results and potential antibiotic residues found in the milk (Breyne et al., 2017). Breyne et al. (2017) demonstrated the use of a *S. aureus* phage cocktail against *S. aureus* in a murine model. The study successfully showed the ability of the phage cocktail (mixture of equal volume

of phage) to reduce the bacterial population from 8.70 log CFU/gland to 4.43 log CFU/gland and revealed an reduced pathological changes in the mastitic mammary gland via histopathological analysis (Breyne et al., 2017).

Phage therapy has been utilized to treat bacterial infections in human models. Sarker et al. (2015) performed oral phage therapy of two coliphages against *E. coli* (ETEC) infections as a randomized trial in children from Bangladesh (Sarker et al., 2016). The primary objective concluded the safety of the phage since no undesirable events were observed in the children treated with the phage. Although an increase in fecal coliphage was observed in comparison to the control children, the phage titer did not show any increase in intestinal phage replication. The authors concluded that even though coliphages showed a relatively safe gut transit, they failed to improve any diarrheal symptoms. It was suggested that a higher phage titer with increased oral dosage and additional in-vivo studies might help in a broader understanding of the phage-bacterial interaction in a complex system (Sarker et al., 2016).

3.5 Phage mediated control of spoilage and foodborne pathogens

A variety of food products are known to pose a risk to human health due to common bacterial contamination, which can result in serious illness and death (World Health Organization, 2015). These foods include meats, seafood, dairy products, poultry meat, and vegetables, which are usually mass-produced through non-diversified farming, bulk co-packing, and multi-product transportation, resulting in an increased risk of contamination (O'Sullivan et al., 2019). Phages have been shown to have a wide application in reducing bacterial contamination on food products leading to improved food safety (O'Sullivan et al., 2019).

3.5.1 Pre-harvest spoilage

Preharvest spoilage of foods, both plant and animal origin, is considered a primary issue in the food industry. Several studies have been conducted concerning the use of phages as a biocontrol for several bacterial plant pathogens (Buttimer et al., 2017). For instance, tomatoes and peppers are susceptible to bacterial infections caused by *Xanthomonas campestris pv. vesicatoria* and *Pseudomonas syringae pv. tomato*, which cause soft rot on fruits and vegetables leading to spoilage and economic loss (Gitaitis et al., 1987; O'Sullivan et al., 2019). The Environmental Protection Agency (EPA) in 2005 approved the use of a phage-based product called AgriPhage^{TM}, which can be used commercially to control the pathogens from infecting young tomato and pepper plants (O'Sullivan et al., 2019).

Berchieri et al. (1991) administered *Salmonella Typhimurium* phage, isolated from sewage, into newly hatched chickens infected with the pathogenic bacteria (Berchieri et al., 1991). The results showed a considerable decrease in the mortality rate among the young chicks, as well as a reduction of the pathogen in the crop, caeca, and small intestine of birds for up to 12 hours (Berchieri et al., 1991). These studies show the capability of phages to be employed as a preventive tool to impede the transfer of disease between animals or plants during the initial processing; thus, acting as an effective biocontrol that can significantly thwart spoilage and economic loss. Phages have also been evaluated for their ability to control infections in foods of animal origin such as lambs, pigs, cattle, and fish (Greer, 2005). Table 3.1, from Greer (2005) summarizes studies conducted on crops and animals using bacteriophage to control preharvest bacterial pathogens.

Table 3.1 - Preharvest bacterial pathogen control using bacteriophages (Greer, 2005)

Food production system	Disease/clinical sign	Bacteriophage host strain
Cultivated mushrooms	Bacterial blotch	*Pseudomonas tolaasii*
Tomatoes	Bacterial spot	*Xanthomonas campestris pv. vesicatoria*
Apples	Fire blight	*Erwinia amylovara*
Stone fruits	Prunus bacterial spot	*X. campestris pv. pruni*
Sprouts	Seed contamination	*Salmonella enteritidis*
Fish	Redfin disease	*Aeromonas hydrophila*
Beef cattle	Bacterial shedding	*E. coli* O157:H7
Calves, piglets, and lambs	Diarrhea, lethal infection	Enteropathogenic *E. coli*
Sheep	Bacteria in rumen, feces, colon	*E. coli* O157:H7
Dairy cattle	Mastitis	*Staphylococcus aureus*
Pigs	Tonsil and cecal *Salmonella*	*Salmonella typhimurium*

3.5.2 Post-harvest spoilage

Bacteriophages have been successfully used for controlling bacterial contamination during post-harvest processing and storage of food products. The fresh-cut produce industry is one of the rapidly growing produce markets, which offers products of convenience (Leverentz et al., 2001). However, the absence or cutting off the peel or rind increases the food safety concerns of fresh-cut fruits and vegetables as this damage can encourage colonization by pathogenic bacteria (Leverentz et al., 2001). Various pathogenic bacteria grow and multiply on the surface of fresh-cut produce such as melons, and lettuce, tomatoes, and apples (Harris et al., 2003). Leverentz et al. (2001) analyzed a biocontrol

method of *Salmonella* on fresh-cut produce using bacteriophages. Lytic *Salmonella*-specific phages were applied to fresh-cut melons in order to demonstrate their ability to reduce the population of the inoculated pathogen. The results indicated that the phage mixture achieved a 3.5 log reduction of the pathogen on the melons (Leverentz et al., 2001). Magnone et al. (2013) studied the capability of a bacteriophage cocktail to inactivate *Escherichia coli* O157:H7, *Salmonella*, and *Shigella* spp. on contaminated fruits and vegetables during a produce wash (Magnone et al., 2013). All the three pathogens were inoculated on broccoli, cantaloupe, and strawberries that were then washed using a bacteriophage cocktail, levulinic acid, or combination of both. The combined produce wash of bacteriophage and levulinic acid achieved more than a 4.0 log reduction of the pathogen even in the presence of a high organic load (Magnone et al., 2013). The findings indicated that a bacteriophage treatment, in combination with a commercial produce wash, could be an effective method in controlling contamination in produce despite the presence of high organic load.

Surprisingly, there is substantially more information published regarding the application of bacteriophage on foods of animal origin. Atterbury et al. (2003) studied the effectiveness of host-specific bacteriophages in reducing *Campylobacter jejuni* contamination on the surface of chicken skin stored at either 4ºC or - 20ºC (Atterbury et al., 2003b). When a high phage titer of 10^7PFU was applied, a significant reduction in the pathogen was observed at each sampling until the end of the study. The difference was clearly evident in the case of chicken skins stored frozen as a log reduction of 2.3 - 2.5CFU was observed in comparison to the control (Atterbury et al., 2003b). The study concluded that the bacteriophages effectively reduced the population of *C. jejuni* on chicken skin even

in the absence of host growth and suggested that further study could help determine more controlling measures for chickens contaminated with this pathogen (Atterbury et al., 2003b). A study conducted by O'Flynn et al. (2004) showcased the most effective use of a bacteriophage cocktail in reducing *E. coli* O157:H7 contamination on beef steaks (O'Flynn et al., 2004). A bacteriophage cocktail containing three different phages was applied to the contaminated beef and reduced the initial pathogen load from 3.0 log CFU to an undetectable level. This study supports the use of bacteriophage as a biocontrol method for reducing *E. coli* O157:H7 contamination on meat and the use of phage therapy as a viable method for controlling pathogens in food (O'Flynn et al., 2004).

The aforementioned studies emphasize the effective use of bacteriophages in reducing the contamination of both spoilage and pathogenic microorganisms of plant and animal origin. Additional studies have also emphasized the ability of phages to control and reduce contamination of different food products listed in Table 3.2 that was summarized from (Greer, 2005)

Table 3.2 Postharvest bacterial pathogen control using bacteriophages (Greer, 2005)

Foods	Bacteriophage host strain
Melon and apple slices	*Listeria monocytogenes* and *Salmonella enteritidis*
Milk	*Staphylococcus aureus* and *Pseudomonas fragi*
Cheese	*Salmonella enteritidis*
Retail chicken	*Salmonella typhimurium* DT104
Chicken frankfurters	*Salmonella typhimurium* DT104
Vacuum-packed beef	*L. monocytogenes*
Pork fat	*Brochothrix thermosphacta* (spoilage control)

3.6 Consideration of bacteriophage as a biocontrol strategy

Bacteriophages have been praised for their extraordinary application in reducing pathogens; however, several issues must be considered before developing a novel application and using them as a biocontrol strategy in food products. Table 3.3 summarized by Greer (2005), lists the various advantages and disadvantages of employing bacteriophages as an effective technique in controlling foodborne pathogens. Employing bacteriophages as a biocontrol strategy must be marketed as a more natural way of food safety and preservation (Greer, 2005). The various studies discussed have demonstrated the use of bacteriophage during pre- and post-harvest phases of food production and have achieved a reduction in both pathogenic and spoilage bacteria.

Table 3.3 Considerations for developing techniques to use bacteriophage as a biocontrol against foodborne pathogens (Greer, 2005)

Advantages	Disadvantages
1. Self-perpetuating	1. Limited host range
2. Selective modification of bacterial flora (specificity)	2. Phage-resistant bacterial mutants
3. Stable in foods and able to survive processing	3. Requires large numbers of target bacteria
4. Natural	4. Barriers in food environments
5. Ubiquitous and readily isolated	5. Transduction of undesirable characteristics
6. Cost-effective	6. Lysogenic conversion (temperate phages)
7. Ease of preparation and application	7. Antigenicity (immune response, allergenicity)
8. Nontoxic to eukaryotic cells	8. Consumer perception of adding viruses to foods
9. No effect on food quality	-

At this time, most experiments that have been carried out using bacteriophages have occurred on a laboratory level and typically focus on using phage for spot treatments on the contaminated surface of the produce. In contrast to the laboratory techniques, the dunk tank method for washing produce is a commonly employed technique in the produce industry. Examining the effectiveness of bacteriophages in a simulated dunk tank for washing contaminated produce could help determine the biocontrol aspect of bacteriophages in controlling foodborne pathogens on fresh produce.

CHAPTER 4. ISOLATION AND ASSESSMENT OF BACTERIOPHAGES OF BOVINE ORIGIN AGAINST *E. COLI* O157:H7

4.1 Introduction

Foodborne illness of microbial origin can range from being mild to life-threatening, depending on the source and type of contamination (Global and Local, 2005). Numerous outbreaks linked to contaminated fruits and vegetables have emerged in recent years (Hussain and Gooneratne, 2017). Outbreaks, particularly associated with raw produce, are a major concern because raw produce harbor foodborne pathogens (Steele and Odumeru, 2004). Several environmental factors contribute to contaminating fresh produce with spoilage and pathogenic microorganisms during pre- and post-harvest processing (Jagannathan and Vijayakumar, 2019). These pathogenic microorganisms include *Campylobacter* spp., *Clostridium botulinum*, *Clostridium perfringens*, enterotoxigenic *Bacillus cereus*, *Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli*, *Listeria monocytogenes*, *Salmonella* spp., *Shigella* spp., enterotoxigenic *Staphylococcus aureus*, *Vibrio cholerae*, *Yersinia enterocolitica*, certain viruses, and protozoa (Steele and Odumeru, 2004). Among those listed above, Enterohemorrhagic *Escherichia coli* (*E. coli*), specifically serotype O157:H7, is a significant pathogen that contaminates fresh produce and is among the leading causes of foodborne outbreaks of gastroenteritis. Although Shiga toxin-producing *E. coli* is primarily associated with the consumption of beef, several outbreaks have been traced back to the consumption of contaminated sprouts and pre-packaged spinach (Berger et al., 2010).

Antibiotics have been used for years against bacterial infections; however, serious medical and social problems have emerged due to the development of antibiotic-resistant strains (World Health Organization, 2014). Prior to the discovery and prevalent use of antibiotics, it was suggested that various bacterial infections could be prevented and/or treated by the administration of bacteriophages (Sulakvelidze et al., 2001). Bacteriophages, informally known as a phage, are bacterial viruses that invade and replicate within bacteria and, in the case of the lytic phage, disrupt bacterial metabolism that causes the bacterium to lyse (Sulakvelidze et al., 2001). Historically, the study of phages suffered from conflicting observations, misinterpretation, and incomplete understanding. Currently, phages are being increasingly used for various purposes, especially in the food industry, due to their antimicrobial potential (Summers, 2012; Zaczek et al., 2015).

In order to meet the growing demand for consumer convenience and variety, fresh produce retail industries have increased their production of pre-packaged salad and fruit (Berger et al., 2010). As a result, there is a parallel increase in foodborne outbreaks linked to the consumption of fresh produce (Berger et al., 2010). Due to the increase in foodborne outbreaks caused by these pathogens, it appears that current technologies employed to prevent the contamination in the food industry are not reliable (García et al., 2008). Additionally, the extensive use of sanitizers has led to the development of resistant bacteria, which has rendered various sanitation procedures less effective (García et al., 2008). Alternatively, some approaches traditionally used in the food industry to reduce contamination by pathogens cannot be directly applied to fresh fruit and vegetables due to their delicate nature and raw consumption. Hence, despite recent

advances to avoid transmission of bacterial pathogens throughout the food chain, novel strategies are still required to fulfill consumer demands for minimally processed foods with fewer chemical preservatives (García et al., 2008).

Optical density measurement, using a microplate reader, is a technique that is widely used to determine the inhibitory effects of antimicrobial agents obtained from plants, spices, and other foods (Vijayakumar and Muriana, 2015). Knezevic and Petrovic (2008) used the microplate technique with crystal violet staining and measurements of optical density to evaluate the ability of *Pseudomonas aeruginosa* phages to inhibit and eradicate biofilm formation (Knezevic and Petrovic, 2008).

The first objective of the current study was to isolate bacteriophages of bovine origin specific to *E. coli* O157:H7 and evaluate their ability, in a cocktail, to infect and kill pathogenic *E. coli* O15:H7; thus, controlling the growth of the pathogen. The second objective was to determine the potential of using bacteriophages in combination with commercial sanitizers such as chlorine and hydrogen peroxide (SaniDate 5.0) at 100-ppm (parts per million) concentration to reduce *E. coli* O157:H7 contamination.

4.2 Materials and Method

4.2.1 Bacteriophage screening, purification, and amplification

Bacteriophages were isolated from the environment by taking a swab of bovine feces collected from the Auburn University College of Veterinary Medicine dairy herd pastures and placing it in brain heart infusion broth (BHI; Bacto Brain Heart Infusion, Becton, Dickinson, and Company, Sparks, MD) containing 20µg/ml novobiocin, and 2.5µg/ml potassium tellurite. After incubation overnight at 37 degrees Celsius (ºC), 1ml of the bacterial suspension in the broth was centrifuged at 12,500 times gravity (x *g*) for 15

minutes, and the resulting supernatant was filter sterilized through a 0.2µm filter (Sterile Syringe Filter with 0.2µm Polyethersulfone Membrane, VWR International). To generate phage plaques, a bacterial lawn of *Escherichia coli* (*E. coli*) O157:H7 (ATCC 43895) was prepared by culturing the strain in a bacteriological incubator with aeration at 37ºC to log phase in Luria-Bertani broth (LB; Difco LB Broth, Miller, Becton, Dickinson, and Company, Sparks, MD) containing 1mM magnesium (LBM). The media was then diluted to an absorbance, optical density, measured at a wavelength of 620 nm (OD_{620}) of 0.8 to 1.0 *E. coli* (ATCC 43895) (0.2ml). The diluted media containing the *E. coli* (ATCC 43895) was then mixed with the phage supernatant, incubated at 37ºC for 20 minutes to allow phage adsorption to the cells, and then mixed with 3.0ml of molten soft agar (LBM with 0.7% Bacto agar). The molten LBM soft agar with *E. coli* (ATCC 43895) and the supernatant were poured onto the LBM underlay, or bottom agar plates (LBM with 1.5% agar-agar), using the double agar overlay technique (Kropinski et al., 2009). The plates were allowed to solidify for one hour prior to overnight incubation at 37ºC. From each plate that showed plaque formation, two plaques were cored using a sterile Pasteur pipette. The cored section was placed in 0.5 ml salts-magnesium (SM) buffer, stored at 5ºC, and allowed to diffuse out of the agar and into the buffer for a minimum of 5 hours (Kropinski et al., 2009). For bacteriophage plaque purification, *E. coli* (ATCC 43895) cells were cultured to log phase, then diluted to an OD_{620} of 0.8 to 1.0. Serial dilutions of each bacteriophage solution were performed, and 0.2ml of the *E. coli* (ATCC 43895) cells were mixed with 10µl of the bacteriophage solution. The cells were incubated with the bacteriophage for twenty minutes before adding 3ml soft agar and pouring the mixture onto an LBM agar plate. The plates were allowed to solidify and were incubated overnight at

37ºC. Isolated bacteriophage plaques were cored, and the cores were placed in 0.5ml SM buffer, stored at 5ºC, and allowed to diffuse for at least 5 hours. The plaque purification procedure was repeated in order to achieve a pure culture of the bacteriophage. To amplify bacteriophage growth to produce high titer stocks, 50ml of log-phase *E. coli* (ATCC 43895) cells growing in LBM broth was inoculated with 0.5ml of the purified phage solution. The lysate was incubated overnight at 37ºC and was then pelleted at 12,500x*g* for 15 minutes. The resulting supernatant was filter sterilized through a 0.2µm filter. To enumerate the phage in each supernatant, a double agar overlay method was used for titration. *E. coli* (ATCC 43895) cells were cultured to log phase, then diluted to an OD_{620} of 0.8 to 1.0. Serial dilutions of each phage solution were performed, and 0.2ml of the *E. coli* (ATCC 43895) cells were mixed with 10µl of the phage solution. The cells were incubated with the phage for ten minutes before adding 3ml LBM soft overlay or top agar and pouring the mixture onto an LBM underlay (bottom agar). Phage plaques were then enumerated to obtain the plaque-forming units per ml (PFU/ml). Bacteriophage isolates were amplified to titers > 10^8PFU/ml (Kropinski et al., 2009). Dimethyl sulfoxide (DMSO) was added to each bacteriophage stock solution until a final concentration of 7% volume to volume was reached. Bacteriophage stocks were then stored at -80ºC (Sambrook and Russell, 2006).

