

**ISOLATION AND CHARACTERIZATION OF  
BACTERIOPHAGE FOR THE CONTROL OF  
ENTEROHEMORRHAGIC *Escherichia coli* ON FRESH  
PRODUCE**

**THESIS**

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By

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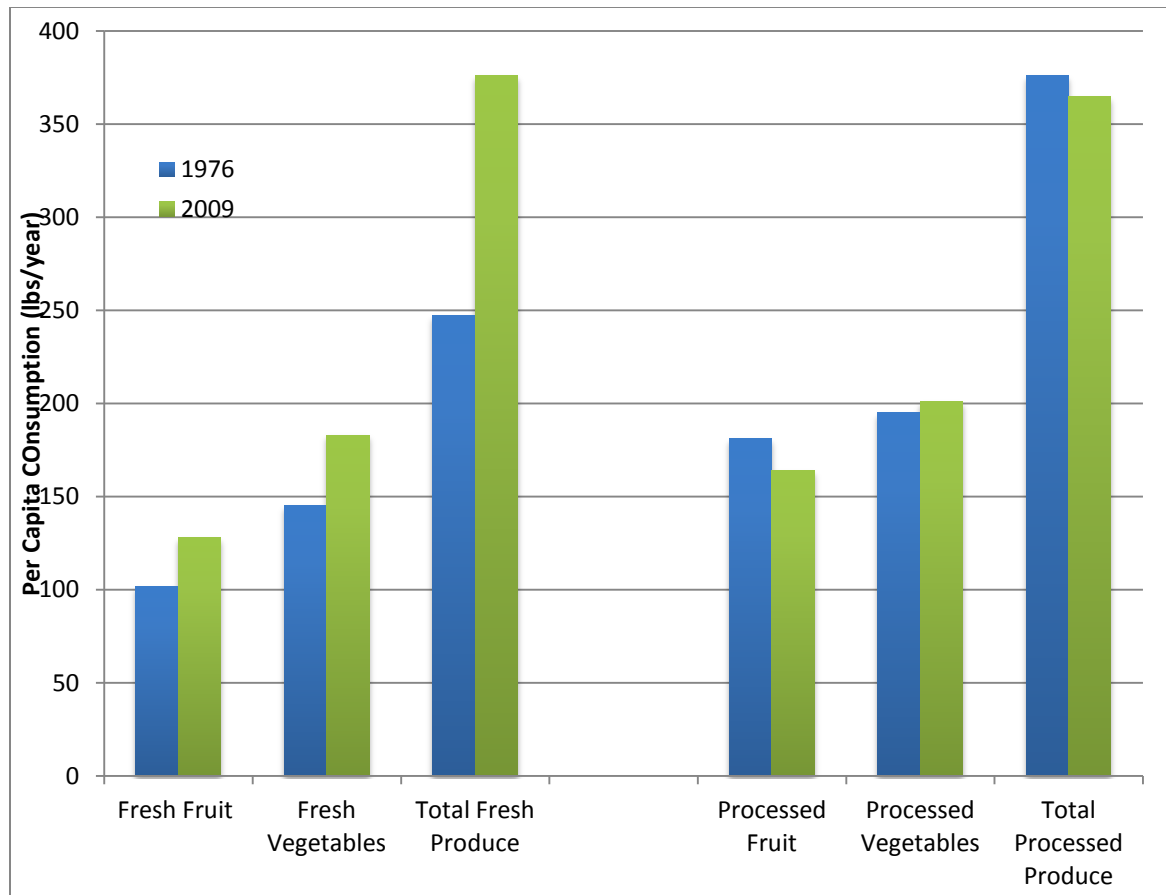
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# CHAPTER 1

## 1.1 Introduction

### Fresh produce safety

American consumption of fresh produce has increased over the past decade and continues to grow as public interest in weight maintenance, functional foods, and minimally processed products has increased (Figure 1.1). While fresh food products have gained popularity, they also represent a significant food safety challenge. Raw fruit and vegetable products have become some of the most heavily scrutinized foods due to recent outbreaks associated with minimally processed produce. The increase in outbreaks associated with these products may be attributed to both the recent increase in consumption and the challenge of ensuring safety in fresh products often served raw.



**Figure 1.1:** Change in American consumption of fresh and processed fruits and vegetables from 1976 to 2009.

Data Adapted from: Cook, Roberta. 2011. Demographics and U.S. Fruit and Vegetable Consumption Patterns

In total, there are an estimated 48 million cases of foodborne illness that occur every year, effecting almost 50 million, or one in six Americans. The Center for Disease Control depends on estimations for these values since many cases are not treated or reported to doctors, and instead handled by the individual at home. Subsequently, these cases are not reported to the CDC for quantification. Of illnesses that do reach the attention of doctors, still some are not diagnosed and attributed to the particular pathogen due to limited availability of the appropriate assay (CDC, 2011). Many cases of foodborne illness, however, are very serious and require medical attention. Annually, 325,000 hospitalizations are attributed to foodborne disease and approximately 5,000 deaths occur each year in the United States as a result of foodborne illness

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(Mead and others, 1999). The Produce Safety Project of 2011 estimates the total cost of foodborne illness at 152 million dollars a year. While this figure represents the collective cost for all foodborne illnesses, a major portion of those are related to fresh produce. About a third of major outbreaks of foodborne disease occurring in 2011 were associated with contaminated fresh produce (Scharff, 2011).

In response to the growing challenge of maintaining food safety in regards to fresh produce, the Food Safety Modernization Act (FSMA) was passed two years ago and contains updated rules for regulating the food supply in order to reduce the spread of contaminated food products to large numbers of people and help prevent outbreaks from even occurring (Kuo, 2011). In response to the FSMA, the Food and Drug Administration (FDA) has announced the development of updated rules for Good Agricultural Practices (GAP) relating to the production of fruits and vegetables (USFDA, 2009). The updated GAP rule is expected to be published sometime in 2012 and is to include guidance on best practices for the production of leafy greens, tomatoes, and melons (FDA, 2012). Fresh produce associated with recent outbreaks has been the target of a lot of the new rules and technologies designed to protect food safety. And, as the demand for fresh produce in the market place continues to grow, the need for practices to ensure the control of food borne pathogens becomes even more relevant.

### **Relevant outbreaks**

Particular kinds of produce are associated with the safety of fresh products because they have been the vehicle for major outbreaks of foodborne disease. Additionally, particular pathogens are of greater concern for food safety both because they have been associated with major outbreaks and because they pose a greater health risk *Escherichia coli* O157:H7 is one of the top five pathogens which contribute to foodborne illness that results in hospitalization in the United

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States (Mead and others, 1999). Of all Shiga toxin producing *E. coli* strains (STEC), the most common one associated with foodborne disease is *E. coli* O157:H7 which is responsible for about a third of all illnesses resulting from pathogenic *E. coli*. While not all illnesses are reported, the CDC estimates that there are upwards of 256,000 cases of illness resulting from STEC contamination of food and water every year in the United States (Division of Foodborne, Bacterial, and Mycotic Diseases, National Center for Zoonotic, Vector-Borne, and Enteric Diseases, 2011).

Leafy green vegetables are some of the most notorious products associated with outbreaks of foodborne pathogens such as *E. coli* O157:H7 (Figure 1.2). They are not only difficult to sanitize or treat to eliminate contaminants; they are also most often consumed raw which makes the risk of foodborne disease resulting from potential contamination all the greater. One of the larger, more recent outbreaks associated with leafy greens is the 2006 outbreak of *E. coli* O157:H7 on baby spinach leaves produced in the Southwestern United States. In September of 2006, doctors in several different states reported cases of *E. coli* O157H7 to the CDC. Eventually, 26 states would be involved in the outbreaks that resulted in 205 cases of foodborne disease, some cases including Hemolytic Uremic Syndrome (HUS). The source was eventually traced to fresh, bagged spinach and by September 14, 2006 the CDC and FDA advised against the consumption of bagged spinach (Grant and others, 2008). This outbreak was only one of 26 outbreaks associated with leafy greens since 1993. While the cost of diagnostics and treatments for these outbreaks were themselves considerable, the long-term effects on consumer confidence in the safety of the food industry are also substantial.

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**Table 1.2:** Recent outbreaks of enterohemorrhagic *Escherichia coli* in leafy greens  
Data collected by the Center for Disease Control

Year	Leafy Green	<i>E. coli</i> Strain	Reported Cases	Effects
2006	Baby spinach	O157:H7	205	3 deaths
2006	Shredded lettuce	O157:H7	71	53 hospitalization
2006	Lettuce	O157:H7	81	26 hospitalization
2010	Shredded lettuce	O145	30	12 hospitalization
2011	Lettuce	O157:H7	60	30 hospitalization

Other fresh produce associated with outbreaks or of food safety concern to manufacturers include sprouts, green onions, melons, tomatoes and peppers. These products have been associated with major outbreaks of *E. coli*, *Salmonella*, and *Listeria*. In the summer of 2008 an outbreak of *Salmonella* gained public attention as over 1,400 cases were reported across North America and resulted in 282 hospitalizations and 2 deaths. The foodborne illness was originally linked to the consumption of contaminated tomatoes. As a testament to the effects of lapses in food safety on public perception and confidence in the safety of food products, tomato consumption drastically decreased costing the industry and estimated 200 million dollars. Later, the source of the outbreaks was identified as jalapeno and serrano peppers imported from Mexico (Maki, 2009). The original confusion was attributed to the frequent consumption of tomatoes and peppers together. The numerous outbreaks associated with fresh produce that have occurred with increasing frequency over the past two decades suggest a need for novel food safety technologies within the unprocessed fruits and vegetables sector of the industry.

## **Processing of leafy greens and peppers**

Currently, fresh produce undergoes minimal treatments after it is harvested before it reaches the consumer. Once harvested, produce is cooled to remove field heat which slows enzymatic reactions and microbial growth. This may be achieved through the use of vacuum cooling, as is often the case with leafy greens, or using cold water or air. Leafy greens may be further processed, undergoing cutting or shredding to become a “ready to eat” product for the consumer (Kadar, 2002). Both leafy green vegetables and vegetables such as peppers and tomatoes, which are actually the fruit of the plant, are often treated with a washing step in a dip tank or conveyor. This aids in the removal of soil and other debris as well as reducing microbial loads (Barringer, 2012; Kadar, 2002). The water used in this washing portion is likely to contain chlorine or a commercial antimicrobial product in addition to a surfactant. The surfactant helps remove solid dirt and debris from the surface of the product while the antimicrobial agents prevent cross-contamination and reduce microbial load (Barringer, 2012). The development of novel antimicrobials applicable to fresh produce is often targeted towards the wash treatment step of processing. Water soluble agents can easily be included in the wash water, a step which is already in place within the industry.

Following the washing step, excess water is removed so that dry product is packaged and sent to distributors. Unlike leafy greens, produce which has a waxy surface like peppers, tomatoes, and apples, are not as disposed to contamination because of the barrier between the environment, where potential contamination could exist, and the nutrient-rich interior of the fruit (Barringer, 2012). And, because of the waxy surface of these products, a rinse with water alone has a much greater effect on eliminating contaminants. However, when these products are sold as pre-cut or “ready to eat” foods, concerns about contamination are more relevant. Pre-cut fruits and vegetables represent the majority of non-leafy green related cases of foodborne illness and,

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given the popularity of cut, pre-prepared salad products, they may represent a significant portion of the total produce related foodborne diseases (Jaykus, 2011; Guenther and others, 2009; Leverentz and others, 2003). These products are susceptible to contamination for several reasons. For cut peppers, the additional processing is often done by hand at the grocer where no additional food safety treatments are applied following processing. Furthermore, any additional processing increases the probability of introducing a contaminant into the food. The cut surface of produce is a much more hospitable environment for microorganisms, containing moisture and nutrients necessary for growth while allowing for the internalization of pathogens which inhibits the effects of many antimicrobial agents (Erickson, 2012).

### **Current intervention strategies for fresh produce**

Current strategies in place to control for foodborne pathogens are mostly limited to antimicrobial agents within the water used during processing prior to packaging of the produce. Although new technologies are being developed and assessed for potential use within the industry and include novel technologies such as ozone treatment, high pressure processing, and UV light, they are not currently in place for treating the majority of fresh produce in the U.S. However, produce may be treated with a washing step that reduces the microbial load through the action of the physical washing. Antimicrobial agents may also be included in the rinse water to reduce the risk of cross-contamination and further reduce microbial load (Barringer, 2012). Common antimicrobial agents include chlorine or commercial products which include a surfactant to help remove debris. This may reduce microbial load by 1 to 2 log (Kreske and others, 2006; Barringer, 2012).

Novel technologies have been explored as potential additions to the processing strategy for fresh produce. Since fresh produce requires non-destructive processing, this limits the

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potential intervention strategies. Exposure to ozone is one such technology that may successfully inactivate pathogens while limiting damage to the food product. Ozone inactivates bacteria by damaging the cell wall, nucleic acids, and enzymes (Khadre and Yousef, 2001). One limitation to ozone treatments, however, is the potential to cause color change in produce products. Excessive exposure to ozone may lead to browning or decolorization of produce, in particular leafy greens such as ozone reacts preferentially with the phenolic compounds. Garcia et al found through sensory analysis that the majority of participants were still willing to purchase leafy greens treated with ozone, although the percentage of consumers who considered the produce visually accepting declined over storage (Garcia and others, 2003). In shredded lettuce and baby carrot vehicles, *E. coli* O157:H7 was reduced by 3 log (Singh and others, 2002). Other limitations of ozone technology include its application as a batch process, slowing continuous production, and the expense.

High pressure processing (HPP) is another emerging technology with potential for application in products that require non-thermal processing. HPP involves the application of pressures  $> 100$  MPa equally to all sides of a food product to inactivate microorganisms including vegetative bacteria, bacterial spores, fungi, viruses, and protozoa. While minimal color loss has been observed with HPP, the treatment can cause textural changes depending on the food product. Model systems like orange juice have been used to assess the potential of HPP treatment to inactivate *E. coli* O157:H7. A 5 min application of pressure up to 500 MPa inactivated 3-4 log of the target (Linton and others, 1999). Liquid products like juices are not subject to the same textural changes as solid food products, making them more ideal for HPP. Similarly, certain food products are better suited to resist quality changes resulting from HPP treatment. Produce and other products that contain fewer air pockets generally maintain better

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structural quality following treatment. As an example, carrots are better suited than strawberries for HPP treatment (Considine and others, 2008). As with ozone treatments, HPP is also limited by its expense in application and its addition of a batch process step in manufacturing.

Perhaps the emerging technology with the least detriment to product quality is UV treatment of fresh produce. UV light kills bacteria on the surface of fresh produce by damaging DNA and preventing accurate replication. However, UV light is only effective against the pathogens on the surface of produce so bacteria that has been internalized or has attached to a protected surface of the produce will not be inactivated by UV. Advantages to UV treatment include the clean label resulting from treatment and relatively simple addition of UV to processing lines (Yousef and Marth, 1988). *E. coli* O157:H7 inoculated on apples, tomatoes, and leaf lettuce showed varying sensitivities to UV treatment. *E. coli* on tomatoes and apples was reduced by ~2.5 log while no significant reduction was observed for *E. coli* on lettuce (Yuan and others, 2004). While UV offers promise for treating surface pathogens, it appears not to be the total solution to food safety challenges in fresh produce.

Non-thermal processing options applicable to fresh produce are limited by cost and effectiveness. Other strategies available for promoting the safety of fresh produce include the use of different antimicrobial agents such as bacteriophage. Bacteriophages (phages), viruses which infect and kill bacteria, have been considered as agents for biocontrol of foodborne pathogens. Phages may be applied in the wash water or applied as a spray and are a potential alternative to conventional processing techniques.