4.2.2 Bacteriophage morphology determination

Bacteriophages were concentrated and purified with Polyethylene Glycol (PEG) (Carlson, 2005). Samples were stained with 2% aqueous (w/v) uranyl acetate adjusted to pH 4.2 and examined with a Philips EM 301 Transmission Electron Microscope operated at 60kV. Bacteriophages were observed at high magnification (x71,000). The images were edited with ImageJ software.

4.2.3 Bacterial culture for microplate study

Pathogenic *Escherichia coli* O157:H7 (ATCC 35150) was obtained from ATCC. Stock cultures were prepared by resuspending cells on to skim - milk media (Difco, Becton-Dickenson Labs) and stored at -25°C. *E. coli* (ATCC 35150) were grown in tryptic soy broth (TSB, Difco, Becton-Dickenson Labs), supplemented with 5 mM of Magnesium sulfate ($MgSO_4$, Fisher Scientific) and Calcium chloride ($CaCl_2$, Fisher Scientific). All (working stock) cultures were held at refrigeration temperature (4°C) for short term storage and -25°C for long term storage.

4.2.4 Bacteriophage titer

Bacteriophage titer was measured before the study for each bacteriophage used in the experiments to measure phage activity. The host strain for all the bacteriophages was *E. coli* (ATCC 35150). Phage titer ranged approximately 10^9PFU/ml for the phage cocktail.

4.2.5 Microplate turbidometric growth inhibition assays and plate count study

E. coli (ATCC 35150) was used as the indicator microorganism for the microplate inhibition assay. An equal volume of C14s, L1, LL15, and V9 phages were mixed in a sterile tube to obtain a phage cocktail. Fresh sterile TSB and TSB in combination with 100µl of *E. coli* (ATCC 35150) were used as a positive control treatment. TSB with a phage cocktail acted as a negative control to prove that bacteriophages do not contribute to turbidity at 660 nm. A volume of 100 µl of overnight grown *E. coli* (ATCC 35150) (~ 1 x 10^8CFU/ml) was inoculated in TSB broth which was distributed to wells in a 96-well flat-bottom microtiter plate (Thermo Fisher Scientific). A bacteriophage cocktail (100µl) was added and mixed by aspiration using a multi-channel micro-pipette contributing to an

MOI (multiplicity of infection) of 1. The settings for the turbidity analysis using a microplate reader (BioTek, Synergy 4) was developed from Vijayakumar, P.P. and P.M. Muriana (2015) (Vijayakumar and Muriana, 2015). The settings for the turbidity analysis were as follows: temperature: 37°C (range: 36.5-37°C); number of flashes: 1; measurement mode: absorbance; measurement wavelength: 660 nm; start kinetic (run: 3:00:00, interval 00:30:00); shake duration (orbital): 10 seconds (s); shake intensity: medium; total measurement time: 24 hours (h); and unit: optical density (OD). In order to prevent evaporation of the liquid and well-to-well contamination, the 96-well plate was sealed with the lid. The OD_{660} values obtained were plotted against time and were used to illustrate the antimicrobial activity of the phage cocktail preparations against *E. coli* (ATCC 35150). Samples from the microplate wells were also collected every three hours in a sterile manner for both control and treatment for up to 12 hours. The obtained samples were then diluted (1:10) using peptone water and plated on pre-made tryptic soy agar (TSA) plates supplemented with 5 mM Calcium chloride and Magnesium sulfate in triplicate. The plates were then incubated overnight at 37°C and the colonies were counted.

4.2.6 Microplate turbidometric growth inhibition assays of bleach/SaniDate 5.0 treated bacteriophage cocktail

The bacteriophage cocktail was exposed to 100-ppm bleach (Sodium hypochlorite, Clorox regular) water for 0, 1, 2, and 3 h. Fresh bleach water (100-ppm) solution was prepared using sterile double distilled water. The concentration of the available chlorine in the bleach water was verified using chlorine test strips (Franklin machine products). A volume of 500µl bacteriophage cocktail (109PFU/ml) was added to 5ml of 100-ppm sterile bleach water and the mixture allowed to sit at room temperature for 3, 2, 1, and 0h. (Fresh bleach water was prepared for every hour of the study). Sterile deionized water (10µl) was

supplemented with Sodium thiosulfate ($Na_2S_2O_3$, Fisher Scientific) (0.5mg/ml) before adding the 100µl of bleach treated phages to the broth, in order to eliminate the effect of bleach on the pathogen from the results. A volume of 100µl *E. coli* O157:H7 (10^9CFU/ml) was added to appropriate wells contributing to an MOI of 1. The microplate study was conducted as previously described and the OD660 values were plotted against time and were used to illustrate the antimicrobial activity of bleach treated phage cocktail preparations against *E. coli* (ATCC 35150). The experiment was repeated with 100-ppm organic sanitizer SaniDate 5.0 (Hydrogen peroxide, Biosafe systems) to determine the ability of the cocktail to survive the organic sanitizer. A study with *E. coli* (ATCC 35150) alone in 100-ppm of each of the sanitizer was performed to determine the ability of the pathogen to survive the sanitizers.

4.2.7 Heat tolerance of bacteriophage cocktail

The effect of temperature on the bacteriophage preparations was studied to understand the ability of the phages to produce plaques under the effects of heat stress. Phage preparations (150µl) were transferred into a sterile Eppendorf tube and placed in a heating block (Techne, DRI- Block, DB-2A) at 35, 45, and 55°C; range±0.2°C in triplicates. An Eppendorf tube containing TSB and a temperature probe acted as a control and was also used for monitoring the temperature. The first phage tube preparations were heated to 35°C, were immediately removed from the heating block, and placed in an ice bath. The phage second tube preparation was allowed to sit at 35°C for 15 min and was then placed in the ice bath. A similar procedure was repeated at temperatures of 45 and 55°C. All the samples were then spotted along with a control (no temperature treatment) onto a lawn of *E. coli* (ATCC 35150).

4.2.8 Statistical Analysis

Generalized estimating equations with Huber-White standard error estimates were used to approximate the mean response for all outcomes. Studies were considered as independent clusters with repeated measures on wells. Because of the non-linear trends of the response over time, time was treated as a categorical factor and Tukey's HSD (Honest Significant Difference) was used to compare treatments at each time point.

4.3 Results

4.3.1 Bacteriophage screening, isolation, and amplification

Four wild bacteriophages (C14s, L1, LL15, and V9) with strong lytic activity for *E. coli* O157:H7 (ATCC 43895) were isolated from dairy calf feces (Auburn College of Veterinary Medicine dairy herd). Examination by transmission electron microscopy (TEM) revealed phenotypic morphology for the four bacteriophages (Figure 4.1). Bacteriophages L1 and LL15 appear as typical members of the family Siphoviridae of dsDNA bacteriophages (Ackermann, 2003), similar to the T5 and T1 morphotype (Ackermann, 2007; Kim and Ryu, 2011; Dalmasso et al., 2016). Bacteriophages C14s and V9 appear as members of the family Myoviridae of dsDNA bacteriophages (Ackermann, 2003), similar to the T4 morphotype and 01 morphotype, respectively (Ackermann, 2007; Yap and Rossmann, 2014; Dalmasso et al., 2016).

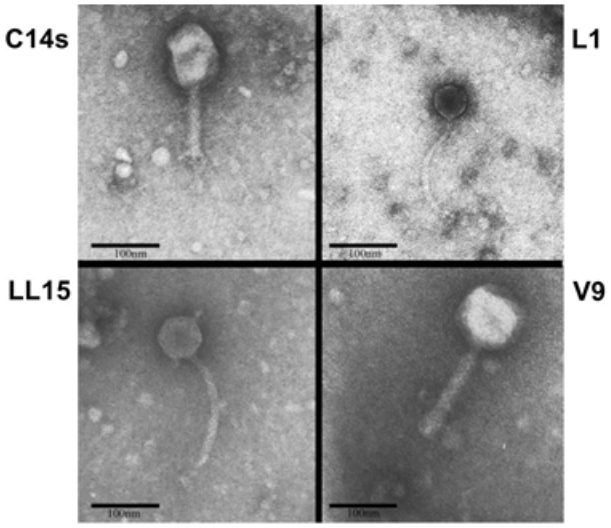


Figure 4.1 Electron microscopic image of the isolated bacteriophages from bovine origin.

4.3.2 Microplate growth inhibition assay and plate count study of bacteriophage cocktail against *E. coli* O157:H7

Positive controls of *E. coli* O157:H7 demonstrated a typical growth pattern. Significant inhibition of the pathogen was observed in the treatment wells containing the bacteriophage cocktail (Figure 4.2); thus, the bacteriophage cocktail preparation decreased the growth of *E. coli* ($P < 0.01$) in a controlled environment. The percent reduction of *E. coli* in the presence of the bacteriophage cocktail at the end of three hours was 99.99%. The bacteriophage cocktail maintained the 5-log reduction (99.99%) until the end of 6 hours; after which there was a subsequent decrease in the reduction percentage to 4-logs (9 hours) and 2-logs (12 hours), achieving 99.93% and 95.81% reduction respectively ($P < 0.01$) (Table 4.1).

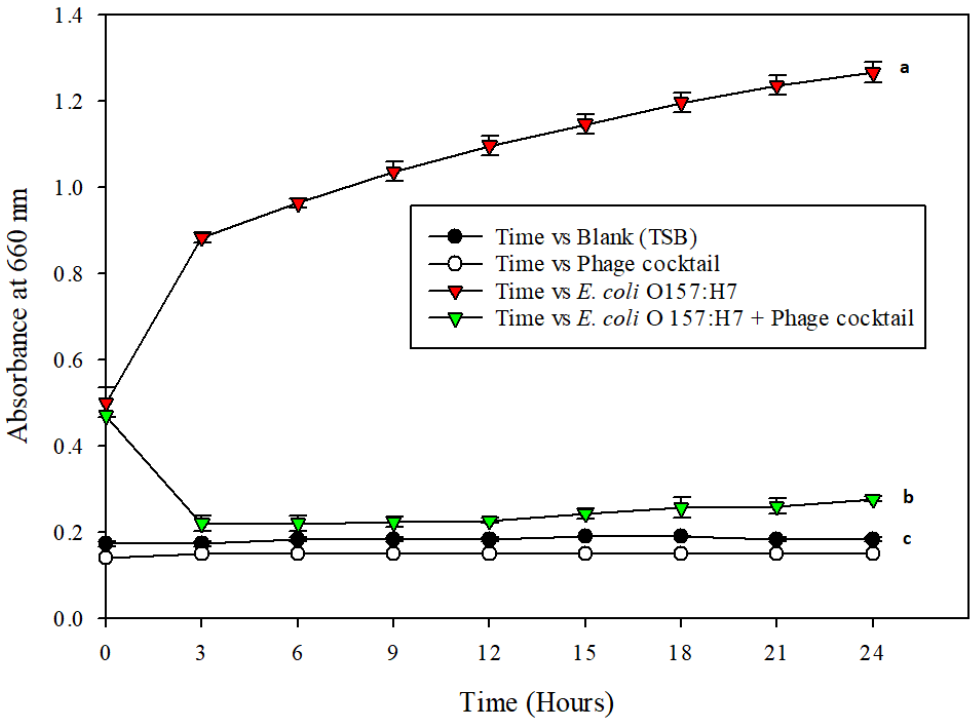


Figure 4.2 Microplate growth inhibition assay showing the activity of bacteriophage cocktail against *E. coli* O157:H7 (ATCC 35150). The data points represent the means of triplicate replication and the error bars represent the standard deviations of three independent experiments. The bacteriophage cocktail reduced the population of *E. coli* O157:H7 (ATCC 35150) significantly ($P < 0.01$) compared to the control.

Table 4.1 Reduction of *E. coli* O157:H7 (ATCC 35150) population in the presence of bacteriophage cocktail (C14s, V9, L1, and LL15). Significant reduction ($P < 0.01$) in the population of *E. coli* O157:H7 (ATCC 35150) was observed between control and treatment.

Hours	Bacterial populations (log CFU/ml)		Percentage reduction (%)
	Control	Treatment	
3	8.99	3.81	99.99
6	9.07	4.68	99.99
9	9.14	5.68	99.93
12	9.31	7.64	95.81

4.3.3 Microplate growth inhibition of bleach / SaniDate 5.0 treated bacteriophage cocktail against *E. coli* O157:H7

A microplate inhibition assay was performed to study the efficacy of a bleach treated bacteriophage cocktail against *E. coli* over time. In spite of the exposure to bleach, the phage cocktail showed inhibition against the indicator microorganism (Figure 4.3) with a significant reduction ($P < 0.05$). At the same time, the pathogen without the phage cocktail, demonstrated a classic growth curve, indicating that 100-ppm bleach had little to no effect against the pathogen (Figure 4.3). In 2002, Vijayakumar and Wolf-Hall studied the bactericidal concentration of bleach on different strains of *E. coli*. They determined that the minimum bactericidal concentration of bleach to be effective against the pathogen was between the range of 1.7 – 2.5% available chlorine in the water. It was also concluded that certain strains of *E. coli* were more resistant to bleach than others (Vijayakumar and Wolf-Hall, 2002). This explains the reason behind the growth of the pathogen in the presence of 100-ppm bleach (Figure 4.3). In the case of the organic sanitizer, 100-ppm SaniDate 5.0 at

0 h resulted in statistically significant inhibition of the pathogen. However, as exposure time increased, the pathogen recovered in the presence of the sanitizer (Figure 4.4). Alternatively, the SaniDate 5.0 treated phage cocktail gave a consistent reduction in the population of *E. coli* compared to control, irrespective of being treated at different time intervals in the presence of the sanitizer (Figure 4.4). These results indicated the ability of the phage cocktail to survive and contribute to the reduction of *E. coli*, despite being exposed to the commercially used sanitizers. These experiments demonstrate the potential of using the bacteriophage cocktail in combination with sanitizers, especially when washing produce where the combination can act as a hurdle technology to reduce the contamination of *E. coli* O157:H7 on fresh produce.