### **Phage application in produce**

Bacteriophage may be particularly well suited to application in fresh produce because of its non-destructive nature. Phages are highly specific to their host, so the treatments can be

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targeted to particular pathogens. Recent work on phage biocontrol of foodborne pathogens have found that inactivation of the target can be achieved up to several logs. Because of the high specificity, many applications of phages for biocontrol of pathogens in produce use a cocktail of phages to safeguard against contamination of a range of particular pathogens. Additionally, synergistic activity has been observed between different phages when applied for pathogen control, another advantage to utilizing several phage strains for food safety. Since phage as so common in the environment, isolation of several different phages active against the target are not difficult to find (Summers, 2004). In fact, even commercial products like Intralytix market cocktails of phages. Synergistic activity has also been observed between phages and bacteriocins, suggesting they might be important components of a hurdle strategy for controlling food pathogens. Additionally, phage and bacteriocins are both produced or found “naturally” and might be appealing alternatives to conventional processing or additives in the eyes of consumers.

Phage cocktails are typically designed to control for one specific target pathogen. That is, either *E. coli*, *Salmonella*, or *Listeria* would be the target for all the phages in the cocktail as opposed to a cocktail with a single phage for each of the different foodborne pathogens. This means that phage treatments target a specific host and would not inactivate normal biota, spoilage or other pathogenic bacteria, or other microorganisms.

A few examples of phage biocontrol of foodborne pathogens include a 2003 study which tested the efficacy of a phage cocktail to control for *Listeria* on pre-cut fresh produce (Leverentz and others, 2003). Cut surfaces of honeydew melon and apple were used as the vehicle for the host and were spot inoculated with the host then treated with a spray-type application of phage lysate. The phage cocktails used in this study came from Intralytix and contained either 6 or 14 different strains of phage. Produce samples were held for seven days, but as with other studies,

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time was not a significant factor when samples were stored at refrigeration conditions. *Listeria* populations on honeydew melon were reduced by ~2 to 4.5 log compared to the control. However, no significant reduction was achieved on the apple slices. This may be a result of the acidity of the apple slices compared to honeydew melon. Acid is known to detrimentally impact phage activity, and may have been the causative agent in this study. It should be noted that although the spray-type application of the phage lysate is described as a kind of “run-off” (that is, one without major physical force), the reductions were determined compared to an untreated control. This might imply that the reductions are a result of both the physical action of spray treatment as well as the lytic activity of the phage.

Another example of *Listeria* control is a study testing phage cocktails on pre-cut leafy greens (Guenther and others, 2009). Samples of cabbage and lettuce were inoculated with *Listeria monocytogenes* and treated with cocktail of three phages. The study was conducted over the course of six days during which samples were stored at refrigeration temperatures. It appears that samples were dip inoculated with *Listeria* in buffer. Treated samples were dip inoculated in the same fashion, but small volumes (0.5-1 ml) of phage lysate were added as well. *Listeria* populations were reduced by ~4 log on cabbage samples and ~2.6 log on lettuce samples compared to an untreated control.

Other produce application examples indicate that pathogenic *E. coli* can also be controlled using phage treatment. In similar fashion to other studies, the produce vehicles used in the studies by Sharma et al were lettuce and cantaloupe. As with the honeydew melon used by Leverentz et al, melon represents one of the few fruits with a pH suitable to phage application. *E. coli* O157:H7 was spot-inoculated onto produce and allowed to dry. Phage lysate suspended in buffer was then applied as a spray. Control samples were treated with a spray of buffer alone. All

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treated produce was stored for a week at both refrigeration and room temperature conditions. Again, time was not a significant factor affecting target population when produce was stored at refrigeration temperatures which achieved lower populations of the target than samples stored at room temperature. Low levels of the target were recorded for the control samples, whether it was a result of low initial level or from loss due to the spray is not discussed. For *E. coli* inoculated on lettuce samples, ~2 log reduction was obtained using phage treatment compared to a spray-treated control. The phage application on cantaloupe was even more successful, achieving a reduction of ~3.5 log (Sharma and others, 2009).

### **Phage application in meat and dairy**

Phage treatments applied to meat and dairy products have likewise been considered. Phage as a form of biocontrol of foodborne pathogens in products like these pose challenges unique compared to those considered in fresh produce. For phage application to be successful, the virus must be able to have contact with the host bacterium and adhere successfully to the target receptor. If components of the food matrix interfere with either of these, the efficacy of phage treatment is reduced. Unlike fresh fruit and vegetable products, meat and dairy products that are susceptible to contamination often contain a more complex make-up. The macromolecules present in meat and dairy, in addition to other ingredients that may be present in products like sausage, pepperoni, flavored milk and yogurts, and cheese, may be problematic if phage application were to be considered. If one of the components of the matrix interacts with the same receptor the phage targets, attachment of the bacteriophage will be reduced since the phage must compete with other potential interactions. Additionally, if the matrix sequesters the bacteria or otherwise limits contact between host and phage, lytic activity will also suffer as a consequence. Fat molecules may provide a protective layer around bacteria and products like

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ground meat or yogurt may pose a difficult matrix in which the virus can be dispersed for access to the target. Despite these limitations, unique benefits to phage treatment exist in these products as well, particularly in fermented products like yogurt. Because bacteriophages are highly specific, a cocktail of phages can be applied to a fermented product without risk of killing the bacteria responsible for fermentation. This selective activity is fairly unique to phage treatments as most other processing technologies and antimicrobials are either completely non-specific or too broad-spectrum to be used to target a specific strain of bacteria.

Despite the previously mentioned challenges, successful phage treatments have been made in these products. A study using phage for biocontrol of *Salmonella* on both cooked and raw beef found significant reductions when samples were incubated at both refrigeration and room temperature conditions (Bigwood and others, 2007). For samples stored in the refrigerator, reductions of 2-3 log were obtained while samples incubated at room temperature had *Salmonella* populations that were nearly 6 log lower than the control. This study also manipulated the concentrations of both the *Salmonella* population as well as the phage titer. Generally, lower concentrations of the target and higher concentrations of the phage prove more successful. Another study using phage treatment on beef steaks measured the increase in shelf-life obtained by including this process in comparison to an untreated control. Steaks were stored at refrigeration temperature and the shelf-life improved between 3.5 and 6.5 days (Greer, 1988).

*Salmonella* has also been the target of phage treatment in a study trying to eliminate *Salmonella* from cheddar cheeses. Both raw and pasteurized milk were used to make the cheese used in the study to assess the difference between the two products once subjected to phage treatment. A notable difference between this food challenge study and the ones previously discussed is that the storage period relevant for cheese is much greater. Here, samples were taken

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and the *Salmonella* population determined after treatment and with less frequency for 99 days following treatment. Phages reduced the target population by 2 log following treatment compared to the inoculation level, but control samples had *Salmonella* populations which increased by 1 log which suggest a total reduction of 3 log. By the end of 99 days, the control cheese had a 3 log CFU/g density of *Salmonella* while the phage treated sample was ~0.5 log CFU/g (Modi and others, 2001).

### **Pre-harvest control using phage**

While some research has been focused on the use of phage for treatment of food products, other researchers have considered the application of phage to agricultural and food production systems to control for foodborne pathogens (Atterbury and others, 2007). This may include application of phage lysate to production or waste management equipment, introduction of the phages to live food animals to reduce the colonization of pathogens, or application of the phage to consumable implements within the production line to prevent contamination and outgrowth of the target. A study conducted to reduce the colonization of *Campylobacter* in broiler chickens using phage therapy found that, compared to the control, feeding phage particles to young broilers reduced the density of *Campylobacter* in the intestine by 0.5-5 log compared to chickens which had not been fed phage. One potential limitation to this technology is the potential for phage resistant mutants to develop *in vivo*. This study found that mutation rate to be less than 4% of the total *Campylobacter* population and that phage resistant mutants quickly reverted back to their wild type once phage treatment was terminated (Carillo and others, 2005).

Another application of phage in production settings is the treatment of waste and consumables. As an example, phage may be applied to drainage equipment or fertilizer to reduce or eliminate cross-contamination of food products. Erickson et al applied a cocktail of phages to

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compost to control for several *Salmonella enterica* strains. Different moisture levels for the compost were evaluated, but across all moisture levels tested a reduction of about 2 log was obtained from phage treatment.

### **Advantages and limitations of phage application in the food industry**

#### **Limitations**

Although phage has shown some promise, limitations still exist in its potential application as a technology for food safety in fresh produce or any product. Some of these limitations became apparent from the results of previous work using phage for biocontrol of foodborne pathogens that were previously discussed. As in the study conducted by Leverentz et al, the acidity of apple pieces proved too acidic for phage activity (Leverentz and others, 2003). Phage have been shown to often be acid sensitive and can be inactivated in environments where the pH is below a certain level (Summers, 2004). From the evolutionary standpoint, it follows that if the pH of the environment were too low for bacterial growth, the activity of the phage would be unnecessary. However, known outbreaks in products such as apple cider, generally considered to be acidic enough to guard against foodborne pathogens, indicate that more food safety controls may be needed. Indeed, fresh fruit, ready to eat cut fruit, and fruit juices have been associated with foodborne outbreaks and are products which merit the attention of food microbiologists. Nonetheless, the protection of these products might not be adequately suited to phage as many fruits have a relatively acidic pH. In addition to acidity, phage application may be limited by other components in the food matrix. If any ingredient has an affinity for the phage's target receptor, it will compete with the phage for binding and effect attachment and as a result, the rate and extent of lysis. Certain food matrices may also limit the potential for phage/host interactions all together if the phage and the bacteria are not distributed equally throughout. Unlike treatments like heat or pressure, phage requires contact with the target.

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Perhaps one of the greatest limitations to phage applications is the degree to which phages are able to reduce the population of pathogenic bacteria. Previous work has indicated that phage cocktails can reduce target population by around 2 to 4 log (Bigwood and others, 2007; Leverentz and others, 2003; Greer, 1988; Guenther and others, 2009). Although an appreciable amount, these reductions may fall short of being the silver bullet solution to the safety of fresh produce. A pasteurization treatment usually requires a reduction of at least 5 log and it is sometimes difficult to persuade a cautious food industry to adopt a novel technology for food safety when conventional treatments like heat have been adopted as the gold standard (Toledo, 1991).

Another limitation to the adoption of this technology is the potential expense involved in its application (Greer, 2005). Large scale production of phage lysate for application in a food system would likely require large fermentation vessels where host bacteria could be grown in the presence of phage. Since the desired phage is specific for a pathogenic microorganism, growth of these bacteria in massive quantities would need to be avoided. Alternatives could include utilizing another sensitive bacterium that is non-pathogenic or developing a strain of an attenuated pathogen which lacks one or more virulence factors. After outgrowth of the host and replication of the phage, purification steps would be necessary to remove bacteria from the media and eventually to purify the phage away from media. Finally, phage could be re-suspended and applied in food. To put the cost of this production in perspective, phage is being proposed as an antimicrobial applied as a spray or dip in a similar way to the low concentrations of chlorine that are currently used.

Concern over the development of phage resistant mutants also exists, particularly in applications to animals or farms where the phage becomes integrated into the environment. As

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reported by Carillo et al, the mutation frequency for phage resistant *Campylobacter jejuni* spp. in broiler chickens treated with phage was around 4%. The potential development of these mutants could render phage treatments ineffective. However, as the authors pointed out, the mutants returned to their sensitive state with relative speed following the removal of phage for treatment from the environment (Carillo and others, 2005). Options for addressing the potential development of these mutants include rotating phage cocktails so that different selective pressure would change periodically and selecting phages that target receptors on the cell surface which are necessary for cell survival or otherwise less prone to mutation. Besides the development of phage resistant mutants, the use of phage treatment may also raise concern about the transmission of virulence factors through transduction. Although primarily associated with lysogenic phages, DNA from the host may inadvertently be packed during phage assembly and transferred to a new host and integrated into the genome (Goodridge and Bisha, 2011). As an example of phage dependent virulence, Shiga toxin producing *E. coli* (STEC) carry a prophage which encodes for production of Shiga toxin 1 and 2. Only lytic phages would be good candidates for phage treatment of food products, but the possibility of transduction still exists although it is low. Transduction rate may be further decreased by selecting phages with fewer receptor sites (Adye, 2004) and targeting lysis from without as the preferable lytic activity.

Consumer acceptability of a product treated with bacteriophage may also prove a stumbling block to commercial application of this technology. Since consumers are most familiar with viruses as human pathogens, it might be difficult to convince the public that bacteriophages are “good” viruses and can aid in food safety measures (Greer, 2005). Consumer education might be an important tool to overcome this challenge. Not only would the idea of viruses need to be addressed, but also the sense that science is interfering with the natural order of things would

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need to be assuaged. With other biotechnologies (GMO's, growth hormones, etc.), a considerable backlash in consumer opinion arose in opposition to the development of so called "Frankenfoods" (Miller, 2004). Unease and mistrust of commercial agriculture necessitate education to alleviate public concerns about the application of a virus to food products.

Finally, the high specificity of phages for their host can be a limiting factor in biocontrol of foodborne pathogens. A single phage isolate may not control for all the potential strains of a target bacterial species. For this reason, a cocktail of phages may be used to account for potential contamination from a variety of relevant strains (Heringa and others, 2007). However, even if multiple strains or species are accounted for by a cocktail, diversity of foodborne pathogens extends over several different microorganisms. A group of phages used to control for several different strains of pathogenic *E. coli* would likely be completely ineffective against *Salmonella* and *Listeria*, two other notable contaminants of fresh produce. Additional phages or phage cocktails could be applied to control for other pathogens, but with each addition there is an increase in cost to produce the lysate for the treatment.

### **Advantages**

Although significant limitations to phage treatments exist and need to be addressed or successful application of this technology to the food industry, phage treatment also provides some unique advantages to food processors. Coincidentally, some of the same characteristics which challenged the successful application of phage treatments also provide benefits to processors in other capacities. As an example, the high specificity of phage was previously cited as a limitation to its application because it made control of all the various foodborne pathogens a challenge when compared to a treatment like heat which was non-specific. This same high specificity confers a unique advantage to phage treatments in that other biota present in the

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product naturally or added by the processor would be unharmed. As previously noted, this is especially important in fermented foods where few other treatments are specific enough to target pathogens while leaving the starter culture unharmed (Greer, 2005; Modi and others, 2001).

Yet another example of dual nature of phage characteristics to serve as both advantages and limitations is the issue of consumer acceptance. In 2002, the FDA approved a bacteriophage preparation for use in raw meat and poultry products (CFR). However, the legality of phage treatments for foods may not be enough to persuade consumers. While consumers may be resistant to having viruses in their food, the idea of phage as a natural antimicrobial may offer some appeal. Trends have indicated a growing interest on the part of consumers for natural products which do not contain synthesized additives and undergo minimal processing. Since phages are naturally occurring components of the environment, consumers may find their application to food products a more desirable alternative to conventional additives.

Bacteriophage treatments may also represent an alternative to conventional additives for use in organic produce. Furthermore, alternative methods for applying phage technologies can be considered and might lessen consumer concern over the issue of viruses in food. The genes involved in secretion of the lysin, the protein responsible for lysis of the host, may be cloned into another cell for expression. Alternatively, the lysin itself may be purified and used as an additive (Greer, 2005). The use of natural antimicrobials like phage may likewise appeal to consumers as news of the increase in antibiotic resistance is on the rise.

A distinct advantage to phage treatments is its applicability to the produce industry. Phage's non-destructive nature makes it one of the few treatments applicable to fresh produce. And, since washes and sprays containing other antimicrobial agents are already in place within processing lines for fresh produce, the addition of phage lysate would not be a difficult change

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(Barringer, 2012). While a limitation to its application is the level of reduction achieved from phage treatments, it may well be applied as part of a hurdle strategy to control for pathogens. As previously discussed, the results reported from experimental phage treatments indicate that a reduction of ~2 to 4 log may be obtained. If phage treatment were combined with other technologies, greater levels of inactivation may be obtained. Furthermore, synergistic activity has been reported between phage and bacteriocin, applications which suggests the potential for phage treatments as part of a larger food safety strategy (Leverentz, 2003).

Overall, phage treatments represent a novel technology which may be a valuable tool for the control of foodborne pathogens, particularly for promoting the safety of fresh produce.