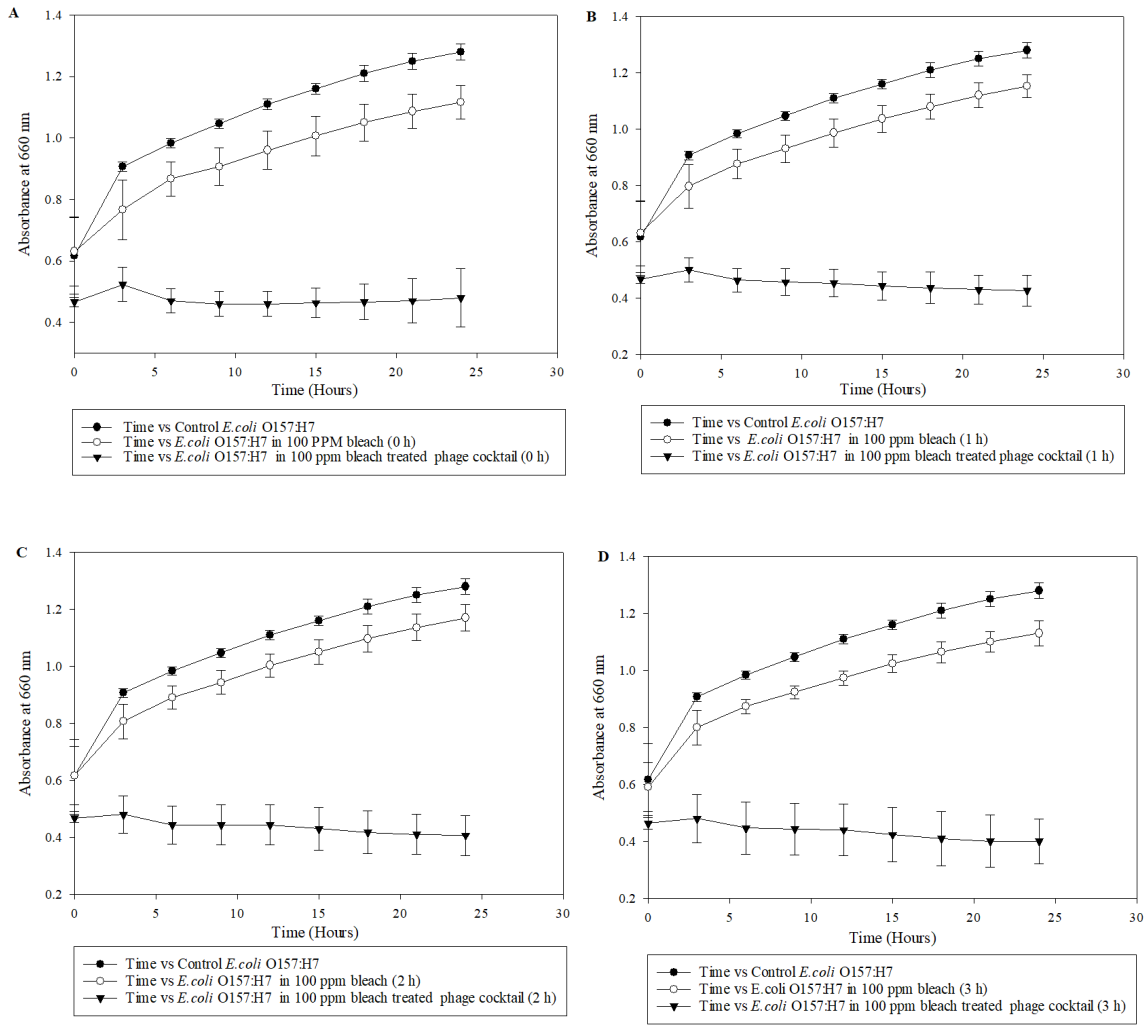


Figure 4.3 Microplate growth inhibition assay showing the activity of *E. coli* O157:H7 (ATCC 35150) in the presence of 100-ppm bleach and 100-ppm bleach treated phages at A) 0-hour, B) 1- hour, C) 2-hours, and D) 3-hours. The data points represent the means of triplicate replication and the error bars represent the standard deviations of three independent experiments. The 100-ppm bleach treated bacteriophage cocktail significantly ($P < 0.05$) reduced the population of *E. coli* O157:H7 (ATCC 35150) at 0, 1, 2, and 3 hours compared to the controls.

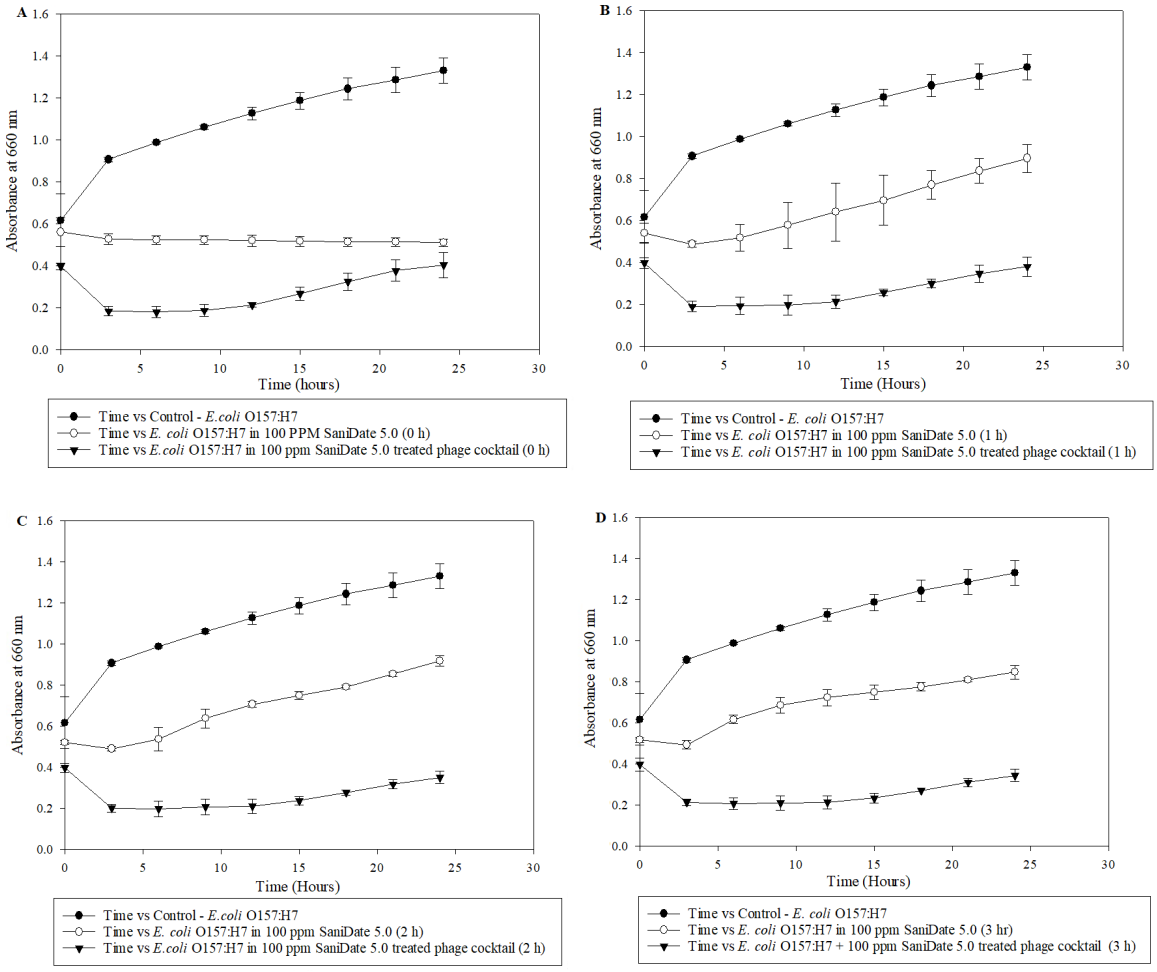


Figure 4.4 Microplate growth inhibition assay showing the activity of *E. coli* O157:H7 (ATCC 35150) in the presence of 100-ppm SaniDate 5.0 and 100-ppm SaniDate 5.0 treated phages at A) 0-hour, B) 1-hour, C) 2-hours, and D) 3-hours. The data points represent the means of triplicate replication and the error bars represent the standard deviations of three independent experiments. The 100-ppm SaniDate 5.0 treated bacteriophage cocktail significantly ($P < 0.05$) reduced the population of *E. coli* O157:H7 (ATCC 35150) at 0, 1, 2, and 3 hours compared to the controls.

4.3.4 Heat tolerance of bacteriophage cocktail

Bacteriophage preparations were examined for heat resistance, both as a potential replacement for filter sterilization and as an indication that the preparations would survive warm environment applications, especially those used on produce during wash treatments. No difference in bacteriophage activity was observed when centrifuged/heat treated bacteriophage preparations were compared to filter-sterilized preparations (Figure 4.5). In subsequent heating trials, temperatures were increased to 45 and 55°C for 0-15 min, with similar results (Figure 4.6). Temperature not only plays a vital role in survivability, but also helps in attachment, penetration, and multiplication of bacteriophages (Jończyk et al., 2011). The ability to survive these heat treatments demonstrates that these bacteriophages may be added to a produce wash or used in combination with mild heat treatment and still retain their ability to infect and reduce the population of *E. coli*.

Figure 4.5 Double agar plate showing the plaques of bacteriophages (C14s, V9, L1, and LL15) against *E. coli* O157:H7 (ATCC 35150).

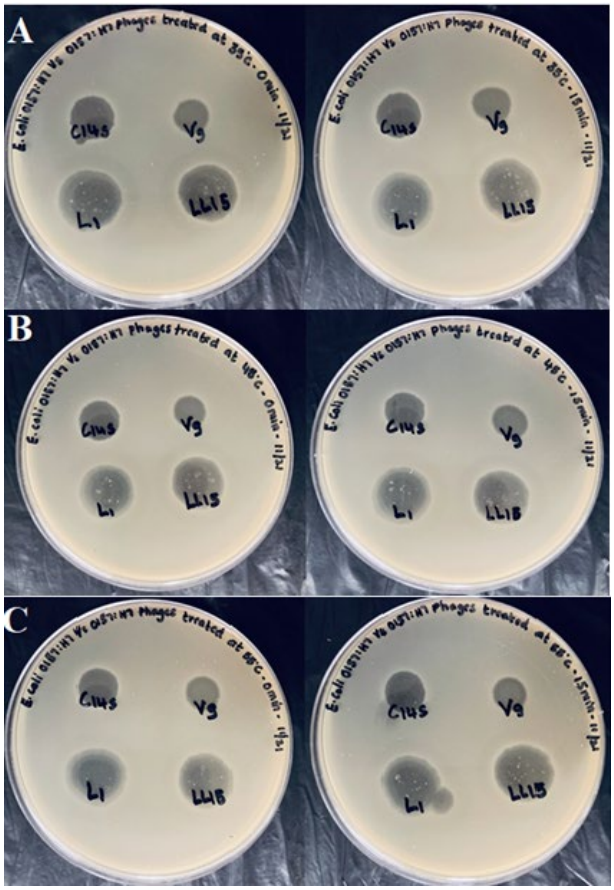


Figure 4.6 Effectivity of heat challenged bacteriophage against *E. coli* O157:H7 (ATCC 35150) at A) 35, B) 45, and C) 55ºC at 0 and 15 mins respectively.

4.4 Discussion

Bacteriophages, specifically those infecting *E. coli* O157:H7, were successfully isolated and identified from bovine feces. The initial microplate study verified the efficacy of the bacteriophage cocktail against the pathogen, which indicates its potential to be used as an antimicrobial. The following study demonstrated that the bacteriophage cocktail could survive 100-ppm SaniDate 5.0 and 100-ppm bleach. Allwood et al. (2005) studied the ability of F-specific RNA coliphage to survive 50-ppm concentration of bleach maintained at different temperatures (4, 25, and 37°C) for up to 28 days. The study demonstrated that F-RNA coliphage had a greater survival rate for 7 to 14 days in 50-ppm chlorine-treated water at all temperatures. It was concluded that the coliphages were relatively resistant to chlorine and can be used as an indicator for virological risk associated with products that are subjected to a high concentration of chlorine-based sanitizers (Allwood et al., 2005). The ability of bacteriophages to survive in the presence of these sanitizers opens new avenues for bacteriophage and sanitizers to be utilized, in combination, by the produce industry. The post-harvest wash process is considered a critical control point in the fresh produce processing industry for removing field-accrued contamination (Warriner and Namvar, 2014). It is well known that the produce industries rely on wash water sanitation to reduce the microbial load, maintain quality, and give an extended shelf life to products (Gil et al., 2009). Many alternative techniques have encouraged the food industries to move away from bleach, due to various issues with maintaining its efficacy, and health problems that are associated with employing this longstanding disinfectant (Gil et al., 2009). The current study also demonstrated the efficacy issue related to long term

sanitizers. The sanitizer solution containing SaniDate 5.0 had a lower disinfectant effect compared to the one at 0h when left to sit at room temperature for 1-3h. In the case of bleach, the 100-ppm concentration had little to no effect on the pathogen's growth. For this reason, continuous monitoring of sanitizer concentration was deemed the most important component of the produce wash procedure (Banach et al., 2015). In contrast, the bacteriophage cocktail gave a consistent reduction in *E. coli* O157:H7 populations from 0-3h irrespective of being exposed to these sanitizers. Therefore, if a deviation occurs, with respect to the concentration of the sanitizer being employed during the produce wash with bacteriophage cocktail/sanitizer combination, the phages would still be able to contribute a reduction of the pathogen population resulting in a safe product.

Dunk/dip/immersion tank washing for produce has been considered one of the most significant practices requiring investigation in the produce industries. Several foodborne outbreaks related to fresh produce have been traced back to improper post-harvest handling. Thus, poor wash water quality and improper sanitation may contribute to the contamination of produce when washed in dunk tanks. It is for this reason that bacteriophages are a promising antimicrobial for use in the food system as an effective bio-preservative, especially in ready-to-eat produce such as spinach, lettuce, and other leafy greens. Due to their ability to act as a natural antimicrobial, they can be integrated as a part of a multi-level sanitation process along with commercially used sanitizers to selectively eliminate pathogens of concern. Crude screening methods, such as plaque and microplate assays, would not be sufficient to forecast their effectiveness in a more complex system such as a produce wash. Therefore, future

studies involving a wash system with a bacteriophage and sanitizer cocktail must be performed to understand their true potential in real-world environments.

CHAPTER 5. EFFICACY OF BACTERIOPHAGE COCKTAIL TO CONTROL *E. COLI* O157:H7 CONTAMINATION ON BABY SPINACH

5.1 Introduction

Fresh fruit and vegetable consumption are often encouraged by government agencies in many countries as an important part of a balanced diet and healthy lifestyle (Berger et al., 2010). Recently, fresh fruits and vegetables that are consumed raw, such as leafy greens, are being recognized as potential vehicles for human pathogens traditionally associated with foods of animal origin (Berger et al., 2010). Current food safety systems are being strengthened by both developed and developing countries around the world to face both real and perceived food safety challenges encountered by their food industries (Henson and Caswell, 1999). Each year, *Escherichia coli* O157:H7 causes 73,000 illnesses in the United States resulting in an estimated 2,168 hospitalizations and 61 deaths (Mead et al., 1999). Infections with *Escherichia coli* O157:H7 are often associated with consumption of meat or meat products. Several outbreaks have been traced back to consumption of contaminated produce such as radishes and pre-packaged spinach (Berger et al., 2010). The first outbreak associated with *Escherichia coli* O157:H7 in produce was reported in 1991(Rangel et al., 2005). Since then, raw produce has been viewed as a potential vehicle for causing various foodborne illnesses. Decontaminating fruits, vegetables, and meat products has always been considered a challenge in the food industry (Abuladze et al., 2008). The most common ways of limiting microbial growth on fruits and vegetables are to wash them with water or to rinse them with a solution containing antimicrobials such as chlorine-based chemicals (Abuladze et al., 2008). Washing produce is considered a vital aspect of post-harvest processing that has a significant influence on

maintaining product quality and safety (Gómez-López, 2012). Wash water quality is one of the most important parameters and plays a crucial role in reducing contamination during post-harvest washing, cooling, and sanitizing operations (Ofor et al., 2009). Although water is a useful tool in reducing contamination, it can also aid in pathogen transfer through cross-contamination during post-harvest activities (Gil et al., 2009). It is well known that produce industries, especially those that handle fresh-cut produce, rely on wash water quality and sanitizers to minimize microbial count and achieve an extended shelf-life for their products (Gil et al., 2009). Chlorine-based sanitizers have long been used by the food industry to maintain the safety of their products (Ölmez and Kretzschmar, 2009). However, recent outbreaks associated with produce have raised concern for traditional sanitizer efficacy in ensuring the safety of the products. Additionally, various concerns over environmental implications and health risks have also risen; (Ölmez and Kretzschmar, 2009) thus, current investigations are seeking alternatives to chlorine based sanitizers, which could provide safety to the products without compromising the quality and shelf life (Ölmez and Kretzschmar, 2009). Bacteriophages (commonly called phage) are bacterial viruses that selectively infect bacteria and disrupt their metabolism resulting in lysis of the host bacterial cell (Sulakvelidze et al., 2001). Since phages are highly specific, they can be used to target a specific pathogen without harming any beneficial microorganisms (Magnone et al., 2013). Phages have been proven to act as a natural antimicrobial to fight against bacterial infections in humans, animals, and crops (Brüssow, 2005). Several studies have focused on phages as a promising alternative that can be used in the food industry to eliminate bacterial contamination, especially on produce (Harris et al., 2001; Leverentz et al., 2003; García et al., 2008; Gragg and Brashears, 2010). The focus of this research was

to investigate the ability of a bacteriophage cocktail to lyse *E. coli* O157:H7 on spinach leaves during a simulated dunk tank wash in the presence and absence of an organic load.

5.2 Materials and method

5.2.1 Bacterial culture for microplate and produce wash study

Pathogenic *Escherichia coli* O157:H7 (ATCC 35150) was obtained from a freezer stock. Working stock cultures were prepared by resuspending cells into tryptic soy broth (TSB, Difco, Becton-Dickenson Labs) and incubated for 48 hours at 37°C before streaking the cultures on MacConkey Agar (MAC, Difco, Becton-Dickenson Labs) and Sorbitol MacConkey Agar (SMAC, Difco, Becton-Dickenson Labs) for isolation. After incubation for 24 hours at 37°C, the characteristics of the colonies were observed and individual colonies picked from SMAC into TSB tubes (supplemented with 5mM of Magnesium sulfate ($MgSO_4$, Fisher Scientific) and Calcium chloride ($CaCl_2$, Fisher Scientific)) using sterile technique. Cultures were grown for 24 hours at 37°C and then stored at refrigeration temperature, 4°C, until needed for propagation. Frozen stock cultures were made and stored at -25°C in skim milk media (Difco, Becton-Dickenson Labs) cryogenic vials for long term storage.

5.2.2 Bacteriophage cocktail preparation

Four bacteriophages (C14s, V9, L1, and LL15), specific to *E. coli* O157:H7, were obtained from bovine feces. The dairy herd bacteriophages were isolated and characterized by the Auburn University College of Veterinary Medicine. Bacteriophages were grown for 24 hours at 37°C with host *E. coli*. Phages were then separated via centrifugation at 20,000

rpm for 20 min in the presence of chloroform. The phages were then filter sterilized through a 0.22µ filter (Miller - Gs) into working stock containers. Equal volumes of individual bacteriophage types were mixed in a sterile test tube and the required volume was pipetted right before every experiment to make the phage cocktail.

5.2.3 Bacteriophage titer

A bacteriophage titer was confirmed prior to ensuring phage activity. The host strain for all the bacteriophages was *E. coli* O157:H7 (ATCC 35150). The phage titer ranged from 7.00 x 10^6 to 1.20 x 10^{10}PFU/ml.

5.2.4 Turbidometric growth inhibition assays in the presence of organic load

An equal volume of C14s, L1, LL15, and V9 phages were mixed in a sterile tube to obtain a phage cocktail. Sterile DE neutralizing buffer broth (Difco, Becton-Dickenson) and DE broth with 100µl of *E. coli* O157:H7 (ATCC 35150) were used as control treatments. DE broth with 100µl phage cocktail acted as a negative control to show that the bacteriophages do not contribute turbidity at 660nm. 100µl of *E. coli* O157:H7 (ATCC 35150) (~ 1.00 x 10^8CFU/ml) was inoculated into DE broth and distributed to wells in a 96-well flat-bottom microtiter plate (Thermo Fisher Scientific). 100µl bacteriophage cocktail was added to the wells and mixed by aspiration using a multi-channel micro-pipette. This ration contributed to an MOI (Multiplicity of Infection) of 1. The settings for the turbidity analysis, using a microplate reader, (BioTek, Synergy 4) was developed from a previously determined procedure (Vijayakumar and Muriana, 2015). The settings for the turbidity analysis were as follows: temperature: 37°C (range: 36.5 – 37°C), number of flashes: 1, measurement mode: absorbance, measurement wavelength: 660 nm, start kinetic

(run: 3:00:00, interval 00:30:00), shake duration (orbital): 10 seconds (s), shake intensity: medium, total measurement time: 24h, and unit: optical density (OD). To prevent evaporation of the liquid and well-to-well contamination, a lid was used to seal the 96-well plate. The OD_{660} values were plotted against time to illustrate the antimicrobial activity of the phage cocktail preparations against *E. coli* O157:H7. Samples from the microplate wells were collected at the end of three hours for both the control and treatment. These samples were then diluted using sterile peptone water (1:10) and plated (100µl) on premade TSA plates supplemented with 5mM Calcium chloride and Magnesium sulfate in triplicates. The plates were then incubated overnight at 37°C.

5.2.5 Initial produce rinse to reduce background microbial contamination on spinach leaves

Fresh baby spinach leaves were purchased from a local grocery chain. Spinach leaves were transferred into a sterile filter bag (Fisher brand – blender bags) and treated with a 2% Lactic acid solution (Fisher Scientific) for 20 mins. The leaves were then treated with 100-ppm bleach water (Clorox) for 20 mins. Leaves were then set under UV light for 20 mins to reduce the background population of microorganisms as well as to dissipate any residual chlorine present on the leaves (Figure 5.1).

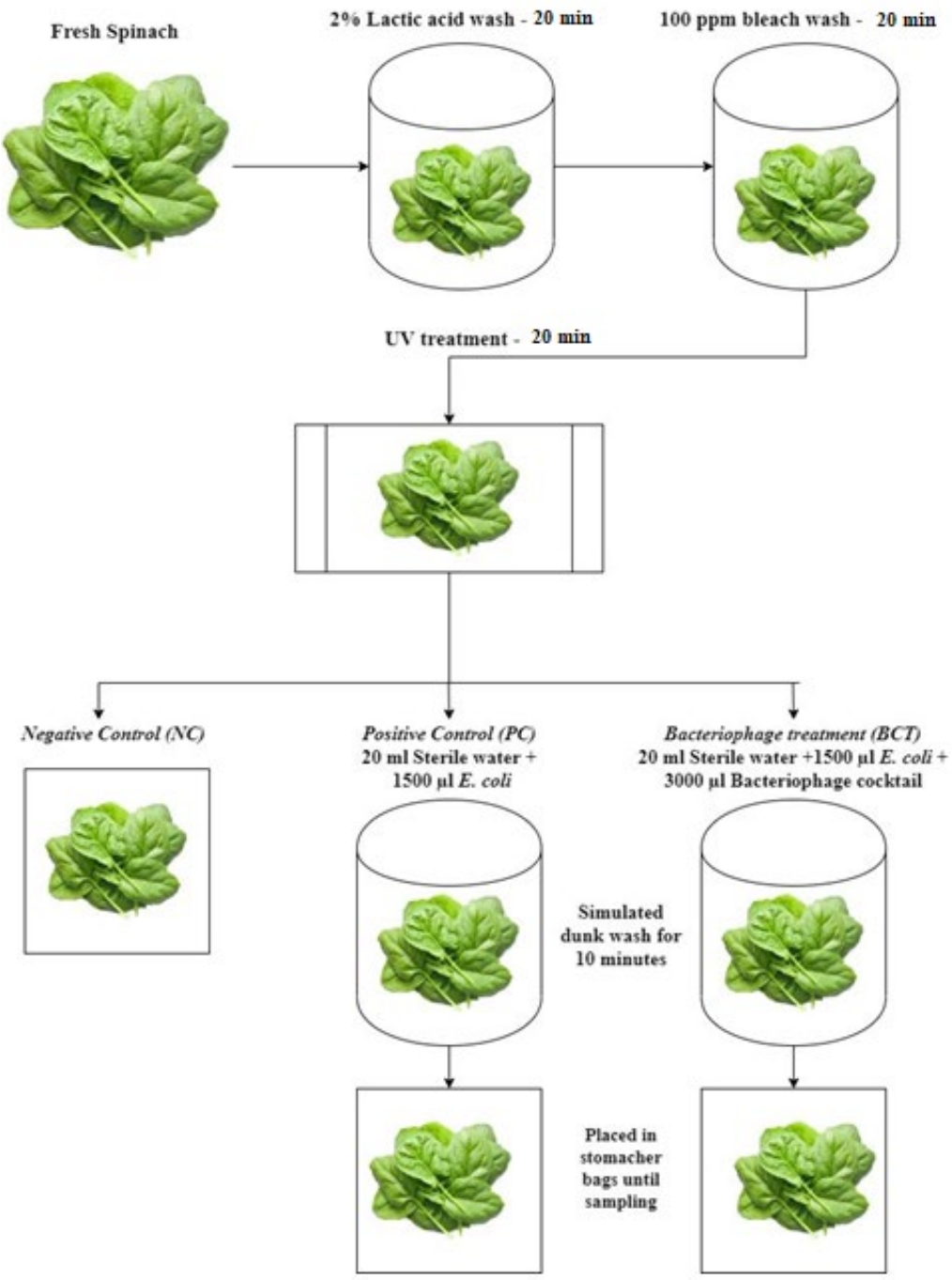


Figure 5.1 Schematic flow of initial produce rinse and dunk wash of spinach leaves.

5.2.6 Wash solution for the simulated dunk tank

20ml double-distilled deionized sterile water was used for the initial experiment to study the efficacy of the bacteriophage cocktail against *E. coli* O157:H7 in the absence of an organic load. For the following study, 20ml of sterilized DE broth containing approximately 9810-ppm of dissolved organic matter (Casein – 1660-ppm, Yeast extract – 830-ppm, Dextrose – 3330-ppm, Tween 80 – 1660-ppm, and Lecithin – 2330-ppm) was used as a wash solution to determine the ability of the bacteriophage cocktail to infect *E. coli* O157:H7 in the presence of an organic load. Control samples were treated similarly with organic load wash water without the bacteriophage cocktail. In both studies, the samples were immersed in the wash solution for the full contact time of 10 minutes.

5.2.7 Application of sterile wash water solution containing *E. coli* O157:H7 and bacteriophage cocktail in a simulated dunk tank

Fresh spinach leaves, after the initial produce rinse step, were separated into three different treatments: Negative control (NC), Positive control (PC), and Bacteriophage cocktail treatment (BCT). The NC had washed spinach without any other treatment. This was used to enumerate the efficacy of the initial wash to observe if any background microorganisms were still present on the leaves. The PC sample had leaves that were dunk washed for 10 min in 20ml sterile water containing 1500µl of *E. coli* O157:H7 (~ 1.0×10^8 CFU/ml). The BCT sample had leaves dunk washed in 20ml sterile water with a combination of 1500 µl of *E. coli* O157:H7 (~ 1.0×10^8CFU/ml) and 3000µl of bacteriophage cocktail (MOI – 2.3). All of the samples were placed in a sterile sampling bag and sampled 0, 3, 6, 9, and 12 hours.

5.2.8 Application of sterile wash solution containing 9810 ppm of organic load comprising *E. coli* O157:H7 and bacteriophage cocktail in a simulated dunk tank

A similar procedure from the above study was applied with DE broth instead of the sterile water to mimic an organic load present in the wash water. All the samples were packed in a sterile sampling bag and were sampled at 0 and 3 hours.

5.2.9 Recovery of bacteria

Produce was rinsed with 1 ml sterile phosphate buffer. Samples were massaged for one minute and serial dilutions of the sample rinse were made in phosphate buffer (pH 7.4 - 7.5). The dilutions were then plated on pre-made TSA plates, supplemented with 5mM Magnesium sulfate ($MgSO_4$, Fisher Scientific) and 5mM Calcium chloride ($CaCl_2$, Fisher Scientific).

5.2.10 Statistical Analysis

The data were analyzed using the GLIMMIX procedure in SAS 9.4. A linear mixed model was used where the response variable was Readings_in_Log10 and the fixed effects were treatment, time and the interaction between treatment and time. Time was treated as categorical since there were two-time points. A random intercept for the subject defined by the spinach with a specific treatment within a study was included in the model. The difference between the Readings_in_Log10 for the treatment at time 0 and time 3, and the difference between the Readings_in_Log10 for the control at time 0 and time 3 were tested using the LSMEANS statement with the slicediff option. The p-values were adjusted for multiple comparisons using the Tukey-Kramer method.

5.3 Results

5.3.1 Microplate growth inhibition assay and plate count study of bacteriophage cocktail against *E. coli* O157:H7 in the presence of organic load

Positive controls of *E. coli* O157:H7 demonstrated a typical logarithmic growth pattern over the test period. The bacteriophage cocktail demonstrated significant inhibition of the pathogen (Figure. 5.2). The bacteriophage cocktail preparation decreased the growth of *E. coli* O157:H7 ($P < 0.01$) in a controlled environment in the presence of a 9810-ppm organic load. The percent reduction of *E. coli* O157:H7, in the presence of the bacteriophage cocktail, at the end of three hours, was 99.99% (Table 5.1). The study demonstrated that phages are highly specific to the host-pathogen despite being in a relatively concentrated organic load. The phages specifically targeted the bacteria, infected, and reduced the host population. This is in contrast to commercially used sanitizers, such as bleach, which are less effective in the presence of an organic load. This is because the chlorine has a higher affinity towards the organic matter, thus, depleting its effectiveness against microorganisms.

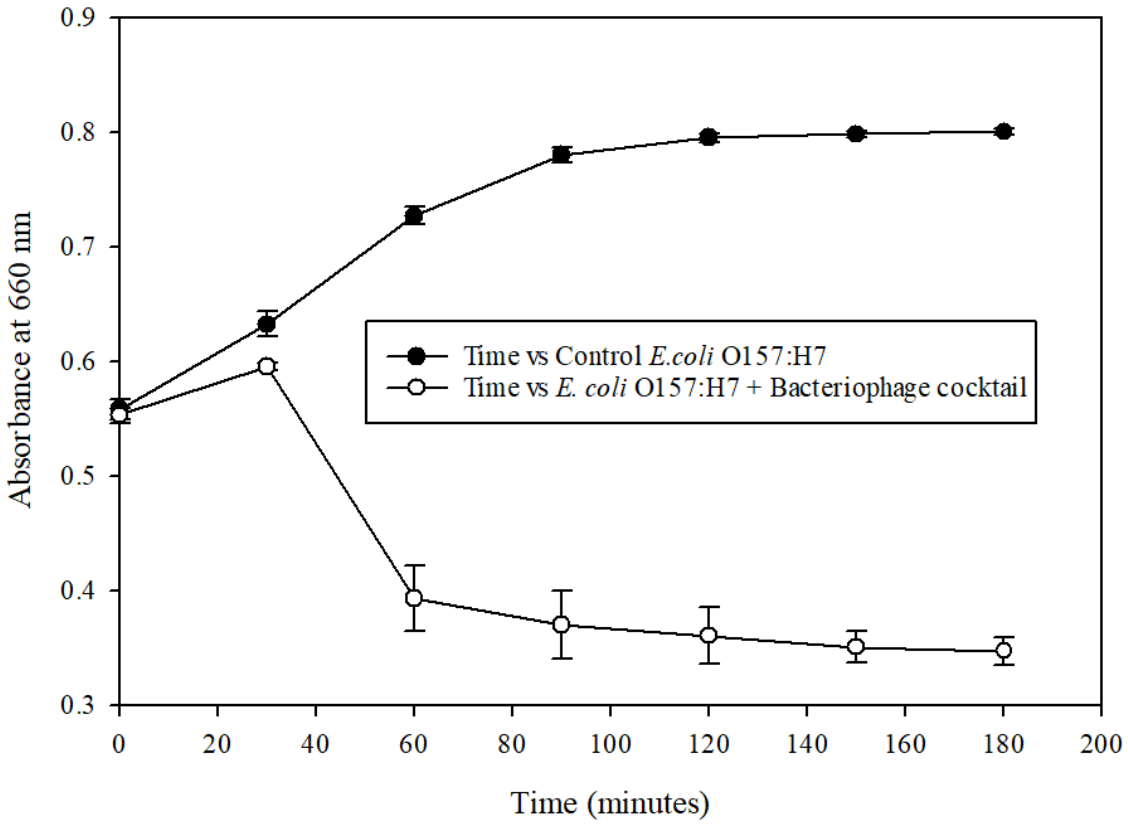


Figure 5.2 Microplate growth inhibition assay showing the activity of bacteriophage cocktail against *E. coli* O157:H7 (ATCC 35150) in the presence of organic load. The data points represent the means of triplicate replication and the error bars represent the standard deviations of three independent experiments. The bacteriophage cocktail reduced the population of *E. coli* O157:H7 (ATCC 35150) significantly ($P < 0.05$) compared to the control.

Table 5.1 Reduction of *E. coli* O157:H7 (ATCC 35150) population in the presence of bacteriophage cocktail (C14s, V9, L1, and LL15) in a sample containing 9810 ppm of organic load. Significant reduction ($P < 0.01$) in the population of *E. coli* O157:H7 (ATCC 35150) was observed between control and treatment.

Hours	Bacterial populations (log CFU/ml)		Percentage reduction (%)
	Control	Treatment	
0		6.82	-
3	9.00	3.26	99.99

5.3.2 Effect of bacteriophage on sterile water wash solution containing *E. coli* O157:H7 inoculated spinach in a simulated dunk tank

The initial produce rinse successfully inhibited the growth of background flora on fresh spinach. The plate count (<1.00 CFU/ml) on the NC indicated that the initial rinse was effective at rinsing the background microflora. Table 5.2 shows the efficacy of the bacteriophage cocktail in the reduction of *E. coli* O157:H7 on spinach washed in potable water containing the phage cocktail compared with the control wash. The 10-minute contact time for the wash solution resulted in a significant reduction ($P < 0.05$) of the pathogen at the end of three hours compared to the PC. A gradual recovery of the pathogen numbers occurred in the samples obtained from BCT after three hours until 12 hours. The statistical analysis indicated that despite the recovery, the BCT was still significantly different from the PC. Therefore, the disinfectant treatment (BCT) was significantly effective ($P < 0.05$) in reducing the population of *E. coli* O157:H7 on the spinach leaves.