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## CHAPTER 2

### ISOLATION OF BACTERIOPHAGE FROM THE ENVIRONMENT AND CHARACTERIZATION OF ISOLATES FOR POTENTIAL CONTROL OF FOODBORN PATHOGENS

#### 2.1 Abstract

Bacteriophages, bacterial viruses, are widely present in the environment, wherever the host bacterium is expected to be found. Some bacteriophages are potentially useful agents in the control of foodborne pathogens. There is a wide array of potential candidate phage isolates; hence, investigations are needed to determine which isolates will likely be the most effective for application in foods. In this study, phages active against pathogenic *Escherichia coli* and *Salmonella* were isolated from sewage and manure samples and compared on the basis of titer, efficacy against target population, stability during storage, and cross-reactivity. The most promising *Salmonella* phage had an average titer of 10.3 log PFU/ml following incubation with the host for 48 hr. However, this phage was not as effective when tested against the target in liquid culture. It had only a bacteriostatic effect on growth of *Salmonella* when incubated in LB broth. After 8 h, the *Salmonella* phage only inhibited the growth of the target without achieving a decrease in *Salmonella* population. The most promising *E. coli* phage isolated from sewage effluent had an average titer of 8.4 log PFU/ml. Incubation of the phage with the host in liquid media decreased the target population by a maximum of 3 log in LB broth and 4 log in 0.1% peptone water and buffer. Stability tests showed ~0.5 log decrease in viable phage particles every 10 days during storage and the phage was active against five of seven *E. coli* O157:H7 strains tested with low to moderate activity against *E. coli* O157:H12 and *E. coli* O157:H1. The phage was also active against some commensal *E. coli* strains, such as K12. Because of *E. coli* phage

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(A)'s strong inhibitory activity in liquid media and relatively broad cross-reactivity spectrum, this phage was selected for future food challenge studies.

## 2.2 Introduction

Bacteriophages, (phages), have been used for the control of pathogens or in phage therapy for almost 100 years. First discovered by Felix d'Herelle in the early 1900s, phages were originally tested as potential agents for the control of diseases in the field of veterinary medicine (17). Phage therapy in these early studies was limited by the inadequate information available on phages at the time. It was not until several decades later when research was done on temperate/virulent life cycles, the narrowness of phage host range, and appropriate purification methods for phage preparations that more effective clinical testing could be conducted. Despite these early limitations, phage therapy has continued with greater interest in Eastern Europe compared with the rather limited work in the United States (4). Phage control has also been evaluated for on-the-farm application to live animals (2) and compost (10) with some demonstrated success in controlling pathogens at the pre-harvest stage of food production. Phage mediated post-harvest biocontrol of foodborne pathogens and spoilage microorganisms has been explored in produce (6, 13, 16), meat (3, 7, 8), and dairy products (5, 9). Given the recent outbreaks of foodborne illness associated with fresh produce, and the limited available options for pathogen control in fresh products, phage treatment may represent a viable option for the food industry to reduce the risk of outbreaks in fresh foods.

Efforts to utilize phage for pathogen control and therapy are aided by the ubiquity of a diverse population of phages in the environment. Although not generally recognized as a living organism, bacteriophages may be the most abundant element in the microbiome, present at even

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greater levels than their bacterial hosts (5). Phage can be found at high levels in environments where the host bacteria can be expected to be found replicating, which include water, soil, feces, and food (and at particularly high levels in fermented food). Phages have been detected at levels of up to  $10^7$  particles/ml in aquatic environments (18) and  $10^8$  viable phage/g of meat product (11). Phage in the environment not only exists in high density, it is also present in great diversity. Additionally, the composition of the phage community present in a given environment varies with the composition of the bacterial community. A single environmental sample may be expected to yield phages of different species, specific for different bacteria present in the environment (18). Additionally, a single environmental sample may contain multiple phage isolates specific for some of the same bacteria, but with a slight variation in cross-reactivity among different strains of the same bacterial species (10).

Initial studies on the application of bacteriophage for control of pathogens require the identification and isolation of an appropriate phage from the multitude of phages that exist in the environment. Phages specific for pathogenic *E. coli* and *Salmonella* have often been isolated from sewage and manure samples (10, 12) and following isolation, characterization of the candidate isolates is necessary to determine the most promising candidate for pathogen control. Spectrum testing is used to evaluate the host range of phage isolates which is generally expected to be fairly narrow, and often limited to specific strains of the target microorganism (4, 5). Since effective control of the target would depend on the sensitivity of multiple strains to phage infection to prevent outbreaks from a wide range of pathogens, cocktails of phages have been used to address the limitation of narrow host range for any individual phage isolate (10, 13, 15). Assuring the lytic nature of phage candidates is also an important parameter to characterize as growth of the pathogen would likely not be inhibited by lysogenic phages (15). The objective of

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this study was to characterize phage isolates from manure and sewage samples to determine the most promising candidate for use in food challenge studies.

## 2.3 Materials and Methods

### **Bacterial culture**

*E. coli* O157:H7 EDL933 and *Salmonella enteric* serovar Typhimurium strain LT2 were used as the target microorganisms. Stock cultures, frozen at -80°C, were transferred to Luria Bertani (LB) broth (BD, Franklin Lakes, NJ) and incubated overnight at 35°C. These cultures were transferred again in LB and incubated at 35°C for 12 h with shaking for use in all isolation and purification experiments.

### **Purification of phage from environmental samples**

Manure samples from cattle, sheep, and horse farms from The Ohio State University and sewage effluent from the Columbus area were screened for the presence of phage active against *E. coli* O157:H7 EDL933 and *Salmonella* Typhimurium strain LT2. Manure samples were added to LB broth supplemented with 2 mM calcium chloride and the mixture was incubated for ~1 hr at room temperature with shaking. Diluted manure and sewage samples were centrifuged (10,000 x g, 10 min). The supernatant was then filter sterilized using a 0.22 µm pore size syringe driven filter unit (Millipore, Billerica, MA). Manure samples were enriched by incubation of the supernatant (20 ml) with the target microorganism at 35°C with shaking for 48 h. Sewage samples were enriched by the addition of equal volumes of the filter sterilized supernatant to 2X LB with 2 mM CaCl<sub>2</sub> and the target, followed by incubation under the same conditions. Enriched samples were again centrifuged and filter sterilized. The filter sterilized phage suspension is known as the lysate.

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### **Isolation of phage candidates**

The presence of phage was confirmed by spotting 5 µl aliquots of the enriched supernatant onto a lawn of the target microorganism on a plate of LB agar supplemented with CaCl<sub>2</sub>. Following overnight incubation at 35°C, zones of inhibition where the supernatant was spotted indicated the presence of lytic bacteriophages. Positive samples were further screened to obtain isolates of a single phage. Serial dilutions of the supernatant were briefly incubated (45 min) at room temperature with the target microorganism then added to of LB soft agar (0.75% agar w/w) supplemented with CaCl<sub>2</sub> and poured over a plate of LB agar. The pour plates were incubated at 35°C for 24 h. Isolated plaque forming units (PFU) were excised and re-incubated and filter sterilized before again pour plating to obtain isolated PFUs. The plaques were excised once more and the process repeated a second time to achieve isolation of a single candidate phage.

### **Phage preparations**

Stock lysates for the phage candidates were prepared by incubation of purified phage with the host in LB broth supplemented with CaCl<sub>2</sub> (2 mM) for 48 h at 35°C with shaking. All *E. coli* phages were incubated with the target *E. coli* O157:H7 EDL933 and all *Salmonella* phages were incubated with the target *Salmonella* Typhimurium strain LT2. Following incubation, the cultures were centrifuged (10,000 x g, 10 min) then filter sterilized and the resulting crude lysate was stored at 4°C.

### **Phage titering**

Serial dilutions of the phage stock lysates were prepared and aliquots of these dilutions were incubated with the host at room temperature for 45 min. The incubated aliquots were added

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to molten LB soft agar supplemented with CaCl<sub>2</sub> and overlaid on an LB agar plate. Plates were incubated at 35°C for 24 h and the resulting PFUs were quantified to determine phage titer.

### **Liquid culture assay**

Bacteriophage candidates were individually incubated with the target microorganism in LB broth supplemented with CaCl<sub>2</sub>, 0.1% peptone water (Becton, Dickinson and Co.) with CaCl<sub>2</sub> (2 mM), and phosphate buffer (pH 7.0) with CaCl<sub>2</sub> (2 mM). Cultures were incubated at 35°C with shaking for 8 hr. Samples were taken every hour, diluted as appropriate in 0.1% peptone water and spread-plated on LB agar. Plates were incubated at 35°C for 48 h and surviving bacteria was enumerated.

### **Phage stability during storage**

Crude lysates of the phage candidates were held in storage at 4°C and the titer value for each lysate was recorded at day 0, 10, 20, and 30 to assess the stability of the phage isolate during storage over time. Titering was performed as previously described.

### **Cross-reactivity**

In addition to the target microorganisms used for phage isolation, activity of phages on closely related isolates was examined. Activity of the phage isolates was first assessed by spotting the crude lysate on a lawn of the potential host bacteria. *E. coli* serotypes (14) and *Salmonella* serovars (15) were activated from a stock culture frozen at -80°C into LB broth and incubated at 35°C for 12 h with shaking (Tables 2.1 and 2.2). Observed inhibition of growth, as marked by clearing where the lysate was spotted, was denoted as susceptibility of the bacteria to

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phage lysis. Susceptible strains were then used as the host microorganism to determine titer, as previously described.

**Table 2.1:** Strains of pathogenic and nonpathogenic *E. coli* used in cross-reactivity work

<b>Microorganism:</b>	<b>Serotype:</b>	<b>Other Information:</b>
<i>Escherichia coli</i>	O157:H7 EDL933	
	O157:H7 GFP B6-914	Source: Dr. LeJeune lab
	O157:H7 EDL933 32C	
	O157:H12	
	O157:H7 EDL933 32A	
	B8508	
	054 220	
	O157:H7 EC88	
	ATCC 25922	
	O157:H7WT	
	O157:H1	
	455	
	K12	
	O157:H7 EDL933 32DB	

**Table 2.2:** Strains of *Salmonella* used in cross-reactivity work

<b>Microorganism:</b>	<b>Serovar:</b>	<b>Other Information</b>
<i>Salmonella enterica</i>	Typhimurium ATCC BAA-185	
	Enteritides 3512H	FDA isolate from potatoes
	Heidelberg	From the lab of Dr. Rodriguez
	Kentucky	From the lab of Dr. Rodriguez
	Senftenberg	OSU# 836
	Typhimurium OSU228	
	Minnesota 00-43	FDA isolate from cantaloupe
	Typhimurium DT109	
	Muenchen ATCC 8388	
	Anatum 68h	FDA isolate from candy-coated pretzel
	Newport H9113	FDA isolate from Mango
	Javiana 7N	FDA isolate from orange juice
	Thompson 2051H	FDA isolate from thyme
Saintpaul 02-109	FDA isolate from cantaloupe	

### Genome size estimation

Pulsed field gel electrophoresis (PFGE) was used to estimate the genomes size for the three bacteriophage isolates (*E. coli* phages (A) and (B) and *Salmonella* phage) according to the protocol published by Linghor et al. Plugs were prepared according the given procedure and gels were run using 6 V/cm for 15 h at 14°C with incremental pulses of 2.2-54.2 s (14).

### Imaging

*E. coli* phage (A) was imaged using procedure adapted from (1). The crude phage lysates were purified by ultracentrifugation (28,000 x g, 3 h) to sediment the phage particles followed by re-suspension of the pellet in ammonium acetate buffer (0.5 M). Phage preparations were stained using the negative stain ammonium molybdate (Electron Microscopy Sciences, Hatfield, PA)

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and loaded on copper microscopial grids (Electron Microscopy Sciences). Transmission electron microscopy (TEM) was conducted at the Wexner Medical Center (The Ohio State University, Columbus, OH).

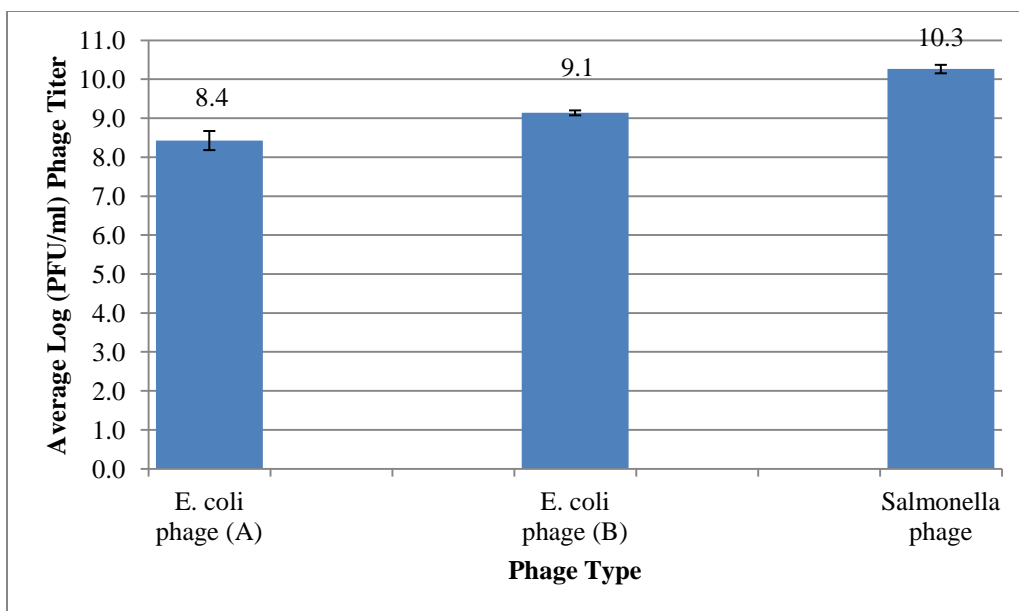
## **2.4 Results and Discussion**

### **Isolation of phage from environmental samples**

Screening of environmental samples yielded a total of five crude lysates active against *Salmonella* and seven active against *E. coli*. From these samples, four bacteriophage isolates active against *Salmonella* were obtained from sewage and one from manure in addition to four unique *E. coli* bacteriophage isolates from sewage and three from manure.

### **Titering**

Titers of isolated phages ranged from  $10^4$  to  $10^{10}$  PFU/ml following 48 h incubation with the host bacteria. Three isolates were found to have a titer above  $10^8$  and three replicates of the titering exercise produced average titer values for the *E. coli* phages (A) and (B) of 8.4 and 9.1 log PFU/ml and 10.3 log PFU/ml for the *Salmonella* phage (Figure 2.1).