Table 5.2 Reduction of *E. coli* O157:H7 (ATCC 35150) on spinach via postharvest pathogen control measures of using bacteriophage cocktail wash solution made with potable water in a simulated dunk tank.

Wash treatment	Wash time (min)	Sampling time (h)	*E. coli* O157:H7 population (log CFU/ml)
Negative Control (NC)	-	0	<1.00
		3	<1.00
		6	<1.00
		9	<1.00
		12	<1.00
Positive Control (PC)	10	0	6.22
		3	6.42
		6	7.10
		9	7.34
		12	7.37
Produce wash + Bacteriophage cocktail (BCT)	10	0	5.81
		3	3.78
		6	4.93
		9	5.30
		12	5.22

5.3.3 Effect of sterile wash solution containing 9810 ppm of organic load comprising *E. coli* O157:H7 and bacteriophage cocktail in a simulated dunk tank

The initial produce rinse was once again effective in reducing the background microflora of the spinach (<1.00CFU/ml). Table 5.3 shows the efficacy of the bacteriophage cocktail in the reduction of *E. coli* O157:H7 on spinach washed in the challenge water (9810 ppm organic load) containing the phage cocktail compared with the control wash. The 10-minute contact time for the wash solution resulted in a significant reduction ($P < 0.01$) of 99.99% of the pathogen at the end of three hours compared to the PC. This study also illustrates the specificity of bacteriophage and its ability to effectively reduce *E. coli* O157:H7 despite being in an environment with a high organic load.

Table 5.3 Reduction of *E. coli* O157:H7 (ATCC 35150) on spinach via postharvest pathogen control measures of using bacteriophage cocktail wash solution made with water containing 9810 ppm of organic load in a simulated dunk tank

Wash treatment	Wash time (min)	Sampling time (h)	*E. coli* O157:H7 population (log CFU/ml)
Negative Control (NC)	-	0	<1.00
		3	<1.00
Positive Control (PC)	10	0	6.46
		3	7.16
Produce wash + Bacteriophage cocktail (BCT)	10	0	6.14
		3	2.94

5.4 Discussion

The post-harvest wash procedure is considered a critical control point (CCP) for removing any field-assimilated contamination in the fresh produce industry (Warriner and Namvar, 2014). Chlorine is one of the most commonly used sanitizers in the produce industry. The internationally recommended concentration for chlorine-based compounds used for rinsing produce is between 50 - 100 ppm of free chlorine (World Health Organization, 2008). This range is reported to achieve a pathogen reduction of approximately 1 - 2 log CFU/g (Ruiz-Cruz et al., 2007). The effectiveness of chlorine-based sanitizers decreases in the presence of organic matter in produce wash water (Park et al., 2009). Thus, pre-treatment removal of organic matter, along with continuous monitoring of sanitizer concentration, is suggested for the effective use of sanitizer in the food industry (Park et al., 2009; Banach et al., 2015). Despite these efforts, bacterial outbreaks in the fresh produce industry continue to be on the rise.

Bacteriophage, as an antimicrobial, has proven to be efficient in reducing the population of *E. coli* O157:H7 in foods. Previous studies have evaluated the effectiveness of bacteriophage cocktails, specific to different pathogens, such as *Salmonella*, *E. coli* O157:H7, and *Listeria*. Leverentz et al. (Leverentz et al., 2001) observed a reduction in the *Salmonella enteritidis* population on fresh-cut honeydew melon after spot treating the infected portion with a bacteriophage cocktail. The pathogen population was reduced 3.5 and 2.5 log CFU/wound after the treated melons were stored at 5-10, and 20^{o}C respectively (Leverentz et al., 2001). Similarly, fresh-cut honeydew melons treated (spray or aliquots) with *Listeria monocytogenes* specific bacteriophages reduced the population of *L. monocytogenes* by 2 to 4.6 log units compared to the untreated controls when stored at

10ºC (Leverentz et al., 2003). Most of these studies either spot or spray treated the samples with bacteriophage to demonstrate their effectiveness against the pathogen.

Although the previous studies demonstrated the efficacy of using bacteriophages against pathogens, they did not apply the results to real-time scenarios. It is for this reason that this study sought to determine the efficacy of bacteriophages in dunk tanks, a commonly used wash procedure. Dunk tanks, also referred to as immersion or dip tanks, carry a significantly higher risk of cross-contamination of pathogens between contaminated and clean produce (Banach et al., 2015). Immersion washers employ techniques such as dumping, submerging, or floating produce in wash water with or without sanitizer (Gómez-López, 2012). The potential of pathogen uptake by produce through infiltration is a major concern for the food industries that use dunk tanks or other immersion techniques (Gómez-López, 2012). Pathogen infiltration can occur through the stem scare, calyx, or other surface openings that are naturally present on fresh produce. Apart from this, if the washing procedure is not monitored or managed properly, it can create produce injury, cross-contamination, or internalization of the pathogen (Gómez-López, 2012). For instance, from 2000-2002, the United States faced a multistate outbreak of *Salmonella* serotype *Ponna* that was associated with the consumption of cantaloupe imported from Mexico. An on-farm investigation of the outbreak revealed that the melons were washed and cooled in contaminated wash water which could have been the possible source for the contamination(Centers for Disease Control and Prevention, 2002; Gómez-López, 2012). A multistate outbreak of *Salmonella enterica* serotype Newport, associated with consumption of mangoes, in the United States led to 78 confirmed cases of salmonellosis in 13 states. Penteado et al. 2004, investigated the recall by recreating the washing scenarios to study

the ability of the pathogen to contaminate the fruit during the washing process. The team tested the ability of *Salmonella* to internalize in fresh mangoes, during a simulated postharvest insect disinfection procedure. Pathogen internalization was observed when heat-disinfected mangoes were cooled using the contaminated water. The study concluded that poor wash water quality and improper chlorination could have served as a vector for contaminating the mangoes (Penteado et al., 2004). These outbreaks emphasize the need for an effective technique during production and post-harvest activities that can mitigate the risk for pathogen contamination on fresh produce. Employing commercial sanitizers alone have not solved the problem of pathogen contamination, since only 1-2 log CFU reduction, under specified conditions, is expected. Employing bacteriophage as a disinfectant has been shown to be effective in reducing the population of *E. coli* O157:H7 in fresh produce without the use of chemical sanitizers. Abuladze et al. 2008, studied the ability of a bacteriophage cocktail to reduce *E. coli* O157:H7 contamination on broccoli, spinach, tomato, and ground beef. Treatment with the bacteriophage cocktail resulted in a significant reduction ($P \leq 0.05$) on the pathogen with a minimal recovery as incubation time increased. The percent reduction on broccoli was 99.5%, 99%, 97%; tomatoes – 99%, 94%, 96%; spinach – 100%, 99.6%, 91% at 24, 120, and 168 hours respectively. Data obtained in the current study were similar wherein the bacteriophage cocktail in sterile wash water reduced the population of *E. coli* O157:H7 by 2.64-log CFU/ml at the end of 3 hours which contributed to 99.77% reduction of the pathogen compared to the control. In the case of wash water containing high organic load, the bacteriophages contributed to a 4-log CFU/ml reduction of the pathogen which corresponds to a 99.99% reduction at the end of 3 hours. The sterile wash water study indicated a minimal recovery of the pathogen as

incubation time increased. The emergence of phage-resistant bacterial mutants, transduction of undesirable characteristics among bacteria and environmental conditions have been suggested as problems that can potentially reduce the effectiveness of a phage treatment (Vidaver, 1976). However, several studies have suggested that employing a cocktail of different bacteriophages could potentially reduce the likelihood of generating a mutant (Kutter and Sulakvelidze, 2004; Tanji et al., 2004). One possible explanation could be the mechanism of phage attachment. Phages tend to attach to different receptors found on the host bacteria, and the mutation of one specific phage receptor would not alter the attachment site for another phage (Tanji et al., 2004). Because phages are ubiquitous, isolating new phages, specific to the pathogen that exhibits a difference in the attachment mechanism, can be used to update phage cocktails to make them effective against the development of phage-mutant strains.

Numerous foodborne outbreaks of *E. coli* O157:H7 have been caused by < 20.00 CFU/g or even < 1.00 CFU/g of the pathogen (Meng, 2001). However, in a real-life scenario, a very high load of *E. coli* O157:H7 contamination on produce is very unlikely to occur (Abuladze et al., 2008). The amount of *E. coli* O157:H7 that was used in this experiment was several thousand-fold higher than that associated with an outbreak. This was performed to better study and visualize the efficacy of the bacteriophage cocktail. Several studies reported by other investigators concluded that a lower bacteria-phage ratio can yield a better reduction of the pathogen (Goode et al., 2003; Abuladze et al., 2008). Therefore, increasing the concentration of the phage might help in achieving a greater reduction in the pathogen during produce wash. Phages employed for the study have also shown to be resistant to 100-ppm chlorine or 100-ppm SaniDate 5.0 for up to three hours.

Thus, developing a multilevel sanitation system that employs both a sanitizer and bacteriophage combination might be one of the solutions to reduce pathogen contamination on fresh produce. Future studies involving combination treatment methods or hurdle technology on large-scale trials might be required to verify this possibility and could help mitigate the exposure of foodborne pathogens on fresh produce.

CHAPTER 6. APPLICATION OF A BACTERIOPHAGE – SANITIZER COMBINATION IN POST-HARVEST CONTROL OF E. COLI O157:H7 CONTAMINATION ON SPINACH LEAVES IN THE PRESENCE OR ABSENCE OF A HIGH ORGANIC LOAD PRODUCE WASH

6.1 Introduction

Consumption of fresh fruits and vegetables continues to increase in the United States due to its association with a healthy lifestyle. Fresh produce remains one of the leading causes of foodborne illness due to contamination with various pathogens such as *Salmonella*, *Listeria monocytogens*, and Shiga Toxin producing *Escherichia coli* (Callejón et al., 2015). A significant portion of the produce is consumed raw and the number of outbreaks associated with it has been increasing correspondingly. The open nature of how raw produce is handled in the food supply chain implies that the contamination can be introduced at any point during production, harvest, and processing (Nüesch-Inderbinen and Stephan, 2016). Hence, disinfecting produce after harvesting is considered an essential step for the post-harvest handling of fruits and vegetables (Feliziani et al., 2016). The minimum requirement for a produce handling facility is to have a disinfection procedure that ensures the commodity is free from fungal and bacterial pathogens (Feliziani et al., 2016). This is usually achieved by using disinfectants such as chlorine, chlorine dioxide, ozone, ethanol, hydrogen peroxide, organic acids, and electrolyzed water (Feliziani et al., 2016). Despite using disinfectants, there continues to be a rise in foodborne outbreaks involving whole and fresh-cut produce. In 2019, a foodborne outbreak of *E. coli* O157:H7 was linked to the consumption of romaine lettuce produced from the Salinas Valley growing region in California (Center for Disease Control and Prevention, 2020b). The outbreak resulted in 167 infections and 85 hospitalizations. A foodborne outbreak of *Salmonella* Carrau linked to the consumption of pre-cut melon left 137 people infected and required 38

hospitalizations in the United States (Center for Disease Control and Prevention, 2020b). The rise in foodborne outbreaks in recent years has made the regulatory agencies, producers, and public increasingly concerned regarding the microbial safety of fresh fruits and vegetables (Sapers, 2001). Washing is defined as rinsing, scrubbing, rubbing, or dipping produce to remove any field acquired contamination from the surface of the product (Gómez-López, 2012). Washing produce is primarily done to improve the physical appearance of produce, but is also used to reduce any microbial or chemical residues which can hasten spoilage, cause product recalls, or result in human illness (Gómez-López, 2012). Immersion washers are one of the widely used techniques for washing produce such as melons, tomatoes, cucumbers, and loose greens (Gómez-López, 2012). Dunk tank immersion washers are mainly employed for removing large debris, biological contaminants, and to reduce physical impact and tissue damage. One significant safety issue with the dunk tank technique of produce washing is the infiltration of water (Gómez-López, 2012). Various factors such as temperature, depth of water, soaking time, wound/scarring, and maturity of the products have to be taken into consideration to avoid cross-contamination or infiltration of contaminated water (Higgins, 2018). It was previously assumed that post-harvest wash/sanitation was adequate to clean and sanitize the produce of potential contaminants (Feliziani et al., 2016). Recent outbreaks and subsequent research have shown that post-harvest washing, under commercial conditions, has a limited efficacy in decontamination of produce and might even lead to cross-contamination of produce during the wash step (Barrera et al., 2012; Gombas et al., 2017). The focus of this research was to investigate the ability of bacteriophages to reduce *E. coli*

O157:H7 contamination on baby spinach in the presence or absence of an organic load along with 100-ppm bleach and SaniDate 5.0.

6.2 Materials and Methods

6.2.1 Bacterial culture for microplate and produce wash study

Pathogenic *Escherichia coli* O157:H7 (ATCC 35150) was obtained from a freezer stock. Working stock cultures were prepared by resuspending cells into tryptic soy broth (TSB, Difco, Becton-Dickenson Labs) and incubated for 48 hours at 37°C before streaking the cultures on MacConkey Agar (MAC, Difco, Becton-Dickenson Labs) and Sorbitol MacConkey Agar (SMAC, Difco) for isolation. After incubation for 24 hours at 37°C, the characteristics of the colonies were observed and individual colonies picked from SMAC into TSB tubes (supplemented with 5mM of Magnesium sulfate ($MgSO_4$, Fisher Scientific) and Calcium chloride ($CaCl_2$, Fisher Scientific)) using sterile technique. Cultures were grown for 24 hours at 37°C and then stored at refrigeration temperature, 4°C, until needed for propagation. Frozen stock cultures were made and stored at -25°C in skim milk media (Difco, Becton-Dickenson Labs) cryogenic vials for long term storage.

6.2.2 Bacteriophage cocktail preparation

Four bacteriophages (C14s, V9, L1, and LL15), specific to *E. coli* O157:H7, were obtained from bovine feces. The dairy herd bacteriophages were isolated and characterized by the Auburn University College of Veterinary Medicine. Bacteriophages were grown for 24 hours at 37°C with host *E. coli* O157:H7. Phages were then separated via centrifugation at 20,000 rpm for 20 min in the presence of chloroform. The phages were then filter sterilized through a 0.22µ filter (Miller - Gs) into working stock containers. Equal volumes

of individual bacteriophage types were mixed in a sterile test tube and the required volume was pipetted just before every experiment to make the phage cocktail.

6.2.3 Bacteriophage titer

A bacteriophage titer was confirmed before each experiment to ensure phage activity. The host strain for all the bacteriophages was *E. coli* O157:H7 (ATCC 35150). The phage titer ranged from 7.00×10^6 to 1.20×10^{10}PFU/ml.

6.2.4 Initial produce rinse to reduce background microbial contamination on spinach leaves

Fresh baby spinach leaves were purchased from a local grocery chain. Spinach leaves were transferred into a sterile filter bag (Fisher brand – blender bags) and treated with a 2% Lactic acid solution (Fisher Scientific) for 20 mins. The leaves were then treated with 100-ppm bleach water (Clorox) for 20 mins. Leaves were then set under UV light for 20 mins to reduce the background population of microorganisms, as well as to dissipate any residual chlorine present on the leaves. Samples (NC) were collected after the initial rinse to analyze whether the wash successfully reduced the background flora.

6.2.5 Wash solution for the simulated dunk tank

Wash solutions were made to simulate the produce industry wash water. The first set of wash solutions were made with 20 ml of sterile doubled deionized water containing 100-ppm bleach (T1) and 100-ppm SaniDate 5.0 (T2). These washes were used to determine their effect against *E. coli* O157:H7, as well as their effect with the addition of the phage cocktail (T3 and T4) (Figure 6.1). Control samples were treated similarly with water and

E. coli O157:H7 (PC). In all of the treatments, the samples were completely immersed in the wash solution for the full contact time of 10 minutes.

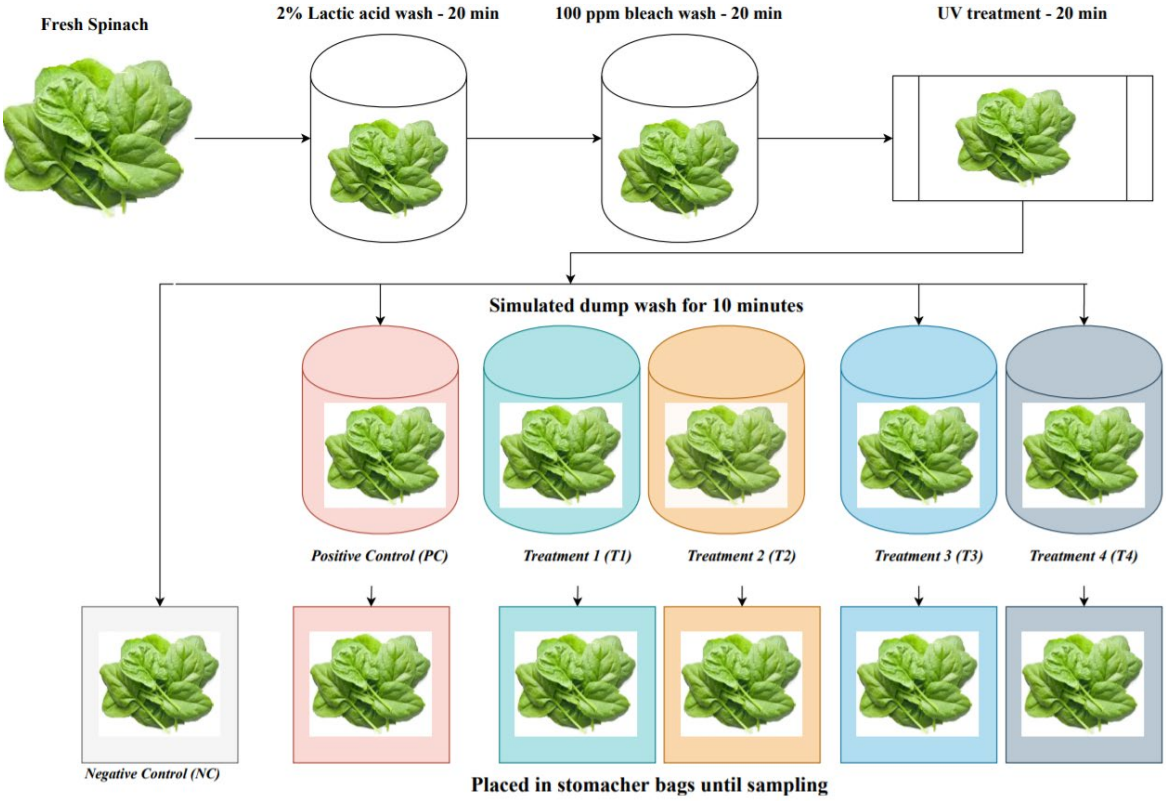


Figure 6.1 Schematic flow of initial produce rinse and dunk wash of spinach in sterile water in combination with the sanitizers and bacteriophage cocktail. NC – No treatment, PC – Leaves washed with *E. coli* O157:H7 (ATCC 35150), T1 – Leaves washed with *E. coli* O157:H7 (ATCC 35150) in water containing 100-ppm bleach, T2 - Leaves washed with *E. coli* O157:H7 (ATCC 35150) in water containing 100-ppm SaniDate 5.0, T3- Leaves washed with *E. coli* O157:H7 (ATCC 35150) in water containing 100-ppm bleach and phage cocktail, T4 - Leaves washed with *E. coli* O157:H7 (ATCC 35150) in water containing 100-ppm SaniDate 5.0 and phage cocktail

6.2.6 Wash solution for the simulated dunk tank with high organic load water

Wash solutions were made similarly with 100-ppm bleach (T1) and 100-ppm SaniDate 5.0 (T2) in 20ml of sterilized DE broth containing approximately 9810-ppm of dissolved organic matter (Casein – 1660-ppm, Yeast extract – 830-ppm, Dextrose – 3330-ppm, Tween 80 – 1660-ppm, and Lecithin – 2330-ppm). These washes were used to determine the effect of the sanitizers against the pathogen, as well as with the addition of the phage cocktail (T3 and T4) to infect *E. coli* O157:H7 in the presence of high organic load (Figure 6.2). Control samples (NCO) were treated similarly with organic load wash water without the bacteriophage cocktail. In all of the treatments, the samples were completely immersed in the wash solution for the full contact time of 10 minutes.

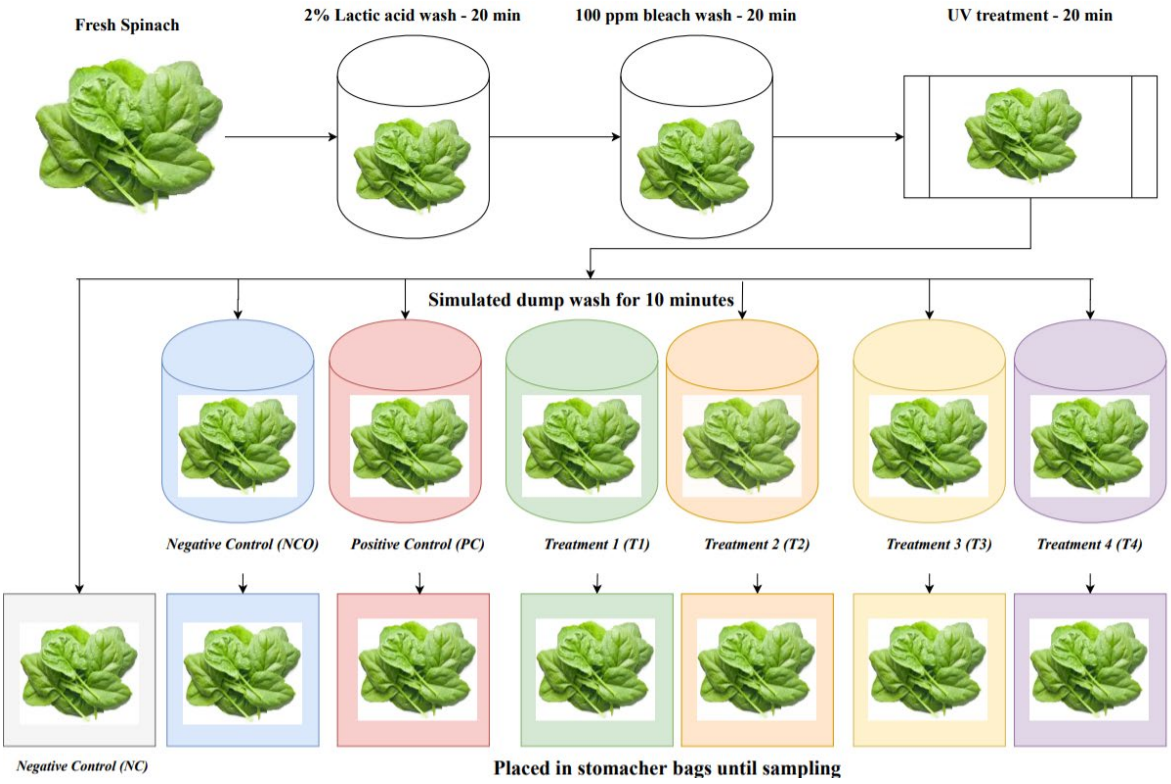


Figure 6.2 Schematic flow of initial produce rinse and dunk wash of spinach in high organic load water in combination with the sanitizers and bacteriophage cocktail. NC – No treatment, NCO – Leaves washed in high organic load water, PC - Leaves washed in organic water containing *E. coli* O157:H7 (ATCC 35150), T1 – Leaves washed in organic water containing *E. coli* O157: H7 (ATCC 35150) + volume of bleach to contribute 100-ppm, T2 - Leaves washed in organic water containing *E. coli* O157: H7 (ATCC 35150) + volume of SaniDate 5.0 to contribute 100-ppm, T3- Leaves washed in organic water containing *E. coli* O157: H7 (ATCC 35150) + volume of bleach to contribute 100-ppm and phage cocktail, T4 - Leaves washed in organic water containing *E. coli* O157: H7 (ATCC 35150) + volume of SaniDate 5.0 to contribute 100-ppm and phage cocktail

6.2.7 Recovery of bacteria

Leaves were rinsed with 1 ml sterile phosphate buffer. Samples were massaged for one minute and serial dilutions of the sample rinse were made in phosphate buffer (pH 7.4 - 7.5). The dilutions were then plated on pre-made TSA plates, supplemented with 5mM Magnesium sulfate ($MgSO_4$, Fisher Scientific) and 5mM Calcium chloride ($CaCl_2$, Fisher Scientific).

6.2.8 Statistical analysis

A linear mixed model was used to analyze the response of log value as a factor of the treatment group, the time (hours 3 and 6), the interaction of treatment and time, and the baseline of log value. Random effects for the subject and study were included in the model. Tukey's method for multiple comparisons was used to test the differences between average treatment means and treatment means at each hour. All analysis was conducted using PROC GLIMMIX in SAS 9.4.

6.3 Results

6.3.1 Effect of bacteriophage on sterile water wash solution containing *E. coli* O157:H7 inoculated spinach in a simulated dunk tank with 100-ppm bleach

The initial produce rinse successfully inhibited the growth of background flora on fresh spinach leaves. The plate count (<1.00 CFU/ml) on the NC indicated that the initial rinse was effective at reducing the background microflora. Table 6.1 shows the efficacy of 100-ppm bleach and 100-ppm bleach/bacteriophage cocktail combination in the reduction of *E. coli* O157:H7 on spinach compared to the control wash. The 10-minute contact time for the wash solution resulted in a significant reduction ($P < 0.01$) of the pathogen at the

end of 3 hours on both the treatments compared to the PC. The 100-ppm bleach treatment by itself contributed to 3.00 log CFU/ml (99.9%) reduction at the end of 3 hours and maintained it until the end of 6 hours despite some recovery. Since there was a parallel increase in the number of the PC at 6 hours, the gradual recovery of the pathogen numbers in T1 still reflected a 3.00 log CFU/ml at the end of 6 hours. In the case of leaves washed with the sanitizer bacteriophage combination, there was a 5.00 log CFU/ml (99.999%) reduction at the end of 3 hours and maintained it until the end of 6 hours. Similar to T1, the T3 reflected a gradual recovery, but a parallel increase to the PC maintained the 5.00 log CFU/ml reduction at the end of 6 hours. The statistical analysis indicated that, despite the recovery, the treatments were significantly different from the PC. Therefore, the disinfectant treatments (T1 and T3) were significantly effective ($P < 0.01$) in reducing the population of *E. coli* O157:H7 on the spinach leaves compared to PC (Figure 6.3 - A).

6.3.2 Effect of bacteriophage on sterile water wash solution containing *E. coli* O157:H7 inoculated spinach in a simulated dunk tank with 100-ppm SaniDate 5.0

The initial produce rinse successfully inhibited the growth of background flora on fresh spinach. The plate count (<1.00 CFU/ml) on the NC indicated that the initial rinse was effective at reducing the background microflora. Table 6.1 shows the efficacy of the bacteriophage cocktail in the reduction of *E. coli* O157:H7 on spinach washed in water containing 100-ppm SaniDate 5.0 along with the phage cocktail compared to the control wash. The 10-minute contact time for the wash solution resulted in a significant reduction ($P < 0.01$) of the pathogen at the end of 3 hours compared to the PC on both the treatments. Recovery of the pathogen was not observed in both the treatments at 0, 3, and 6 hours. The statistical analysis indicated that both treatments (T2 and T4) were significantly different

from the PC. Therefore, treatments T2 and T4 were significantly effective ($P < 0.01$) in reducing the population of *E. coli* O157:H7 on the spinach leaves (Figure 6.3 - B).

6.3.3 Effect of sterile wash solution containing 9810 ppm of organic load comprising *E. coli* O157:H7 and bacteriophage cocktail in a simulated dunk tank with 100-ppm bleach

The initial produce rinse was once again effective in reducing the background microflora of the spinach (<1.00CFU/ml). The secondary negative control (NCO) also had no recovery (<1.00CFU/ml) on the studies which indicated that the organic load did not influence the growth of any underlying microflora. Table 6.2 shows the efficacy of the bacteriophage cocktail in the reduction of *E. coli* O157:H7 on spinach washed in the challenge water (9810 ppm organic load) containing the phage cocktail compared with the control wash. The 10-minute contact time for the wash solution resulted in a significant reduction ($P < 0.01$) (99.99%) of the pathogen at the end of 3 and 6 hours. Compared to T1 and PC, the bacteriophage treatment (T3) resulted in 4.00 log CFU/ml and 5.00 log CFU/ml reduction and the end of 3 and 6 hours respectively. In the case of T1, the obtained data were not significantly different from PC and the pathogen had a similar growth pattern. This study illustrates the specificity of bacteriophage and its ability to effectively reduce *E. coli* O157:H7 despite being in an environment with a high organic load (Figure 6.3 - C).

6.3.4 Effect of sterile wash solution containing 9810 ppm of organic load comprising *E. coli* O157:H7 and bacteriophage cocktail in a simulated dunk tank with 100-ppm SaniDate 5.0

The initial produce rinse was once again effective in reducing the background microflora of the spinach (<1.00CFU/ml). Table 6.2 shows the efficacy of the bacteriophage cocktail in the reduction of *E. coli* O157:H7 on spinach washed in the

challenge water (9810 ppm organic load) containing the phage cocktail compared with the control wash. The 10-minute contact time for the wash solution resulted in a significant reduction ($P < 0.01$) (99.99%) of the pathogen at the end of 3 and 6 hours. Compared to T2 and PC, the bacteriophage treatment (T4) resulted in 3.00 log CFU/ml and 5.00 log CFU/ml reduction and the end of 3 and 6 hours respectively. In the case of T2, the obtained data were not significantly different from PC and the pathogen had a similar growth pattern. This study also illustrates the specificity of bacteriophage and its ability to effectively reduce *E. coli* O157:H7 despite being in an environment with a high organic load (Figure 6.3 – D).

Table 6.1 Reduction of *E. coli* O157:H7 (ATCC 35150) on spinach via postharvest pathogen control measures of using bacteriophage cocktail in combination with commercially used sanitizer wash solution made with potable water in a simulated dunk tank.

Wash treatment	Wash time (min)	Sampling time (h)	*E. coli* O157:H7 population (log CFU/ml)
Negative Control (NC)	-	0	<1.00
		3	<1.00
		6	<1.00
Positive Control (PC)	10	0	6.43
		3	6.62
		6	7.42
Treatment 1 - 100-ppm bleach water (T1)	10	0	3.24
		3	3.49
		6	4.04
Treatment 2 - 100-ppm SaniDate 5.0 water (T2)	10	0	<1.00
		3	<1.00
		6	<1.00
Treatment 3 - 100-ppm bleach water + bacteriophage cocktail (T3)	10	0	4.35
		3	1.11
		6	2.10
Treatment 4 - 100-ppm SaniDate 5.0 water + bacteriophage cocktail (T4)	10	0	<1.00
		3	<1.00
		6	<1.00

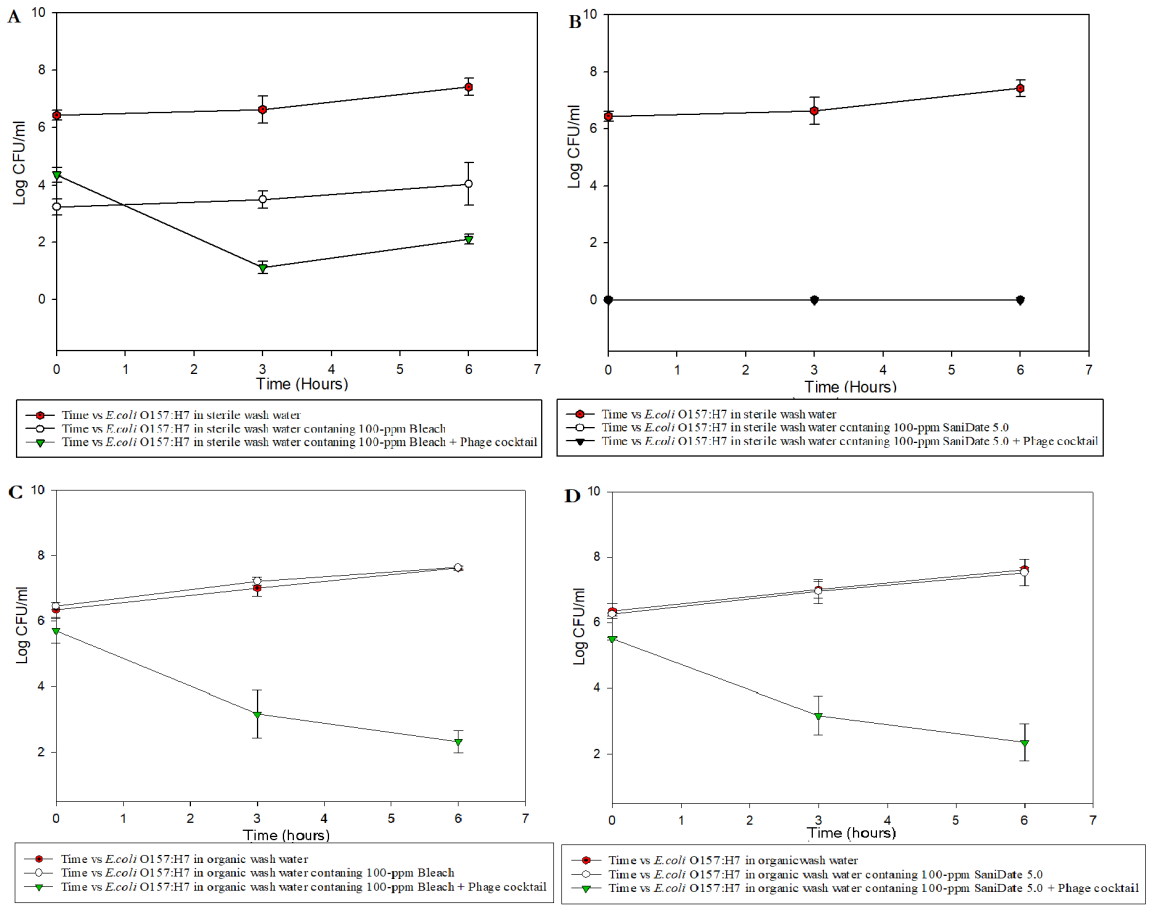


Figure 6.3 Reduction of *E. coli* O157:H7 contamination of spinach leaves treated with bacteriophage cocktail in combination with sanitizer. (A) 100-ppm bleach and 100-ppm bleach + bacteriophage cocktail in sterile wash water, (B) 100-ppm SaniDate 5.0 and 100-ppm SaniDate 5.0 + bacteriophage cocktail in sterile wash water, (C) 100-ppm bleach and 100-ppm bleach + bacteriophage cocktail in high organic wash water, (D) 100-ppm SaniDate 5.0 and 100-ppm SaniDate 5.0 + bacteriophage cocktail in high organic wash water

Table 6.2 Reduction of *E. coli* O157:H7 (ATCC 35150) on spinach via postharvest pathogen control measures of using bacteriophage cocktail in combination with commercially used sanitizer wash solution made with high organic load water in a simulated dunk tank.