**Figure 2.1:** Average (three replicates) of titer values for two unique *E. coli* phage isolates and a *Salmonella* isolate

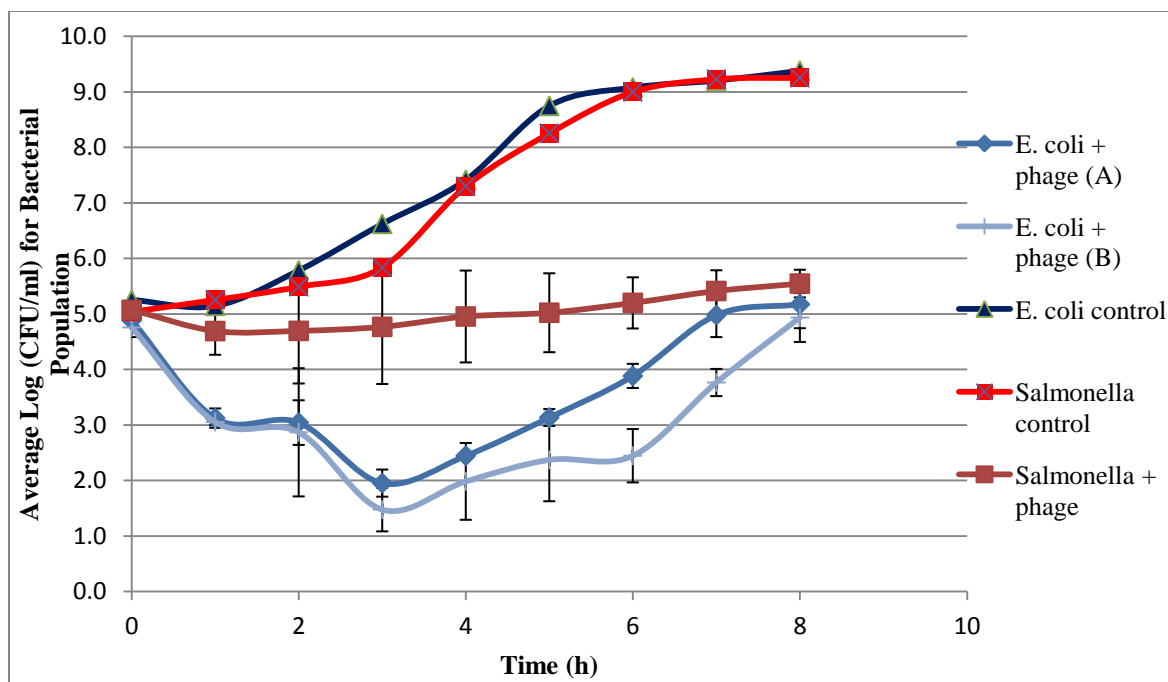
Although titer values are only one method for predicting phage efficacy in pathogen control, they provide some indication about the phage's interaction with the target pathogen. Generally, a higher titer is better since it indicates more replication of the phage during incubation and growth of the host. Greater replication, as indicated by high titer, suggests more phage particles will be produced that subsequently may infect more bacteria. It may also be indicative of a high affinity of the phage for the target receptor displayed by the host. Conversely, a low titer may indicate low affinity of a phage candidate for a particular host. Under low-titer conditions, the probability of an individual phage attaching to the target is decreased and may require more time for infection to occur or a higher multiplicity of infection (MOI) to increase the probability of attachment. Titer values may also be dependent, in part, on burst size. During the lytic life-cycle, phage replication by the host cell machinery will occur until a certain number of phage particles have been synthesized. Once this number has been

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achieved, secretion of the lysin, a pore-forming protein, occurs which causes lysis of the cell and release of the daughter phages. Concentration of phages in the crude lysate is dependent on many factors, but higher titer values indicate the sensitivity of the target to phage infection and for this reason, only phage isolates with titer values above  $10^8$  were selected for further testing.

### **Liquid culture assay**

The effect of phage on the growth of the target pathogens in different media is perhaps an even better indicator of an isolate's potential as an agent of pathogen control than comparison of titer value. Growth curves of the two target microorganisms, *E. coli* and *Salmonella* in LB broth show the expected lag, exponential, then stationary shape (Figure 2.2). The curve for *Salmonella* incubated with its respective phage shows stationary levels throughout the 8 h period while the *E. coli* cultures incubated with phage show a 3-log decrease, approximately, in population within the first several hours but the population returns to starting levels by the end of the 8 h holding period (Figure 2). Based on these results, the *E. coli* phages were pursued as the more promising candidates since they achieved an initial decrease in population compared to the *Salmonella* phage's bacteriostatic effect. Although both *E. coli* and *Salmonella* phage cultures returned to the same population at the end of 8 hr, the *E. coli* phages initial kill showed promise for future food applications since the growth conditions for surviving target bacteria would likely not be as accommodating on a food product as in LB incubated at 35°C.



**Figure 2.2:** Effect of bacteriophages on growth of *Escherichia coli* O157:H7 EDL933 and *Salmonella* Typhimurium LT2 growth curves when incubated in LB broth for 8 hours

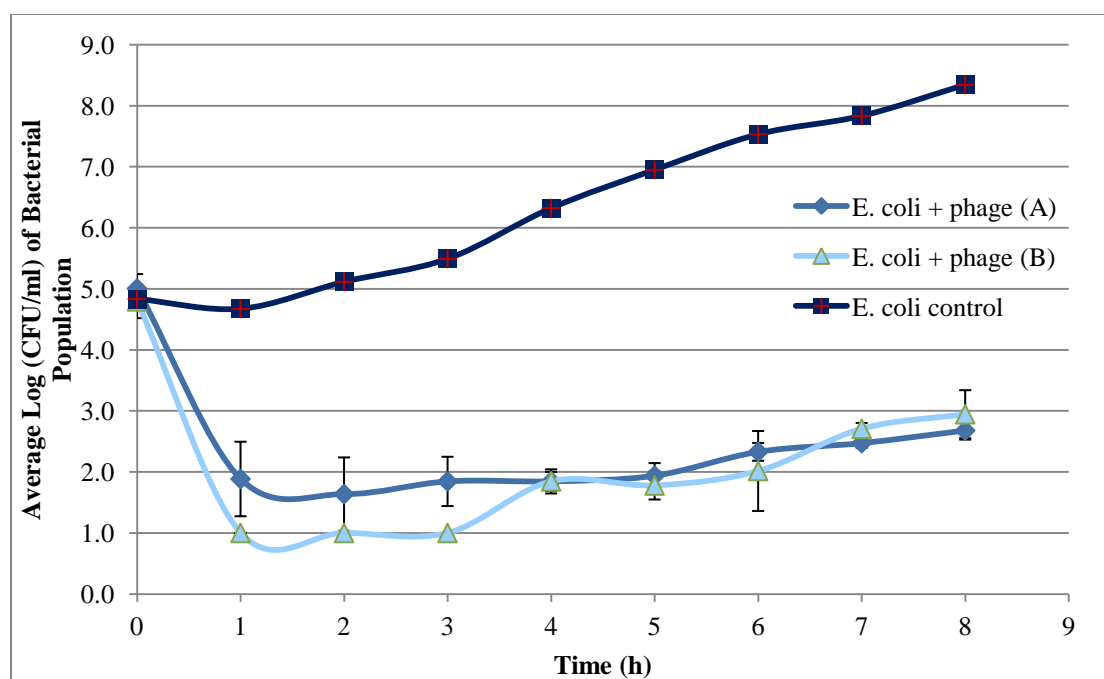
\*Error bars denote standard deviation among three replicates

To more rigorously assess the bacteriocidal activity of the *E. coli* phages, the same experiment was repeated using 0.1% peptone water as a growth medium instead of LB broth. As expected, the outgrowth of the target was negatively impacted in peptone (Figure 2.3) when compared to growth of the control in LB broth (Figure 2.2). The phases of growth (lag, exponential, stationary) are less clearly defined and the total population at the end of 8 h is close to 1 log lower for the control grown in peptone compared to the control grown in LB, which is the result of limitation of available nutrients in peptone, perhaps more representative of the nutritional conditions available to pathogen contaminants on fresh produce. During the first hour of incubation, the *E. coli* phages decreased the target population by about 4 log. This initial kill was 1 log greater than that obtained in the LB experiment and the regrowth of the target at the end of 8 h was also less when the host was incubated in peptone. At the end of 8 h, the *E. coli*

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population incubated with phages recovered by about 2 log, reaching a level of 3 log less than the overall starting population for the cultures.

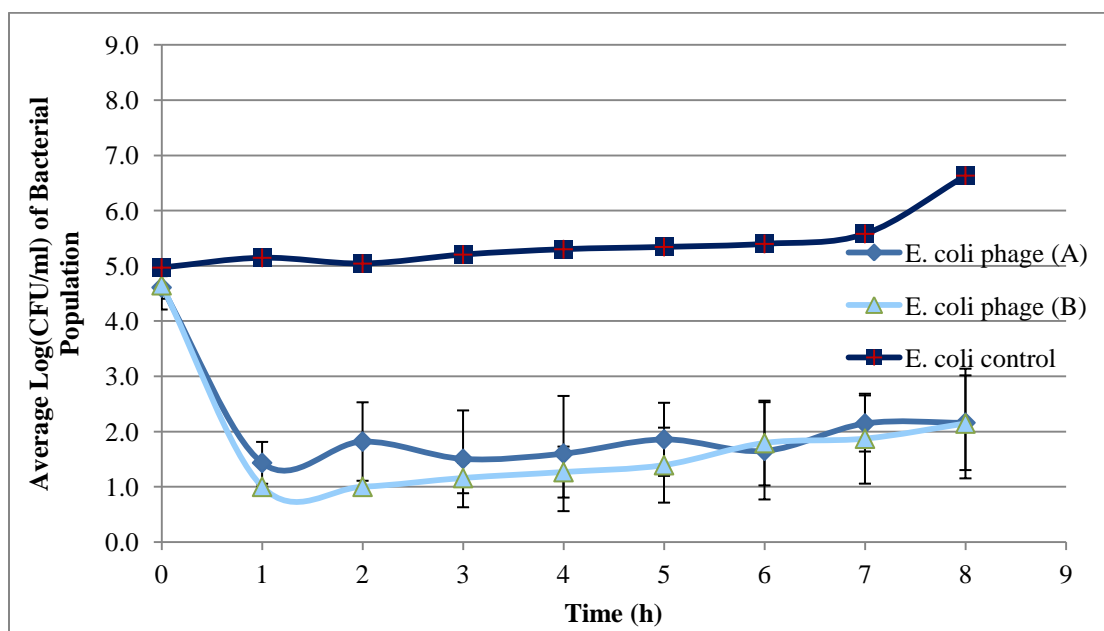


**Figure 2.3:** Effect of phages on *Escherichia coli* O157:H7 EDL933 and *Salmonella* Typhimurium LT2 growth curves when incubated in peptone for 8 hours

\*Error bars denote standard deviation among three replicates

Finally, the experiment was repeated in phosphate buffer, a medium which lacked essential growth factors for the target bacteria. As a result, the control culture shows little or no growth over the 8 h incubation period while the initial kill achieved from incubation with phages is nearly sustained at the end of the holding period (Figure 2.4). Similar to incubation in peptone (Figure 2.3), the initial kill of about 4 log CFU/ml was obtained within the first hour. However, unlike the peptone experiment, incubation in PBS showed minimal regrowth of 1 log, approximately. Based on the similarity of the results from the liquid culture work, homology was suspected between the two *E. coli* phage isolates.

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**Figure 2.4:** Effect of phages on *E. coli* O157:H7 EDL933 and *Salmonella* Typhimurium LT2 growth curves when incubated in buffer for 8 hours

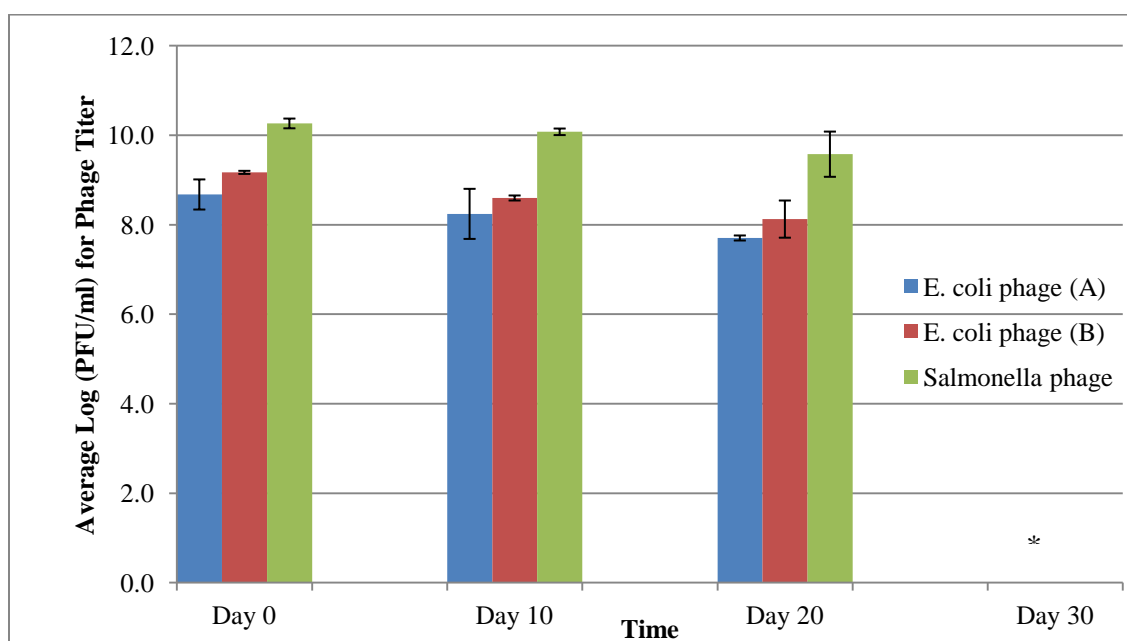
\*Error bars denote standard deviation among three replicates

### Phage stability during storage

Since potential application of candidate phage isolates as agents of pathogen control would likely be subjected to holding period prior to distribution and application, the stability of the phage particles under refrigeration conditions becomes a parameter of relative importance to the food industry. To mimic industrial storage conditions, crude lysates were held in refrigeration (4-6°C) in the dark for about a month. Titer values for all three candidate phages originally identified showed a marginal decrease over each ten-day sampling period (Figure 2.5). For instance, *E. coli* phage (A) decreased in viable phage count by about half a log/ml every ten days. However, no PFU/ml value was reported at day 30 or beyond since the plaques resulting from the pour plates were too small and ill-defined to accurately enumerate. As discussed

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previously, this suggests that using titer as an assessment of phage efficacy against a target is only a limited estimation. While the titer values decrease relatively slowly, the appearance of the plaques indicates that interactions between the phage and the host may be more greatly impacted by storage than the titer indicates. The stability of the titer value over 20 days is an indication that these isolates are relatively stable under storage conditions as a crude lysate, but further research is required on phage in the purified form in which it would be applied in industry. Additionally, using metrics in addition to titer determination to assess viability is necessary to more fully understand the stability of phage preparations over time.



**Figure 2.5:** Concentration of crude phage lysate held at 4°C over 30 days of storage

\*Plaque forming units could not be enumerated

†Error bars represent the standard deviation for the averages obtained from three replicates

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### **Cross-reactivity**

Initial spectrum testing was conducted for both *E. coli* phage (A) and the *Salmonella* phage isolates. The *Salmonella* phage showed moderate to low activity against 6 of the 14 *Salmonella enterica* strains tested (Table 2.4). The *E. coli* phage was active against 11 of 14 strains tested. For the strains showing sensitivity to phage infection, titer values ranged from 6.9 to 8.9 log PFU/ml (Table 2.3). Three of the strains which showed sensitivity when spotting the lysate onto a lawn of the host were not able to be titered because the resulting plaques could not be enumerated. As concluded previously, this result indicates the limitation of using titer as a proxy for activity. Sensitivity was observed in both pathogenic and nonpathogenic isolates. Of the *E. coli* O15:H7 strains tested, five of seven showed sensitivity to this phage isolate. However, the *E. coli* O157:H12 and *E. coli* O157:H1 strains showed moderate sensitivity to phage treatment. The nonpathogenic strain *E. coli* K12 also showed sensitivity to phage infection, which indicates the potential for phage interaction with commensal *E. coli* strains as well. For application within the food industry, it is likely that a cocktail of phages active against a broader spectrum of pathogenic *E. coli* strains would be necessary.

**Table 2.3:** Sensitivity of pathogenic and nonpathogenic *E. coli* strains to bacteriophage (A)

<b>Serotype:</b>	<b>Titer (Log PFU/ml)<sup>¥</sup></b>	<b>Inhibition<sup>*</sup></b>
O157:H7 EDL933	8.4	++++
O157:H7 GFP labeled	8.9	++++
O157:H7 32C	8.0	++++
O157:H12	Not visible <sup>†</sup>	++
O157:H7 32A	-	-
B8508	Not visible	+++
054 220	6.9	++++
O157:H7 EC88	8.5	++++
ATCC 25922	-	-
O157:H7WT	-	-
O157:H1	Not visible	+/-
455	7.0	++++
K12	8.3	+++
O157:H7 32DB	8.8	++++

\*Inhibition determined by spot on lawn method. Positive and negative designators were qualitatively determined based on the appearance of relative inhibition.

†Plaques too small or ill-defined to be accurately enumerated

¥ Titer values were determined from an average of three replicates

**Table 2.4:** Sensitivity of *Salmonella* serovars to bacteriophage

<b>Serovar:</b>	<b>Inhibition*</b>
Typhimurium ATCC BAA	+
Enteritides	-
Heidelberg	+
Kentucky	++
Senftenberg	-
Typhimurium OSU228	+
Minnesota	-
Typhimurium DT109	-
Muenchen	++
Anatum	-
Newport	-
Javiana	-
Thompson	-
Saintpaul	++

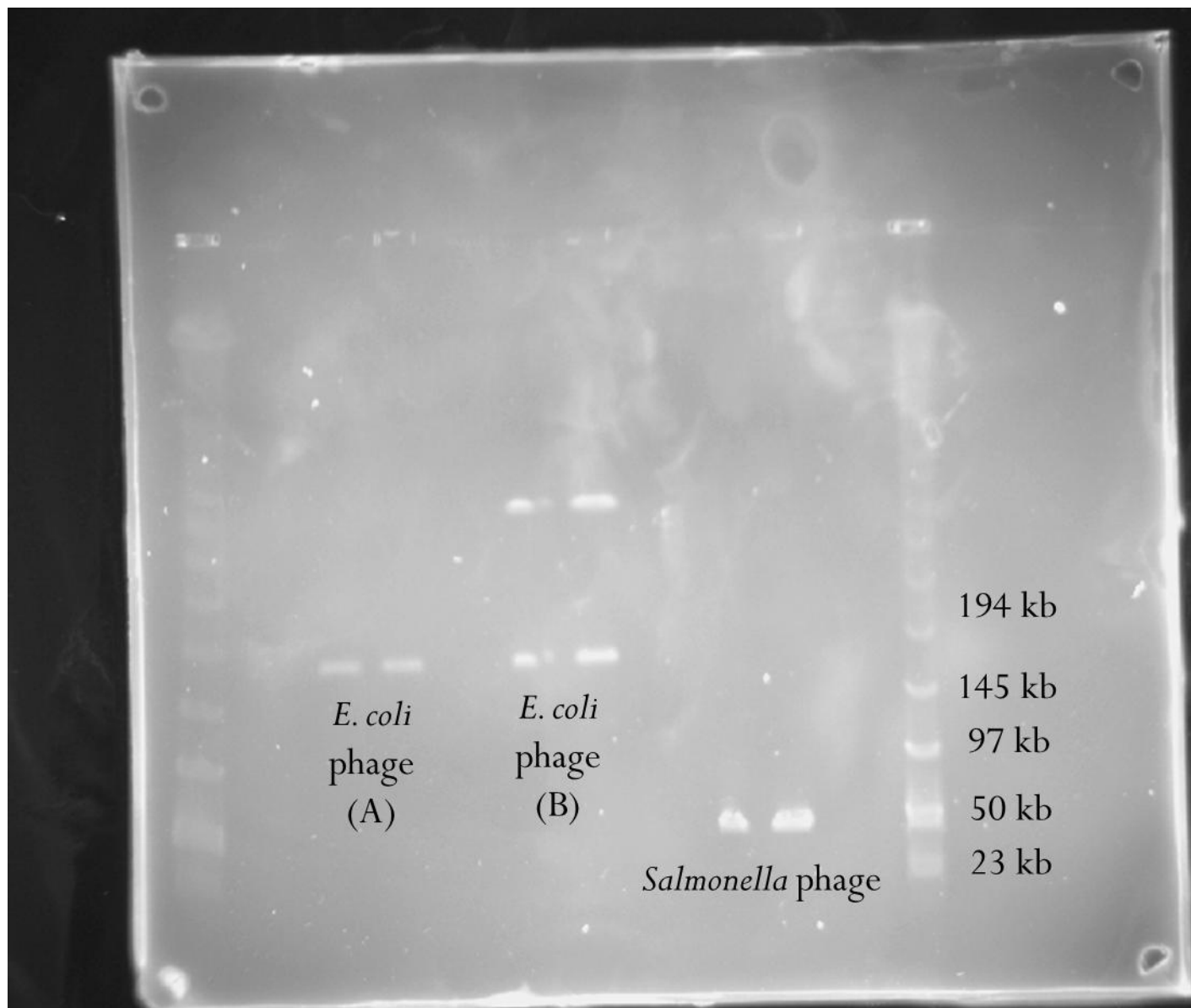
\*Inhibition determined by spot on lawn method. Positive and negative designators were qualitatively determined based on the appearance of relative inhibition.

### **Genome size estimation**

Bacteriophage genomes have been found ranging from 15 to 500 Kb (1). The three isolates examined in this study ranged from 50-150 Kb (Figure 2.6). *Salmonella* phage had a genome size of ~50 Kb while the two *E. coli* phages had a band at ~150 Kb. *E. coli* phage (B) also had another band of greater weight appear in its lane. Potentially, this could indicate a segmented genome but it could also suggest an incomplete isolation of a single phage.

Additionally, the similarity between the bands just above the 150 Kb marker potentially suggest that the two *E. coli* phages are very similar if not the same isolate. These results coupled with the similar data from the prior characterization work further support the decision to eliminate *E. coli* phage (B) from consideration for food challenge studies.

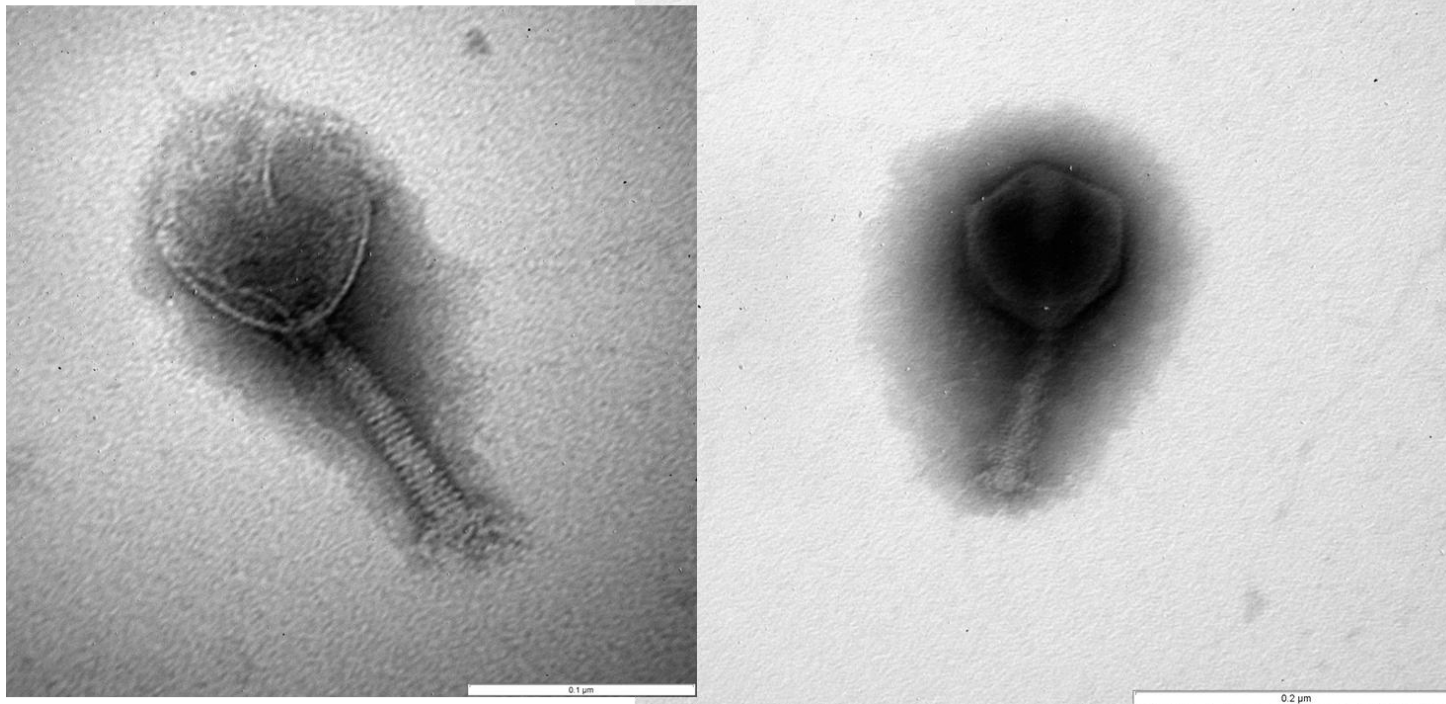
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**Figure 2.6:** Bands of three bacteriophage genomes ranging from 50-150 Kb

### Imaging

*E. coli* phage (A) was imaged using TEM (Figure 2.7). The images obtained indicate that *E. coli* phage (A) is a tailed bacteriophage belonging to the family Caudiovirales.



**Figure 2.7:** Transmission electron microscopy images of *E. coli* phage (A)

## 2.5 Conclusion

Isolation and purification of phages from the environment yielded 12 candidates for potential use in future food safety studies. Titers for crude lysates generated after 48 h of incubation with the target indicated that three of the isolates were most promising for future characterization studies. Two *E. coli* phages and one *Salmonella* phage had average (n=3) titer values above  $10^8$  PFU/ml which indicated high replication when incubated with the target. Incubation of the phages with the target in liquid media and quantification of the bacterial survivors indicated that the *E. coli* phages had greater activity against their host than the *Salmonella* phage. Incubation in LB broth provided an initial kill of about 3 log for the *E. coli* phages while the *Salmonella* phage exhibited only a bacteriostatic effect. Further liquid culture

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experiments wherein the *E. coli* phages were incubated with the target in 0.1% peptone water and buffer showed even more promising results as the target bacterial population was not able to recover as successfully in the less supportive media. The similarity in the results of the liquid culture studies indicated potential homology between the two *E. coli* phages; therefore, only *E. coli* phage (A) was used in cross-reactivity work. Preliminary assessment of the activity spectrum for *E. coli* phage (A) and the *Salmonella* phage showed the *Salmonella* phage to be active against six of fourteen pathogenic serovars tested. The *E. coli* phage showed strong activity against five of seven *E. coli* O157:H7 strains; however, the *E. coli* O157:H12 and *E. coli* O157:H1 strains showed only moderate sensitivity. The nonpathogenic strain, *E. coli* K12, also showed sensitivity to phage infection, thus both pathogenic and non-pathogenic *E. coli* may be susceptible to lysis by this phage. Although the storage stability work showed a minimal decrease in phage titer over time, further characterization about the activity of the phages against the target would need to be conducted to more fully report on their relative stability. Based on these findings, the *E. coli* phage (A) was selected as the most promising isolate to carry forward into food challenge studies because of its relatively broad spectrum of reactivity and bacteriocidal effect in liquid culture experiments.

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## CHAPTER 3

### USE OF BACTERIOPHAGE TO CONTROL ENTEROHEMORRHAGIC *Escherichia coli* ON GREEN BELL PEPPERS AND BABY SPINACH LEAVES

#### 3.1 Abstract

Bacteriophage is a potentially applicable agent for the control of foodborne pathogens. It is particularly relevant to the safety of produce and other minimally processed products since phage application is a non-destructive treatment. The purpose of this study was to evaluate a bacteriophage isolate's ability to control the host pathogen on produce. A phage active against *E. coli* O157:H7 EDL933, with a titer of  $10^8$  PFU/ml, was isolated from waste water. Application of phage for biocontrol was tested on green peppers and baby spinach leaves. Cut peppers were treated with UV light to eliminate background biota, then spot inoculated with *E. coli* O157:H7 EDL933 on cut edges and allowed to dry. Baby spinach leaves were spot inoculated with a strain of *E. coli* O157:H7, that has a green-fluorescent protein (GFP) marker, and no efforts were made to eliminate background biota. Green pepper pieces were treated with a 5-min dip in purified phage lysate while baby spinach leaves were dipped for only 2 min. Treated produce was stored at 4°C, with or without pre-incubation for 4 h at 25°C. Samples were taken periodically over three days to quantify bacterial survivors and phage titer. A control treatment was applied to other samples in the form of a buffer dip, and analyzed at the same time intervals. An additional control set of produce, simply spot inoculated and incubated, were also included. Compared to phage-free rinse, the phage treatment reduced *E. coli* population on peppers and spinach by 1.5 log CFU/g. The rinse treatment alone reduced *E. coli* by 1 log; therefore, the total reduction from the rinse with phage lysate was 2.5 log CFU/g, compared to the untreated control. The phage

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titer on fresh produce samples initially increased, but then declined over the remainder of the three-day storage period. These findings suggest the possible utility of using bacteriophage to selectively control pathogens on fresh produce without damaging the product. Further testing is warranted to evaluate phage biocontrol in other food products as well.

### 3.2 Introduction

Produce consumption has been increasing among American consumers and is often associated with many health benefits (1). In contrast to the nutritional benefits, fresh produce has also been associated with several notable outbreaks of foodborne pathogens. By definition, fresh produce is exposed to minimal processing which renders the elimination of potential contaminants challenging. Conventional methods for food preservation such as heat treatments, drying, or the addition of some antimicrobials are not viable options. Moreover, fresh produce is often consumed raw which means that, unlike other food items such as meat, the preparation of produce by the consumer does not include a final heat treatment to eliminate pathogenic microorganisms. As a result, fresh produce safety is perhaps one of the most researched areas in food microbiology, requiring novel technologies to effectively prevent outbreaks of pathogens.

The effects of foodborne disease include not only health risks for the consumer, but pose an economic burden as well. A 2011-study estimates the cost of foodborne illness to be \$152 million annually (9). Similarly, the Center for Disease Control (2) estimates that there is an annual 76 million cases of foodborne disease which result in 325,000 hospitalizations and 5,000 deaths (8). *Escherichia coli* O157:H7 is a major contributor to these totals. Although the exact numbers are difficult to tabulate since many illnesses are not reported, the CDC estimates that there are 265,000 cases of illnesses resulting from Shiga toxin-producing *E. coli* (STEC) every year. Of those cases, approximately 36% are attributed to the strain *E. coli* O157:H7 (3).

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*Escherichia coli* O157:H7 was determined to be one of the top five pathogens contributing to domestically acquired foodborne illnesses that resulted in hospitalization for 2011 (8).

Bacteriophage treatment may be an effective form of biocontrol for pathogens such as *E. coli* O157:H7 on produce. Previously, researchers isolated phages from the environment and evaluated their potential for biocontrol of *E. coli* O157 in liquid culture based on the receptor specificity of the phages for the O157 antigen (5). O'Flynn et al., have published preliminary work showing that a cocktail of three phages isolated against *E. coli* O157:H7 was effective at controlling *E. coli* on the surface of beef steaks, reducing the *E. coli* population below detectable limits (7). The efficacy of phage treatment has also been evaluated on fresh-cut produce for the control of *Salmonella* by Leverentz et al (6). Reduction of *Salmonella* ranged from 2.5-3.5 log CFU/g of cut melon, depending on the storage temperature, while virtually no reduction was observed on cut apple slices. The ineffectiveness of phage treatment on apple slices was attributed to low pH, a limiting factor for many phage applications (6). While many fruits have an acidity level that may render phage treatment ineffective, fresh vegetables' pH is often more suitable for bacteriophage. Additionally, the non-destructive nature of phage treatment also lends itself to application within the fresh vegetable industry. The objective of this study was to evaluate the efficacy of a phage lysate dip to control *E. coli* O157:H7 on fresh green peppers and baby spinach leaves.

### **3.3 Materials and Methods**

#### **Bacterial culture**

*E. coli* O157:H7 EDL933 was used in all experiments using green pepper as the vehicle.