Wash treatment	Wash time (min)	Sampling time (h)	*E. coli* O157:H7 population (log CFU/ml)
Negative Control (NC)	-	0	<1.00
		3	<1.00
		6	<1.00
Negative Control Organic (NCO)	10	0	<1.00
		3	<1.00
		6	<1.00
Positive Control (PC)	10	0	6.35
		3	7.00
		6	7.62
Treatment 1 - 100-ppm bleach in organic water (T1)	10	0	6.46
		3	7.20
		6	7.63
Treatment 2 - 100-ppm SaniDate 5.0 in organic water (T2)	10	0	6.25
		3	6.94
		6	7.52
Treatment 3 - 100-ppm bleach in organic water + bacteriophage cocktail (T3)	10	0	5.70
		3	3.17
		6	2.32

Table 6.2 (Continued).

Wash treatment	Wash time (min)	Sampling time (h)	*E. coli* O157:H7 population (log CFU/ml)
Treatment 4 - 100-ppm SaniDate 5.0 in organic water + bacteriophage cocktail (T4)	10	0	5.50
		3	3.17
		6	2.35

6.4 Discussion

Increased microbial contamination in vegetables has led to several foodborne outbreaks which have created a growing concern for producers, consumers, and public health organizations with regards to the safety of the products that are being produced. Water is considered as one of the major routes through which pathogens can cross-contaminate produce. Hence, treating wash water with sanitizer is necessary to prevent the accumulation of pathogens during produce wash. Proper sanitation, especially during post-harvest washing of produce, in a recirculated wash water system, such as dunk tanks, is crucial for producing safe food for consumers (Sargent et al., 2000). Chlorine-based sanitizers are deemed as one of the most commonly used sanitizers in the fresh produce industry (Chen and Hung, 2017). Although bleach is relatively inexpensive and can eliminate a broad range of microorganisms, it is also considered highly corrosive and has a greater affinity to bind with available organic load (Sargent et al., 2000). Consequently, maintaining an adequate concentration of free chlorine in produce wash water, especially in the presence of high organic load, is a great challenge for the produce industry (Chen and Hung, 2017). The recommended concentration for chlorine-based compounds used for rinsing produce is between the range of 50 - 100 ppm free chlorine (World Health

Organization, 2008). Akbas and Olmez (2007), studied the effect of chlorine solution on reducing the population *E. coli* and *L. monocytogens* on contaminated lettuce (Akbas and Ölmez, 2007). The lettuce samples were dipped in 100 mg/L of free chlorine water for 2 and 5 minutes. The results indicated that the treatment resulted in 1.0 and 2.0 log CFU/g reduction of the population of *L. monocytogens* and *E. coli* respectively. Chen and Hung (2017), studied the effect of organic load on the chlorine demand for fresh produce wash water system using romaine lettuce. The team studied the chlorine demand on wash water with different organic loads, pH, and concentrations of chlorine. The results indicated that chlorine demand significantly increased with an increase in organic load (Chen and Hung, 2017). Additionally, various studies have also supported the presence of organic matter reduces the efficacy of any chlorine-based sanitizers (Park et al., 2009). Similar to these results, bleach and SaniDate 5.0 were both capable of reducing the population of *E. coli* O157:H7. The 100-ppm bleach treatment in sterile wash water gave a 3.13 and 3.38 log CFU/ml reduction of the pathogen at 3, and 6 hours on the spinach leaves. In contrast, the 100-ppm bleach and phage cocktail in sterile wash water gave a 5.51 and 5.32 log CFU/ml reduction of the pathogen at 3 and 6 hours respectively. The phage-bleach combination achieved a 2.38 log CFU/ml more on the reduction of the pathogen compared to bleach treatment alone at the end of 3 hours. The 100-ppm SaniDate 5.0 in sterile wash water, both in the presence or absence of the phage cocktail, led to an undetectable amount on the pathogen. However, once the organic load was introduced into the wash water, both the sanitizers were severely limited in reducing the pathogen. The 100-ppm bleach and 100-SaniDate 5.0 in organic water had an extremely restricted effect on the growth of the pathogen. Despite the presence of a high organic load, the 100-ppm bleach - phage cocktail

treatment gave a 3.83 and 5.30 log CFU/ml reduction of the pathogen at 3 and 6 hours respectively and the 100-ppm SaniDate 5.0-phage cocktail treatment gave a 3.83 and 5.27 log CFU/ml reduction at the end of 3 and 6 hours respectively. This corresponds to a 4.03 and 5.31 log CFU/ml more reduction on the bleach-phage combination treatment and 3.77 and 5.17 log CFU/ml more reduction on the SaniDate 5.0-phage combination treatment. Despite the presence or absence of the sanitizer, the phage cocktail demonstrated a consistent reduction (99.99%) of *E. coli* O157:H7 at 3 and 6 hours. This study demonstrated the phage's ability to selectively eliminating the contamination despite being subjected to a complex wash solution.

Survival of the phage cocktail in the presence of sanitizers might open new avenues of using phage-sanitizer combination as an effective method in eliminating select pathogens in the food industry. The emergence of phage-resistant bacterial mutants, transduction of undesirable characteristics among bacteria, and environmental conditions are potential problems that can reduce the effectiveness of phage treatment. The discovery of new phage and rotational phage application might help to prevent the formation of any phage-resistant mutants. Phages are one of the most abundant microorganisms with an estimated range > 10^{30} particles found in our biosphere (Brüssow and Hendrix, 2002). Phages are also found in food and water that are commonly consumed by humans (Abuladze et al., 2008). For instance, phages have been isolated from a variety of food products such as pork sausage, poultry, ground beef, freshwater fish, marine fish, oysters, cheese, and raw skim milk (Whitman and Marshall, 1971; Kennedy et al., 1984; Kennedy et al., 1986; Gautier et al., 1995; Atterbury et al., 2003a). Therefore, the technique of using phage to reduce contamination on food products might be one of the most natural ways of

eliminating specific pathogens. Apart from its application on food products, phages can also be used selectively for eliminating spoilage microorganisms, cleaning food and non-food contact surfaces, and equipment naturally, or in combination with sanitizers. The data presented in this report suggest that a phage-based approach might help prevent disease caused by foodborne bacteria, such as *E. coli* O157:H7. Additionally, the study supports their ability to reduce the pathogen in the presence of a high organic load; thus, ultimately lowering the possibility of bacterial related foodborne outbreaks in the produce industry.

CHAPTER 7. CONCLUSION

The incidence of foodborne outbreaks involving fresh fruits and vegetables is a concern for consumers worldwide. Developing a novel technique and frequently updating sanitation methods are necessary for not only controlling pathogens, but also to prevent the occurrence of foodborne outbreaks. The microplate technique used in this study helped in analyzing the effect of the phage cocktail on *E. coli* O157:H7 that resulted in a 4 log CFU/ml (99.99%) reduction of the pathogen for up to 9 hours. The ability of the phage cocktail to survive 100-ppm bleach and 100-ppm SaniDate 5.0, opened new avenues for testing the sanitizer treated phage cocktail on *E. coli* O157:H7. The results indicated the efficacy of the phage cocktail to eliminate the pathogen was significantly different despite being subjected to a 100-ppm concentration of the sanitizers. The temperature study proved that phages were capable of withstanding a temperature of 45°C and 55°C for 0-15 minutes. The ability to survive these heat treatments demonstrates that the phages could be added to a produce wash, or used in combination with mild heat treatment, and still retain their ability to infect and reduce the population of the pathogen. The produce wash study proved their effectiveness in reducing the contamination both in the presence and absence of sanitizer, even when subjected to a complex wash system containing a high organic load. Results from these studies indicate that bacteriophages can be effectively used in reducing *E. coli* O157:H7 contamination on fresh produce that is exclusively washed in dunk tanks. It is possible that with further optimization of the dosage, delivery mechanism, and formulation, the effectiveness of phage can be further improved in specifically reducing *E. coli* O157:H7 contamination on fresh produce. The ability of phages to selectively infect bacteria can be utilized to formulate cocktails which can then be selectively used against

pathogens or spoilage microorganisms depending on the type of food products. A bacteriophage biocontrol strategy would be an acceptable technique and a natural alternative to food safety and preservation.

APPENDICES

Figure A1. Spot assay of bacteriophages $C14_s$, V9, L1, and LL15 against *E. coli* O157:H7

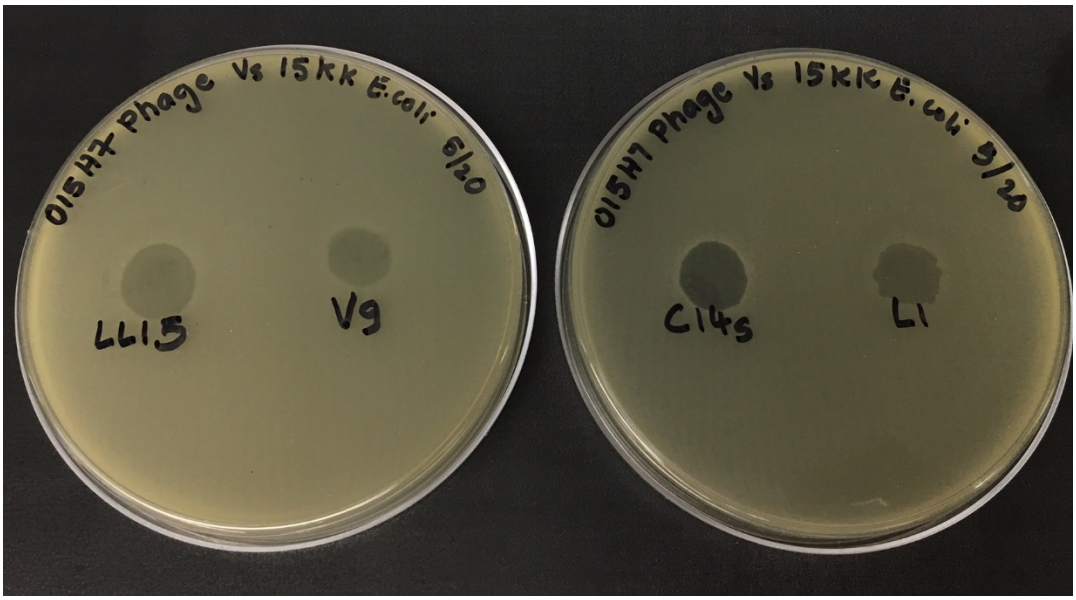


Table A1. Plaque forming units (PFU) of bacteriophages $C14_s$, V9, L1, and LL15 against *E. coli* O157:H7

Phage	Countable dilution range	Plaque Forming Unit*
$C14_s$	10^5	7.00 x 10^6 PFU/ml
V9	10^5	2.20 x 10^7 PFU/ml
L1	10^8	1.20 x 10^{10} PFU/ml
LL15	10^6	1.48 x 10^9 PFU/ml

* PFU Calculation : Example - Plaques formed = 148, Dilution factor = 10^6, Inoculum volume = 0.1 ml (or 100 µl)

Titer = Plaque formed x Dilution factor / inoculum volume

= 1.48 x 10^9 PFU/ml

Table A2. Phage Score based on plaque appearance

Phage	Scores
$C14_s$	4
V9	3
L1	4
LL15	4

Figure A2. Microplate growth inhibition assay showing the activity of bacteriophage $C14_s$ against *E. coli* O157:H7 (ATCC 35150)

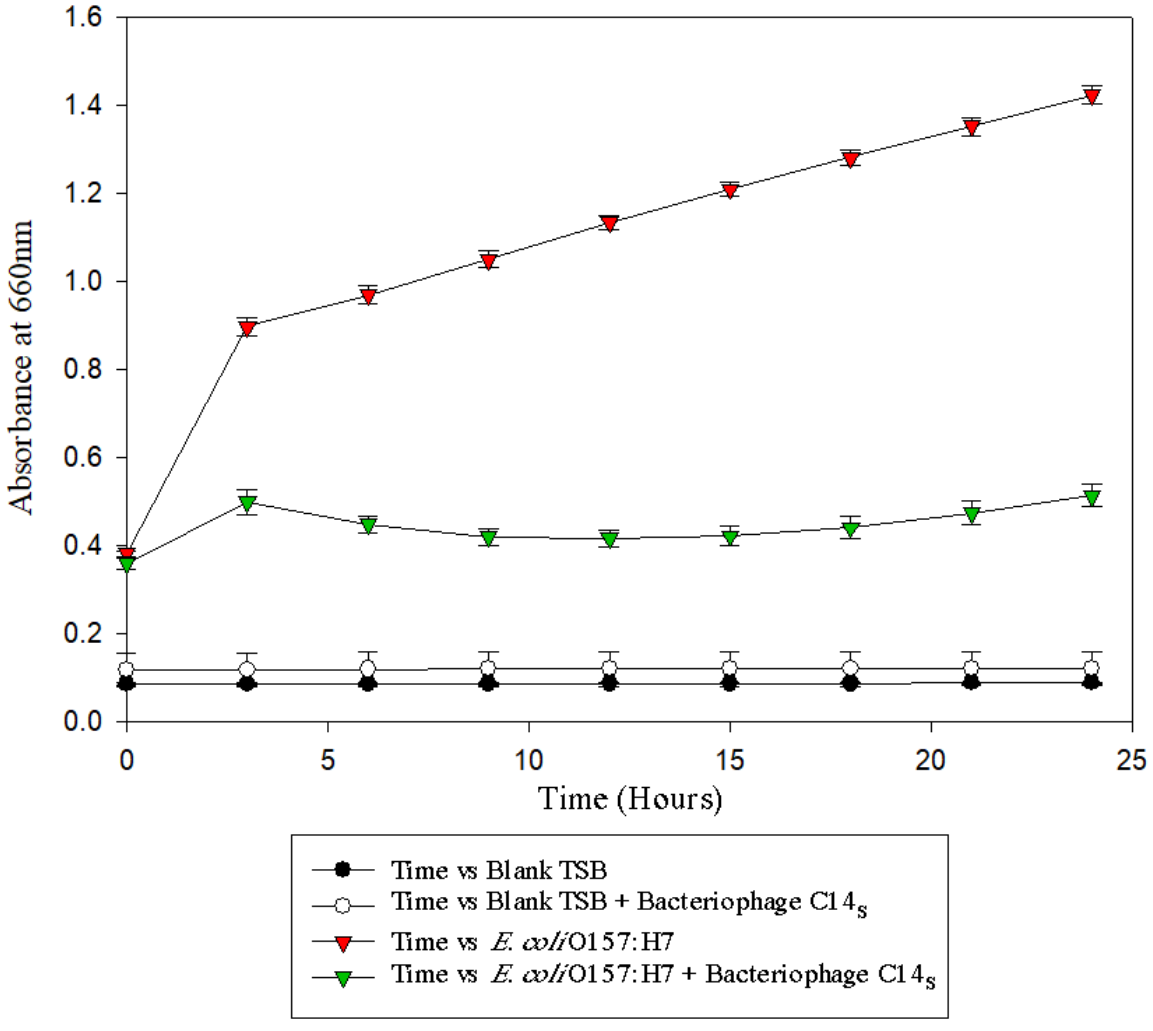


Figure A3. Microplate growth inhibition assay showing the activity of bacteriophage V9 against *E. coli* O157:H7 (ATCC 35150)