*E. coli* O157:H7 GFP B6-914 was kindly provided from the lab of Dr. Jeffery LeJeune (The Ohio State University, Ohio Agricultural Research and Development Center) and was used in [Type text]

experiments for baby spinach. This strain does not produce Shiga toxin1 or Shiga toxin2, is resistant to ampicillin and cycloheximide, and produces the green fluorescent protein which allows for discrimination of the bacterium from normal biota under ultra-violet (UV) light. A loop-full of the frozen stock culture, stored at  $-80^{\circ}\text{C}$ , was used to inoculate LB broth (Becton, Dickinson and Co., Franklin Lakes, NJ) and was incubated at  $35^{\circ}\text{C}$  for 12 h with shaking. This activated overnight culture was used to inoculated fresh LB broth and incubated again at  $35^{\circ}\text{C}$  for 12 h for use within experiments. Cells of *E. coli* cultures were harvested by centrifugation ( $10,000 \times g$ , 3 min). The cells were re-suspended in phosphate-buffered saline (PBS, pH 7.0) for use in final experiments and the concentration of these cultures was determined to be  $\sim 10^9$  CFU/ml. However, to increase the load of initial bacterial population on spinach samples, the optimized spinach treatments used a 10X concentrated culture. Following centrifugation, the cell pellet was re-suspended in PBS 0.1times the volume of LB in which the culture had been grown. The concentration of this culture was determined to be  $\sim 10^{10}$  CFU/ml.

### **Phage preparations**

Crude phage lysates were prepared by incubation of the phage stock with the host microorganism, *E. coli* O157:H7 EDL933 in LB broth supplemented with  $\text{CaCl}_2$  (2 mM) for 48 hr at  $35^{\circ}\text{C}$  with shaking. The cultures were then centrifuged ( $10,000 \times g$ , 10 min) to separate solid matter from the supernatant. The supernatant was then filter-sterilized using a  $0.22 \mu\text{m}$  filter (Millipore, Billerica, MA). The filtrate (i.e., crude lysate) was used in preliminary studies. For the final experiments, the crude phage lysates were purified by ultracentrifugation ( $28,000 \times g$ , 3 h) to sediment the phage particles, followed by resuspension of the pellet in PBS. Phage preparations were titered prior to use and were determined to be  $\sim 10^8$  PFU/ml.

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**Fresh produce**

Green bell peppers were obtained from a local grocery store (Columbus, OH) and held at 6°C until use. Peppers were cut into large, flat slices (~2 in x 3 in) and transferred to Petri dishes. Sliced pepper was treated with UV in a biosafety cabinet for 1 hr on each side to eliminate background biota (4). The population of microorganisms prior to UV treatment was determined using the method described below and was found to be around 4 log CFU/g and following treatment population was found to be around 1 log CFU/g. Peppers were then aseptically cut into small pieces (<1 g) immediately prior to inoculation and 5 pepper pieces were used for each sampling point. Organic baby spinach was obtained from a local grocer and stored at 6°C. Spinach was divided into 50-g samples and no attempt was made to eliminate background biota.

**Inoculation of samples**

Produce was spot-inoculated with 10 µl of the 12-h bacterial culture in a biosafety cabinet. Preliminary testing used the overnight culture for inoculum, while optimized tests used overnight cells which had been pelleted and re-suspended in PBS. Spinach samples were inoculated with a 10-X concentrated culture. Green pepper pieces were inoculated on cut-edge surfaces to facilitate absorption while spinach leaves were spot inoculated on uninjured surfaces. Inoculated produce was allowed to dry for 1 h at ambient temperature prior to treatment.

**Treatments**

Phage treatment in preliminary testing was administered as a crude lysate. Green pepper pieces were dipped in a beaker of lysate with mixing for either 2 or 5 min. Pepper pieces were also treated with a control treatment to account for reduction in bacterial population due to the action of the rinsing, but not the phage. This control was a dip for either 2 or 5 min in a beaker of LB broth with mixing. Another set of control samples were simply transferred directly to incubation without treatment was also in place. Pepper pieces were stored at either 4°C or 25°C

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for both 2 and 5 min dip treatments. Preliminary spinach treatment was administered as a dip in crude lysate for 2 min with mixing. A 5-min treatment was not used with spinach due to the potential for damaging the fragile leaves. Spinach leaves were then incubated at either 4°C or 25°C. The controls used in green pepper experiments similarly were used for spinach experiments. Produce samples were transferred to stomacher bags for incubation following treatment.

Optimized conditions were determined based on the preliminary findings. For the final experiments, purified lysate re-suspended in PBS was used in phage treatments while the control treatment dip was PBS alone. Pepper pieces were dipped for 5 min and were stored at either 4°C during incubation, or were incubated with a combination of 4 h at 25°C then transferred to 4°C for the remainder of the incubation time. Spinach samples were dipped for 2 min and stored under the same conditions, either 4°C during incubation, or were incubated with a combination of 4 h at 25°C then transferred to 4°C for the remainder of the incubation time. The same controls used in preliminary testing were in place under the optimized experiments as well. Samples were plated immediately following treatment, at 4 h following treatment, 24, 38, and 72 h following treatment to enumerate bacterial population. Phage titer was also determined at each of these time points as well.

### **Recovery of bacteria**

At periodic sampling time points, 0.1% peptone water was added to produce samples. Pepper samples were stomached for 2 min and the fluid was diluted and spread-plated onto LB plates (Becton, Dickinson and Co., Franklin Lakes, NJ). Spinach samples were similarly prepared, but were instead plated on LB supplemented with 100 µg/ml each ampicillin and cycloheximide (Sigma-Aldrich, St. Louis, Mo). All plates were incubated at 35°C for 24 h.

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### **Recovery of phage**

After produce samples were stomached at periodic time points, fluid from pepper samples was serially diluted and used as the phage lysate. Fluid from spinach samples was filter sterilized using a 0.22  $\mu\text{m}$  pore size syringe driven filter unit (Millipore, Billerica, MA) and used as the phage lysate. Serial dilutions of the phage lysates were prepared and aliquots of these dilutions were incubated with the host at room temperature for 45 min. The incubated aliquots were added to molten LB soft agar supplemented with  $\text{CaCl}_2$  and overlaid on an LB agar plate. Plates were incubated at 35°C for 24 h and the resulting PFUs were quantified to determine phage titer.

### **Statistical analysis**

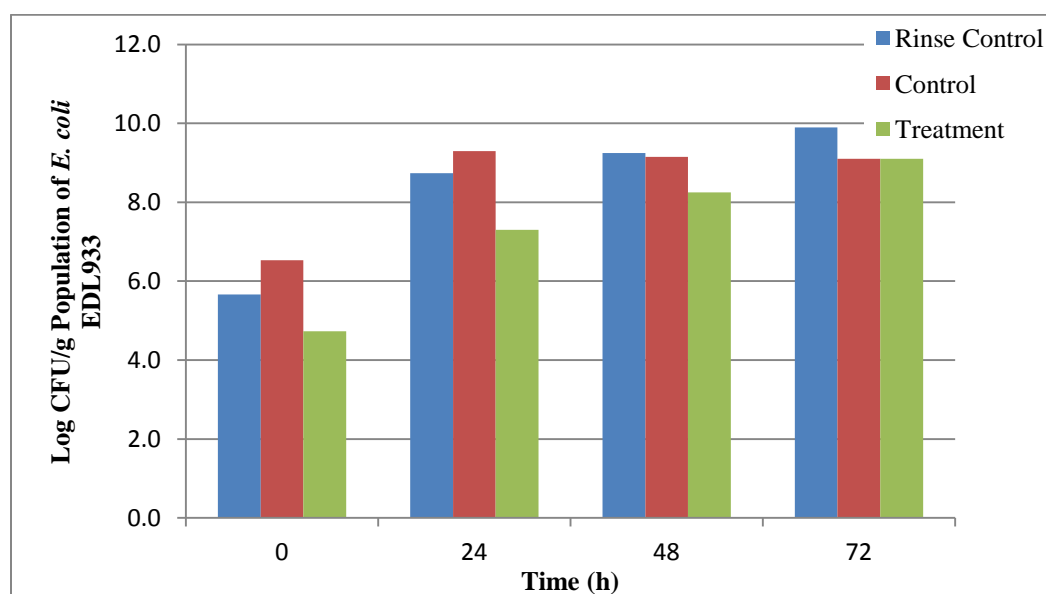
The final, optimized experiments were run in triplicate for each treatment condition (i.e. combination of dip time and incubation temperature). Total *E. coli* populations and phage titers were averaged for the three replicates and converted to logarithmic values prior to statistical analysis. Comparisons between treatments, controls and phage dip, were made using Statistical Analysis Software (SAS9.2, SAS Institute Inc., Carey, NC). Differences were analyzed using the model: population =  $\mu$  + treatment + refrigeration + time + treatment\*time + treatment\*refrigeration + refrigeration\*time + treatment\*time\*refrigeration + error, where  $p < 0.05$  was considered significant.

## **3.4 Results and Discussion**

### **Preliminary Green Pepper Treated with a 2 Minute Dip**

The preliminary results using a 2-min dip in crude lysate as a phage treatment and a 2-min dip in LB as a “rinse control” indicate that the phage treatment caused 1 log reduction and the rinse-alone provided an additional 1 log reduction (Figure 3.1). The reduction due to rinse  
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alone was obtained from the difference between the “control” sample which was inoculated and immediately plated following drying and the “rinse control” sample which was inoculated, dried, and dipped for 2 min with mixing in LB. The population of *E. coli* EDL933 was lower on the treated sample compared to the controls until 72 h of storage when the population of the phage treated sample and the untreated sample were approximately the same.

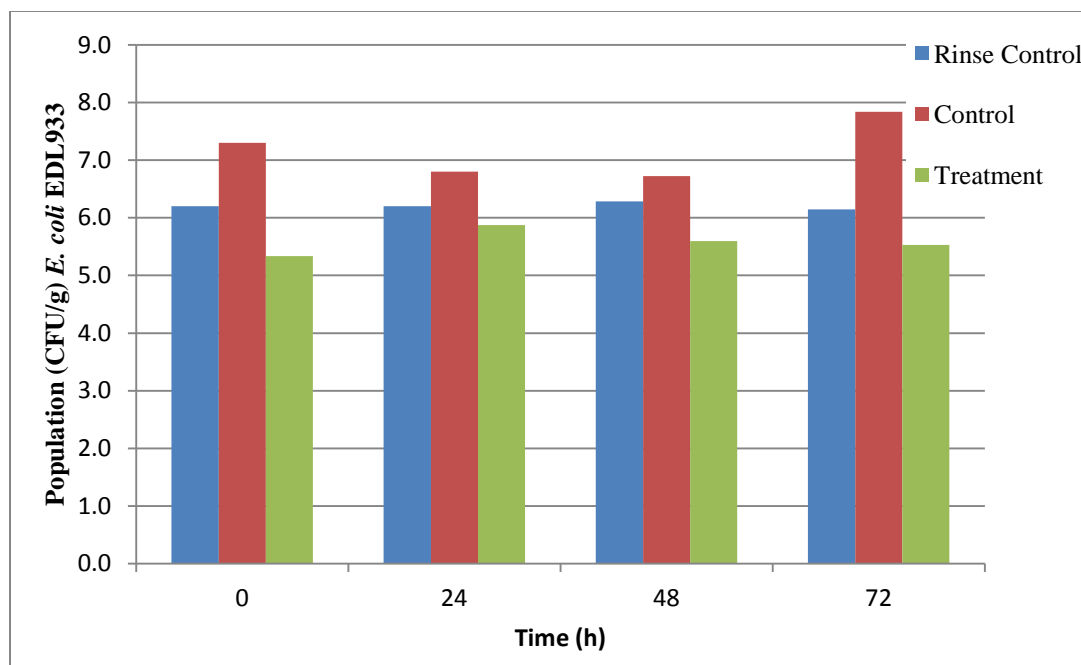


**Figure 3.1:** *Escherichia coli* EDL933 population on green pepper pieces following a 2-min dip in crude lysate and incubation at 25°C

When this same experiment was repeated but with a storage temperature of 4°C instead of 25°C the initial difference between the phage treated sample and the rinse control was again approximately 1 log which was, again, in addition to the approximately 1 log reduction achieved from washing alone (Figure 3.2). However, comparison of the populations of the treated samples and the controls at 24 h reveals a potential effect of storage temperature. The difference in population between the treated sample and control at 24 h for the sample held at room temperature increased by about half a log from the populations of the samples at time 0. By

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contrast, the difference between the 24 h and 0 h populations on the samples incubated under refrigeration conditions actually decreased by about half a log.

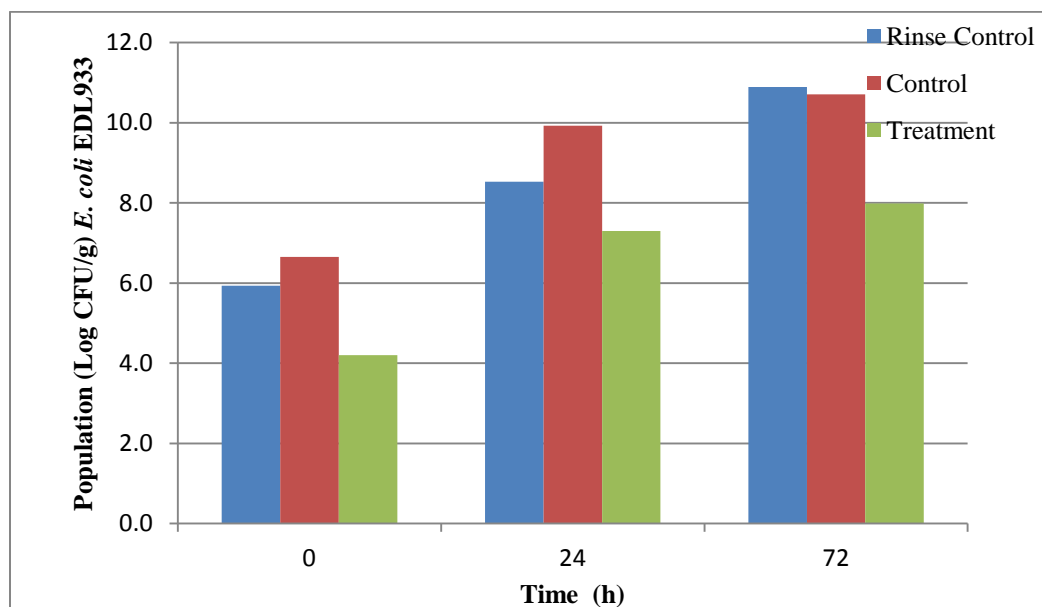


**Figure 3.2:** *Escherichia coli* EDL933 population on green pepper pieces following a 2-min dip in crude lysate and incubation at 4°C

### Green Pepper Treated with a 5-Minute Dip, Preliminary Experiments

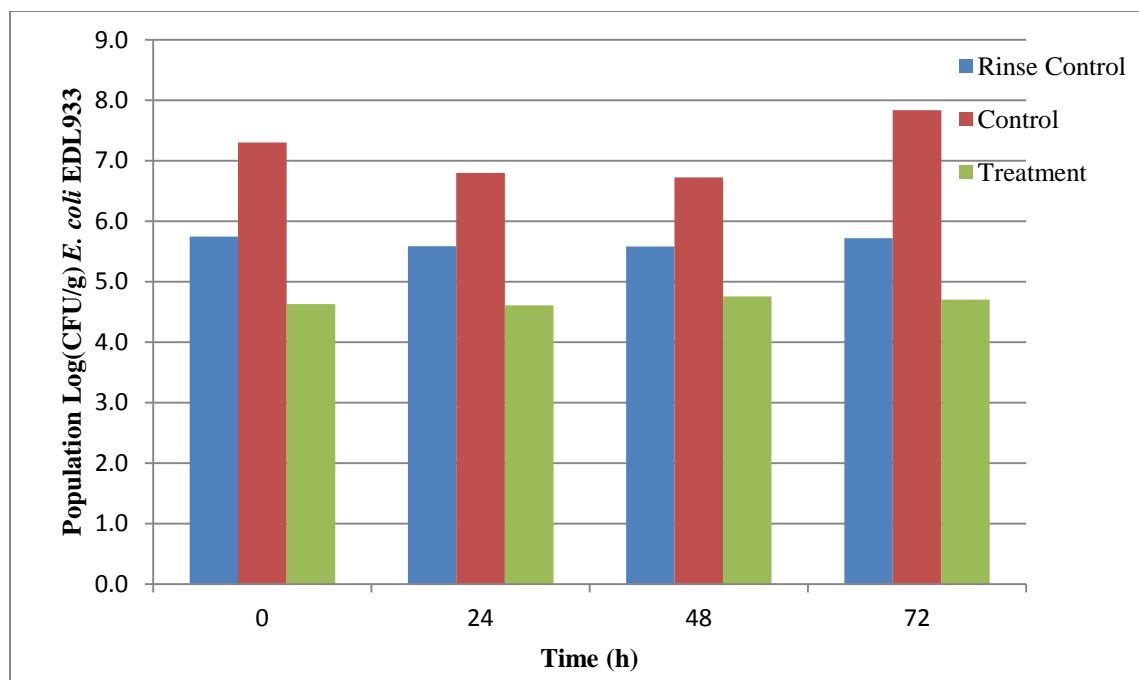
Increasing the dip time to 5 min and storing samples at 25°C increased the initial reduction obtained from phage treatment, compared to the rinsed control, to nearly 2 log (Figure 3.3). Perhaps more intriguing is the sustainability of this reduction over the storage period when compared to the same storage condition with the 2-min dip. After 3 days, the population of the target pathogen on pepper pieces treated with a 2 min dip was approximately the same as the population on pepper pieces without any treatment at all. The 5 min dip, however, decreased the level of recovery over the holding period and since the population on the control samples actually grew, as they were incubated at 25°C, the difference between phage treated and the controls increased over 72 h. storage.

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**Figure 3.3:** *Escherichia coli* EDL933 population on green pepper pieces following a 5-min dip in crude lysate and incubation at 25°C.

A 5-min dip with incubation at 4°C also had a greater initial reduction in target population, but storage over time decreased the difference to approximately 1 log after 24 h which was sustained throughout the remainder of the holding period (Figure 3.4). When compared to the same conditions with a 2-min dip, however, the differences between samples were reduced even further indicating that a 5-min dip not only increased the initial reduction of bacteria but also increased the reduction that was sustained over the course of 3 days. As no appreciable damage was observed to the quality of the pepper, a 5-min dip was established as an effective treatment to be used in the optimized studies with the purified lysate treatment.



**Figure 3.4:** *Escherichia coli* EDL933 population on green pepper pieces following a 5-min dip in crude lysate and incubation at 4°C

#### **Baby Spinach Treated with a 2 Minute Dip, Preliminary Experiments**

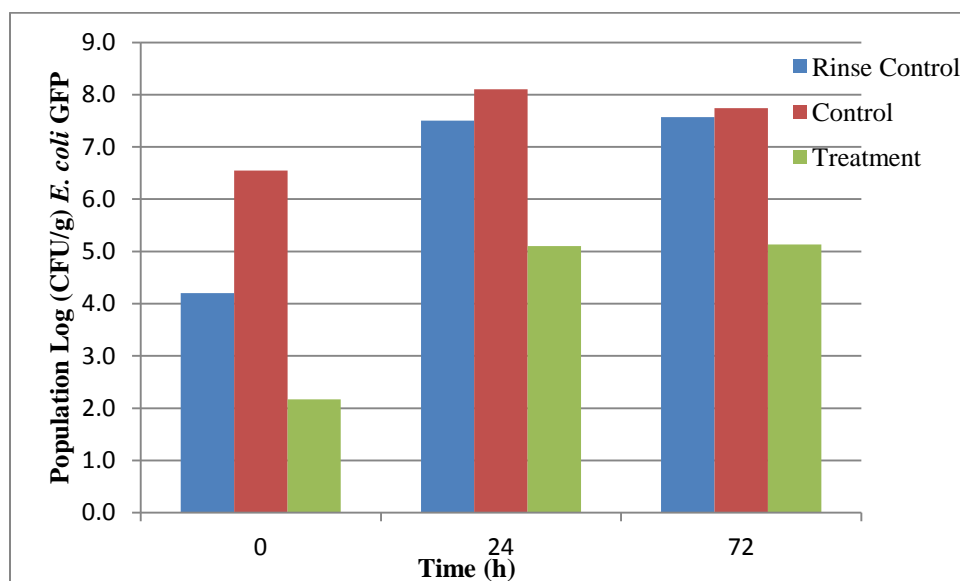
Preliminary testing on baby spinach leaves inoculated with GFP-labeled *E. coli* O157:H7

included a 2-min dip in crude phage lysate. A dip of 5 min was not used because of the fragility of the spinach leaves compared to the hardness of the green pepper. Even with only a 2 min dip, the reductions in target populations approached the detection limit of the enumeration method (2 log CFU/g). The action of the washing alone, determined by comparison of the untreated control (“control”) with the rinse control was responsible for a 2 log reduction (Figure 3.5). Compared to results using pepper as the vehicle, there was a greater loss of initial *E. coli* population due to rinsing of spinach leaves. This is perhaps a result of the difference between the inoculation methods between the two experiments. Green pepper pieces were spot-inoculated on cut-surfaces and inoculum clearly diffused into the tissue of the pepper. Conversely, spinach leaves were spot inoculated on undamaged surfaces and the inoculum was dried as a film on leaf surface. A 2-min dip with agitation proved more effective at removing the cells attached to the surface of the

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spinach leaf as opposed to the cells which may have been internalized into the tissues of green pepper. This difference may also contribute to the effectiveness of the phage treatment since attachment of the phage to the host bacterium is a crucial step in lysis and is impacted by the ease of contact between the phage lysate and the target.

The initial reduction in target population resulting from the phage treatment, compared to the rinsed control, was approximately 2 log. Storage at 25°C lead to growth of *E. coli* on both the treated spinach samples and the controls. At the 72 h mark, the difference between the controls and the treated samples was approximately 2.5 log. Although, the population on the treated sample increased from the initial value of ~2 log CFU/g to ~5 log CFU/g as the samples were incubated at room temperature for 3 days.

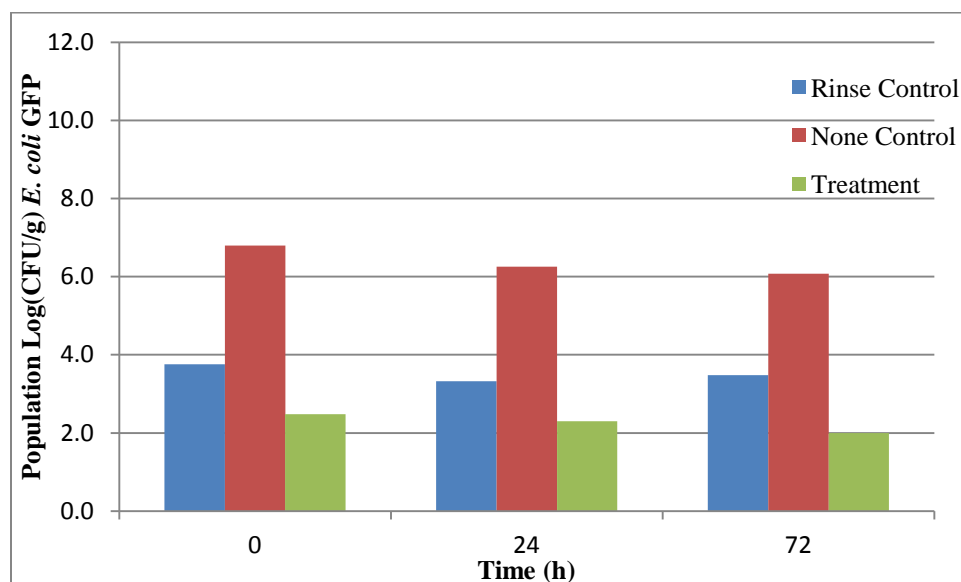


**Figure 3.5:** *Escherichia coli* GFP population on baby spinach leaves following a 2 min dip in crude lysate and incubation at 25°C

Treatment of spinach leaves with a 2 min dip in crude lysate and storage at 4°C caused similar decreases in *E. coli* population as that of rinsing (Figure 3.6). As previously observed, the subsequent reduction due to phage treatment yielded an initial population on treated samples that

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approached the detection limit of this method. To avoid this problem in optimized studies, 1 ml of stomached fluid was plated (instead of 100  $\mu$ l) and a 10X concentrated overnight culture was used in inoculation. While the initial reduction was not as great with samples immediately transferred to refrigeration for storage, the total population of *E. coli* was maintained at far lower levels than that achieved under storage conditions of 25°C. Based on these differences in the preliminary observations, it was determined that a 4°C storage condition would be most appropriate for optimized studies. The 25°C storage condition was eliminated since storage of fresh produce for 3 days at ambient temperature would not have practical application. However, to account for the increased initial reduction in population observed at 25°C, another storage condition combining an initial holding period for 4 h at 25°C prior to transfer and continued storage at 4°C was selected for optimized studies as well. The preliminary results suggest that an initial storage temperature which allows for replication of the target may contribute to greater efficacy of the phage.



**Figure 3.6:** *Escherichia coli* GFP population on baby spinach leaves following a 2 min dip in crude lysate and incubation at 4°C

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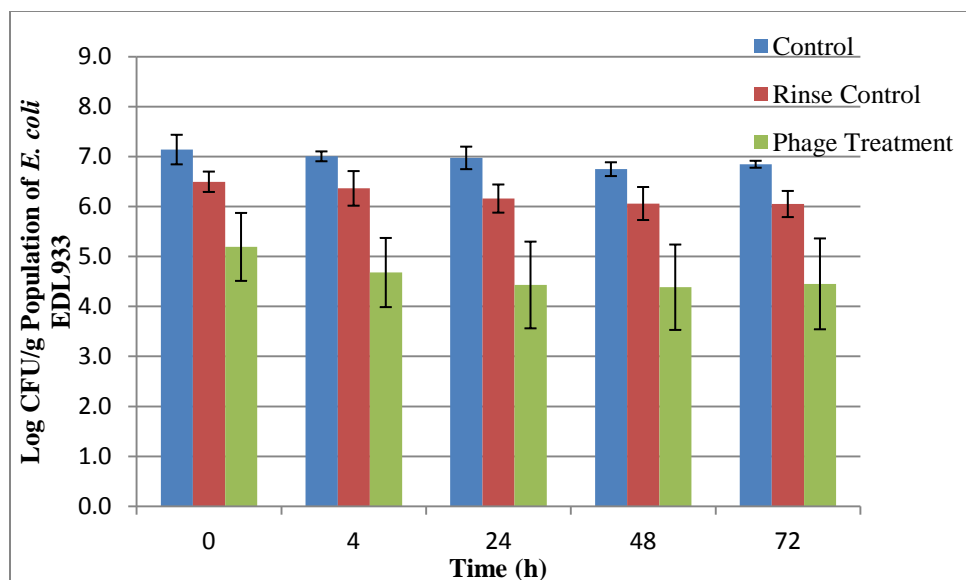


From the preliminary studies using crude lysate the final, optimized conditions to be tested using a purified lysate treatment were determined. For green pepper, a 5 min dip was selected over a 2 min dip and the storage conditions of 4°C (refrigeration) for the entire holding period in addition to samples initially held at 25°C (room temperature) for 4 h then transferred to 4°C for the remainder of the 72 h hold time were to be tested. Since baby spinach has delicate leaves, only a 2 min dip was considered. Samples were also to be treated under the two aforementioned storage conditions used for green peppers. Additionally, the method of inoculation and enumeration would be adjusted to account for detection limits. The inoculum would be concentrated and a greater sample volume would be plated so that differences in population could be more accurately identified. The change in inoculum medium may also be a contributing factor to loss due to rinsing as previous studies on the treatment of leafy greens with a rinse have indicated a 1 log reduction in bacterial population to be a reproducible finding when the culture is suspended in buffer (10).

#### **Green Pepper Treated with a 5 Minute Dip, Optimized Experiments**

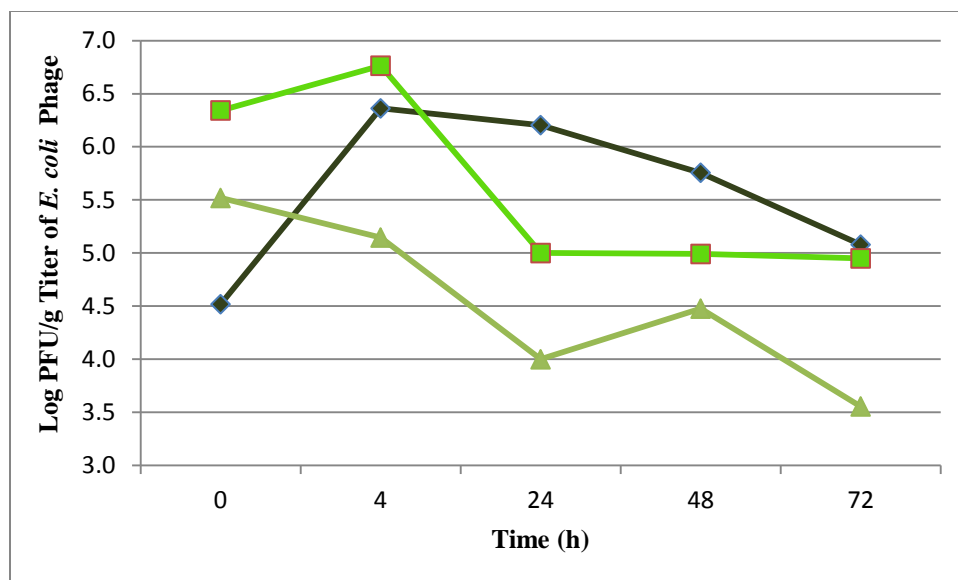
In all optimized studies, green peppers were treated with a 5 min dip in purified phage lysate as the treatment and a dip in buffer as a rinse control treatment. Pepper pieces incubated at 4°C without either dip treatment had an *E. coli* population of ~7 log CFU/g throughout the duration of the study (Figure 3.7). For pepper pieces rinsed in buffer, that load was reduced between 0.5 and 1 log so that the population was ~ 6 log CFU/g pepper for the rinse control pieces. Finally, the *E. coli* populations on phage treated samples were additionally reduced between 1 and 1.5 log compared to the rinse control samples so that the final *E. coli* population on treated pepper pieces was ~4.6 log CFU/g which was sustained throughout the holding time.

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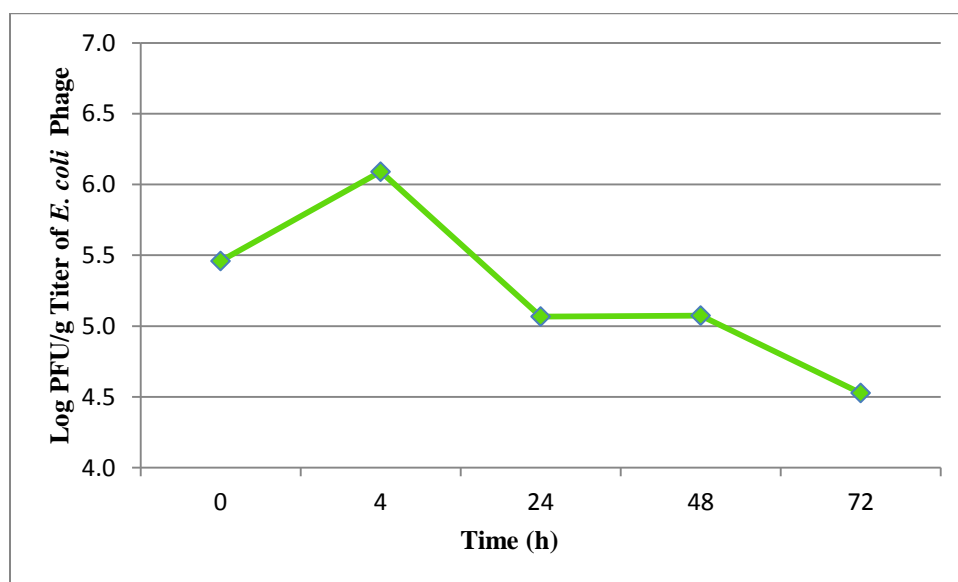


**Figure 3.7:** *Escherichia coli* EDL933 population on green pepper pieces following a 5 min dip in purified lysate and incubation at 4°C

The phage titer was also determined at each of the sampling points (Figure 3.8). A great deal of variability exists in phage titers among the three trials, not only in terms of starting titer, which varied by about 2 log, but also in terms of how the titers change over time. Despite this variability, the trend was an increase in titer during the first few hours followed by a steady decline during the remainder of the storage (Figure 9). However, the time point at which the titer starts to decline is not consistent among the trials, increasing error for the average titer.