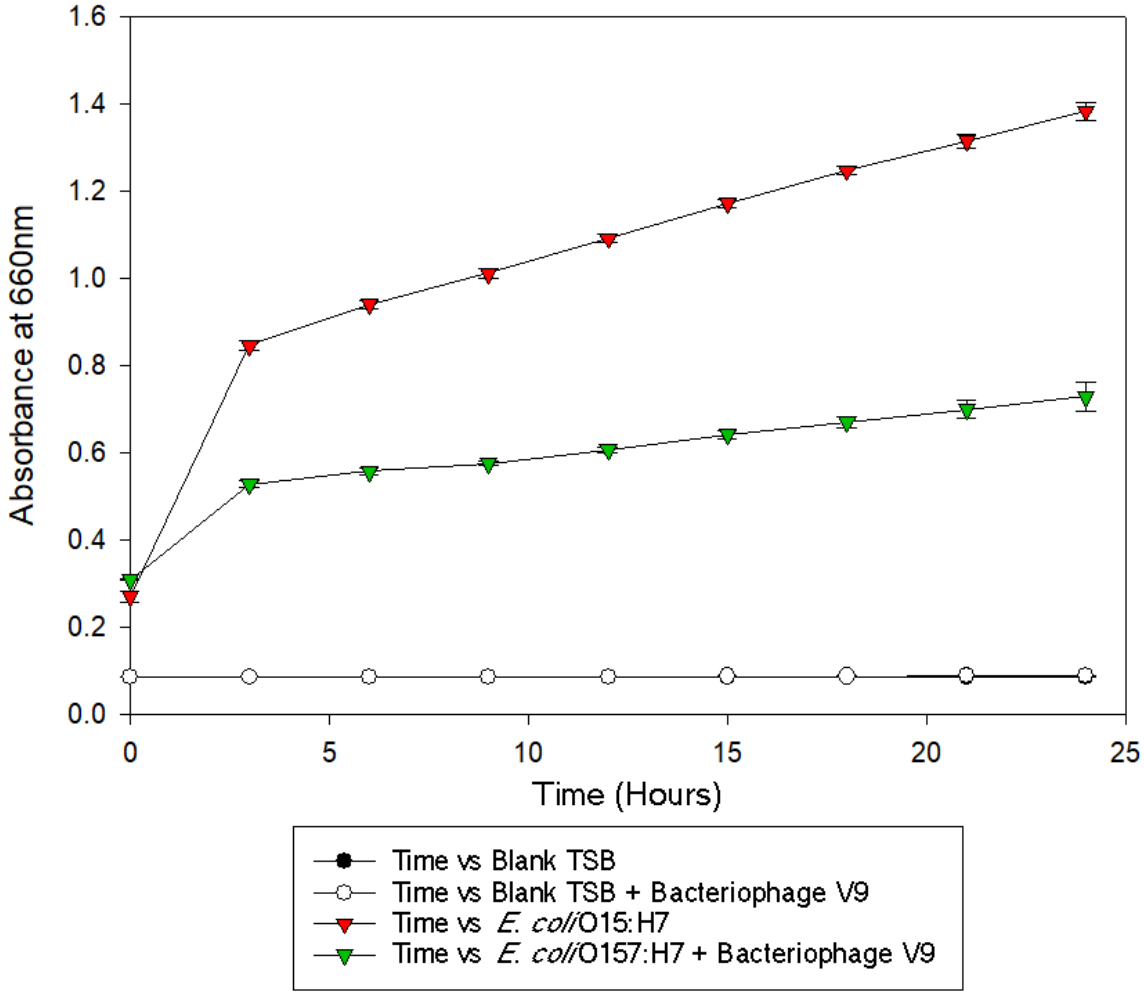


Figure A4. Microplate growth inhibition assay showing the activity of bacteriophage L1 against *E. coli* O157:H7 (ATCC 35150)

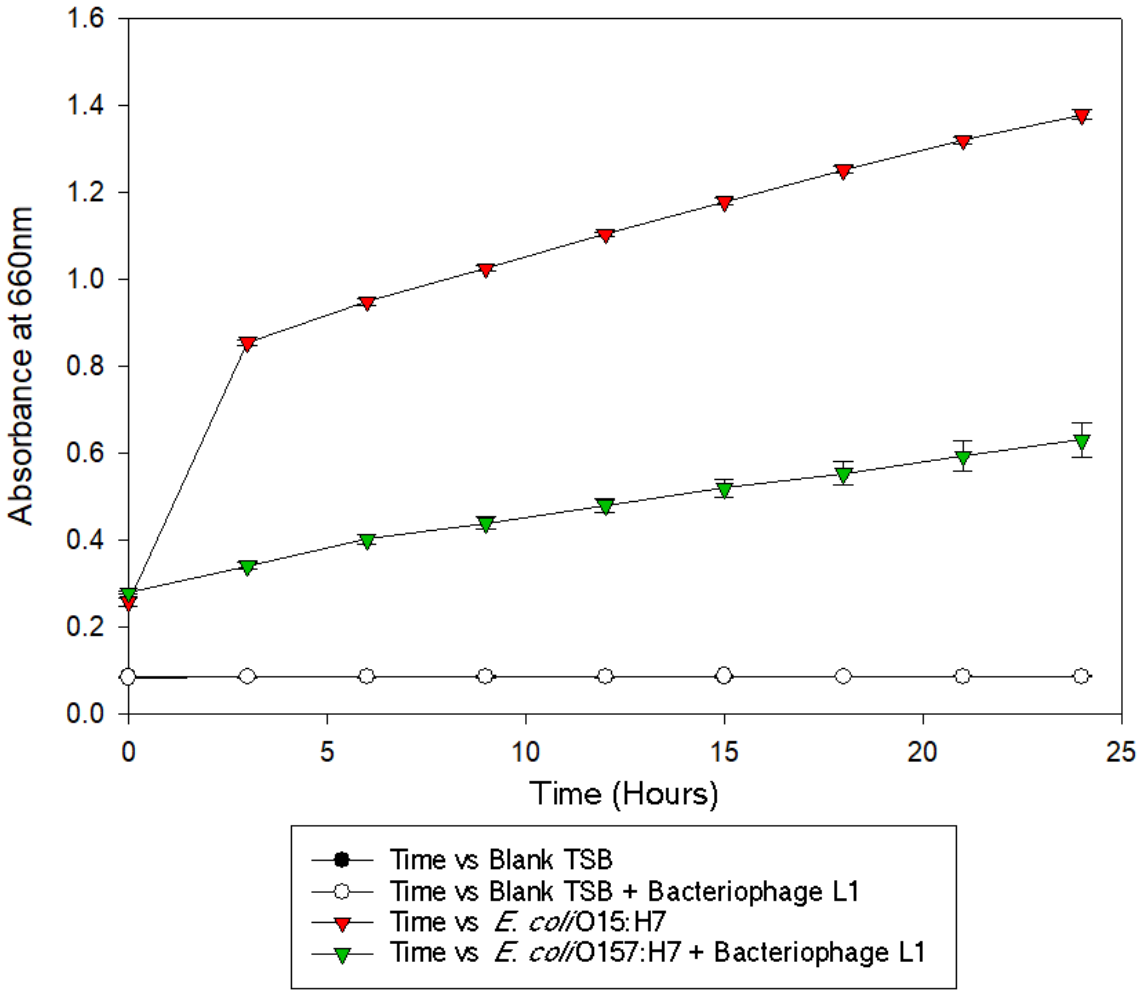


Figure A5. Microplate growth inhibition assay showing the activity of bacteriophage LL15 against *E. coli* O157:H7 (ATCC 35150)

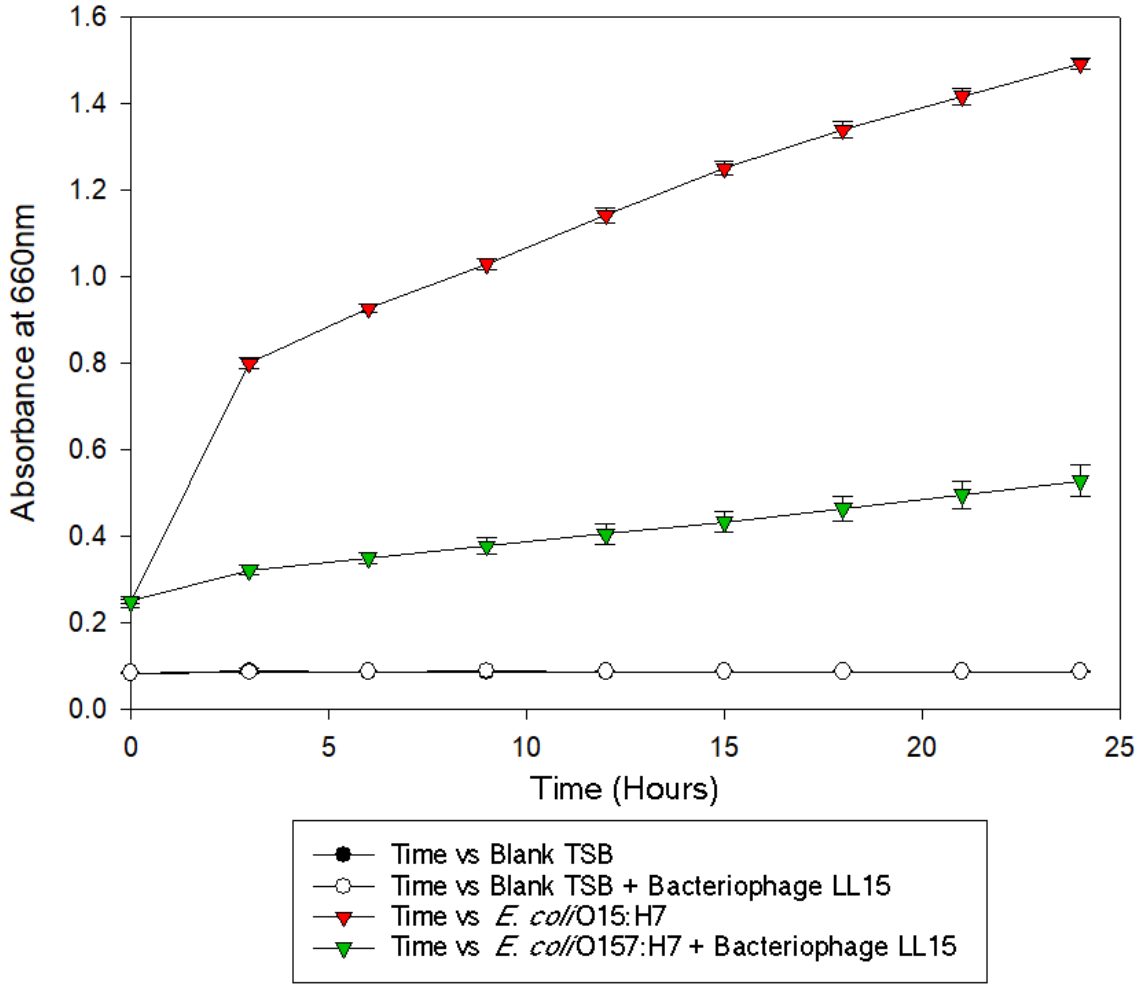


Figure A6. Spot assay of 100-ppm bleach treated phage at 0, 1, 2, 3 hours

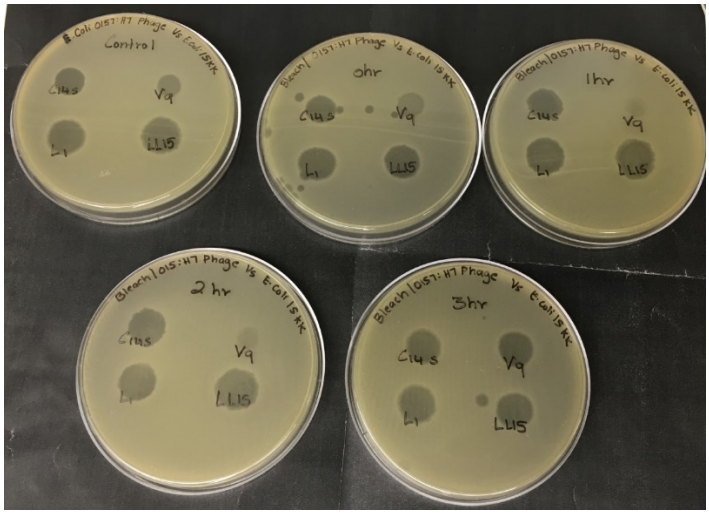


Figure A7. Spot assay of 100-ppm SaniDate 5.0 treated phage at 0, 1, 2, 3 hours

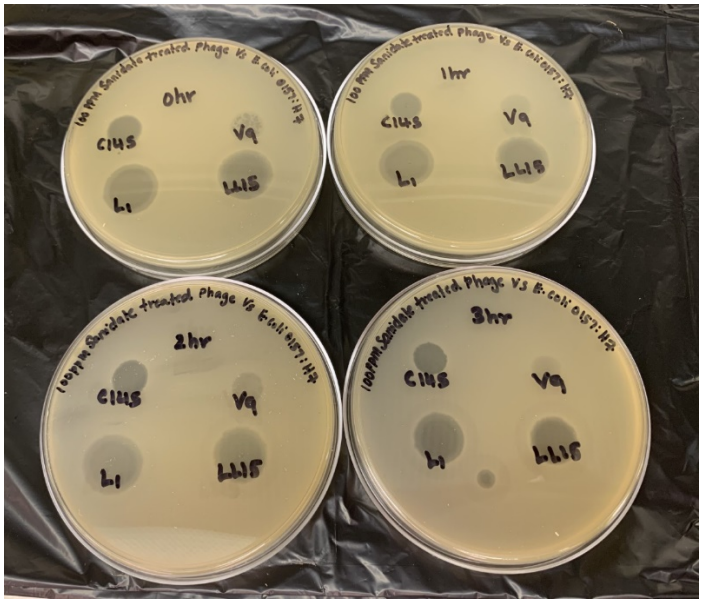


Figure A8. Spot assay of 100-ppm bleach treated phage cocktail at 0 hour

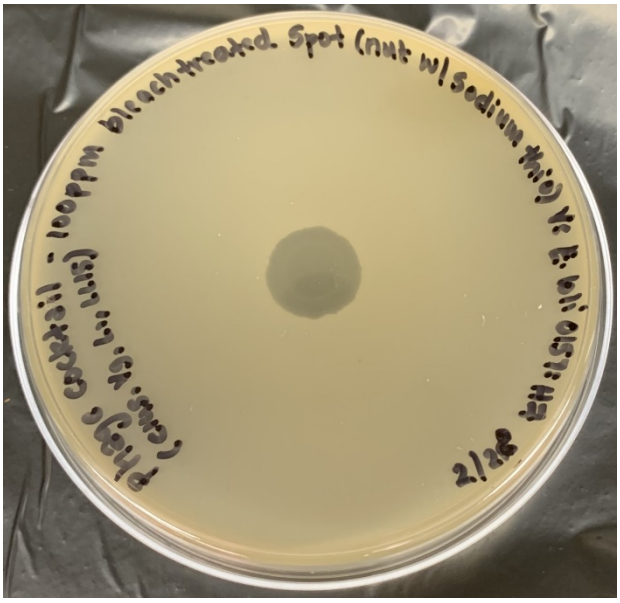
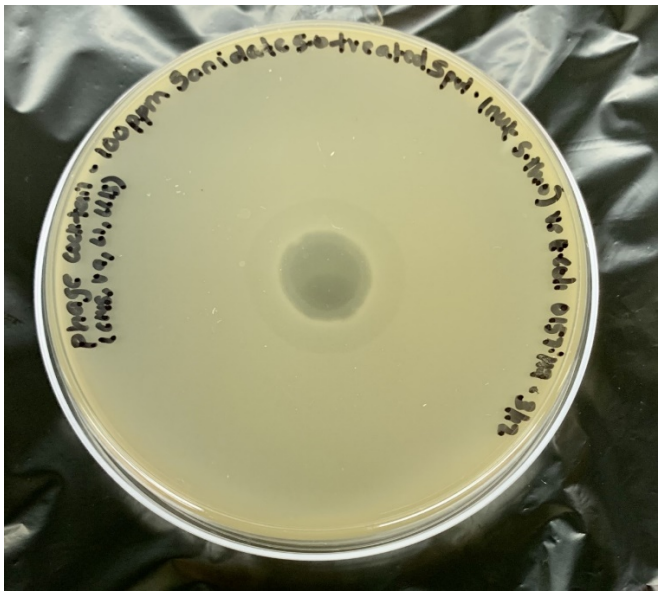


Figure A9. Spot assay of 100-ppm SaniDate 5.0 treated phage cocktail at 0 hour



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