**Figure 3.8:** *Escherichia coli* phage titer on green pepper pieces following a 5 min dip in purified lysate and incubation at 4°C for 3 trials

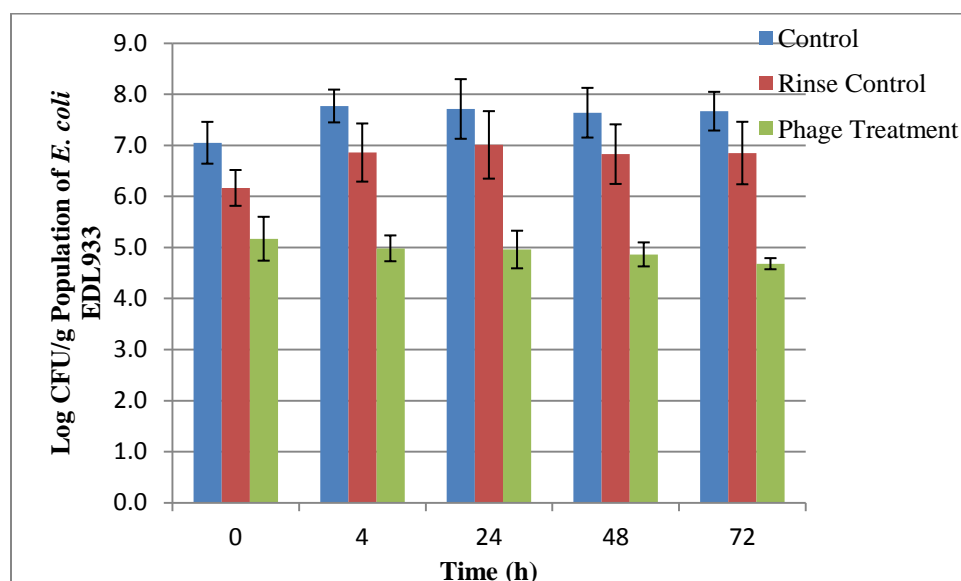


**Figure 3.9:** Average *Escherichia coli* phage titer on green pepper pieces following a 5 min dip in purified lysate and incubation at 4°C for 3 trials

It seems that incubation of phage-treated green pepper at room temperature for 4 h allowed for growth of the target microorganism which may better facilitate the lytic activity of the phage. However, this temperature also promotes the growth of the target microorganism on

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both controls, and the growth of other bacteria present on the product as well. Although the initial populations on all samples were similar to those in the green pepper study incubated at 4°C immediately following treatment, the control populations increased following the 4 h incubation at 25°C. At the 4 h time point, the control and the rinse control populations had increased by more than 0.5 log (Figure 3.10). The pepper pieces treated with a phage lysate dip had an *E. coli* population that was slightly lower than the initial count. However, this count was greater than that for the treated pepper pieces incubated under refrigeration conditions following treatment (Figure 3.7).

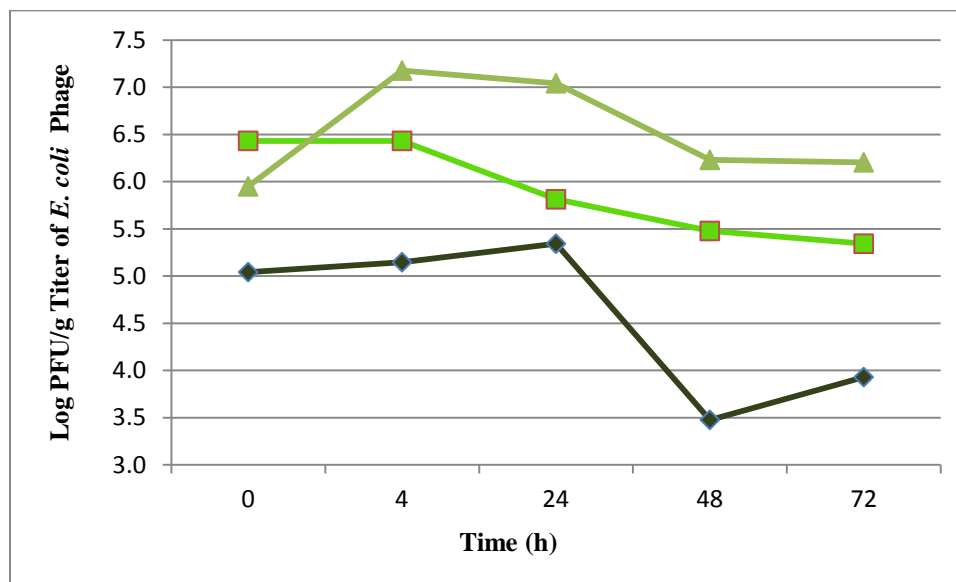


**Figure 3.10:** *Escherichia coli* EDL933 population on green pepper pieces following a 5 min dip in purified lysate and incubation at 25°C for 4 hours followed by incubation at 4°C

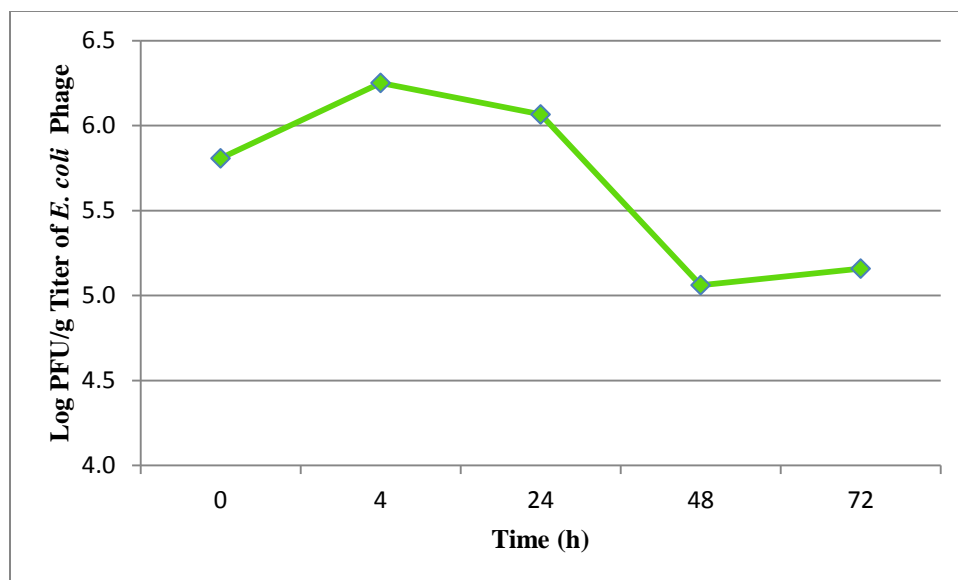
Phage titers for three trials with a preliminary room temperature incubation (Figure 3.11) showed a great deal of variability in initial titer and time point at which titer started to decrease, similar to the trends and variability observed in trials incubated under refrigeration conditions for the entire holding period (Figure 3.8). The average for the three trials incubated at 25°C followed by storage at 4°C also initially increased in titer before a decrease in phage titer as storage

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progressed. Perhaps a notable difference between the phage titer for pepper pieces with an initial room temperature holding period and titers for pepper pieces stored entirely under refrigeration is that pepper pieces with an initial room temperature holding period maintain a higher titer for a longer period of time before concentration begins to decrease (Figure 3.12) The average titer for the combination storage experiment shows a considerable decrease in titer concentration after 24 h while titer concentration for refrigerated samples declines after the 4 h time point.



**Figure 3.11:** *Escherichia coli* phage titer on green pepper pieces following a 5 min dip in purified lysate and incubation at 25°C for 4 hours followed by incubation a 4°C for 3 trials



**Figure 3.12:** Average *Escherichia coli* phage titer on green pepper pieces following a 5 min dip in purified lysate and incubation at 25°C for 4 hours followed by incubation at 4°C for 3 trials

The optimized green pepper experiments stored at both conditions showed a significant decrease in *E. coli* EDL933 population from samples inoculated but not dip treated (“control”) and samples dipped in buffer (“rinse control”) indicating that the action of the rinsing alone accounted for a significant reduction in bacterial load. However, there was also significant reduction in bacterial load between samples dipped in buffer (“rinse control”) and samples dipped in purified phage lysate (“phage treatment”) for both storage conditions, indicating that the action of the phage had a significant effect on reducing target population separate from the effect of the washing itself.

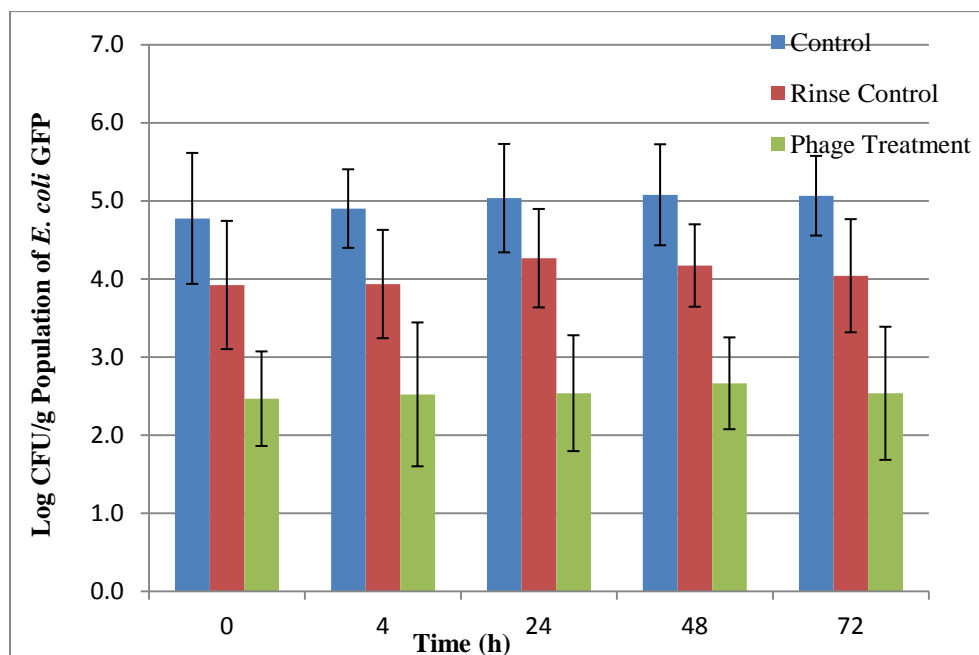
The storage conditions also impacted the bacterial populations. Control pepper pieces stored initially at 25°C increased ~0.7 log for both control and rinse control samples during 4 h following treatment. There was no significant increase between these same time points for pepper pieces incubated at 4°C. Although the increase was not as great for phage treated samples, there was still a significantly greater *E. coli* population on pepper pieces incubated with

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an initial room temperature holding period. Following transfer to refrigeration, there was no significant increase in target population for any treatment during the duration of storage.

### Optimized Baby Spinach Treated with a 2 Minute Dip

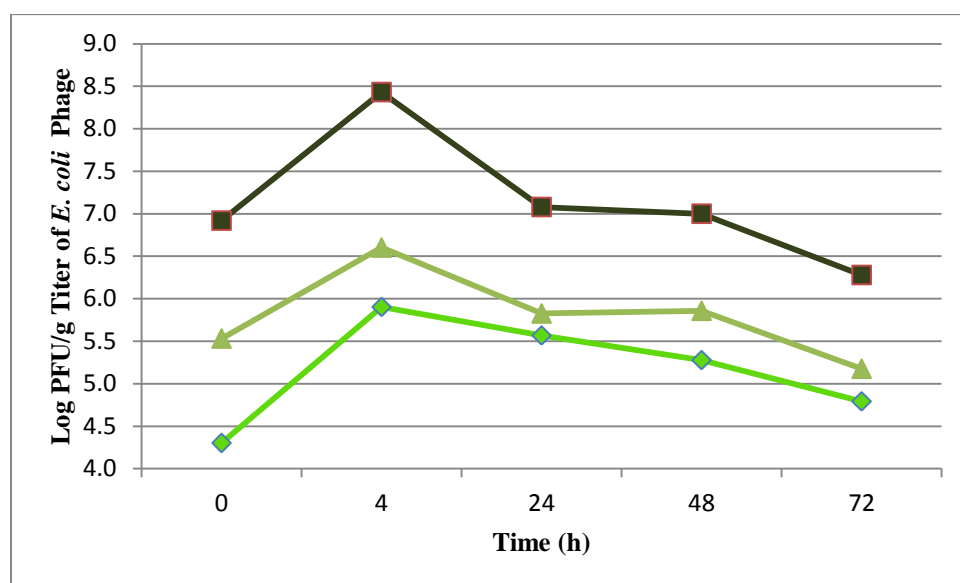
All optimized spinach treatment included a 2-min dip in purified phage lysate, . To increase initial population from the preliminary work, the inoculum was also concentrated and more of the stomached sample was plated. For spinach leaves stored at 4°C during the entire incubation, the population of GFP labeled *E. coli* was ~5 log CFU/g (Figure 3.13). Rinsing the baby spinach leaves in buffer decreased the *E. coli* population by ~1 log for a rinse control population of ~ 4 log CFU/g spinach. Compared to the rinse control, the phage treated samples reduced target population by an additional 2.5 log for a phage treated sample population of ~2.5 log CFU/g spinach.



**Figure 3.13:** *Escherichia coli* GFP population on baby spinach leaves following a 2 min dip in purified lysate and incubation at 4°C

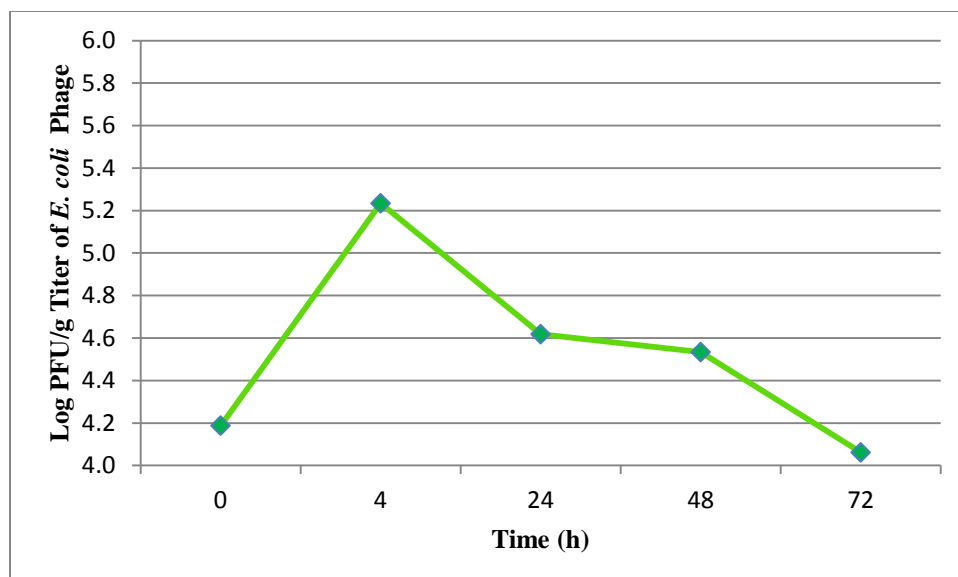
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While the spinach studies showed more variability in bacterial population, the phage titer taken throughout the course of the experiments showed more consistency in terms of the pattern of change (Figure 3.14). Initial titer levels still varied by more than 2.5 log, but for spinach incubated at 4°C the titer initially increased but following the sampling point at 4 h after treatment, the titer progressively decreased for the remainder of the study. The average of these three trials (Figure 3.15) shows a similar pattern for phage titers from the green pepper studies. Both start with an initial increase in phage titer followed by a prolonged decline. However, unlike the green pepper phage titers, the phage concentration on baby spinach increases and decreases much more uniformly over storage.



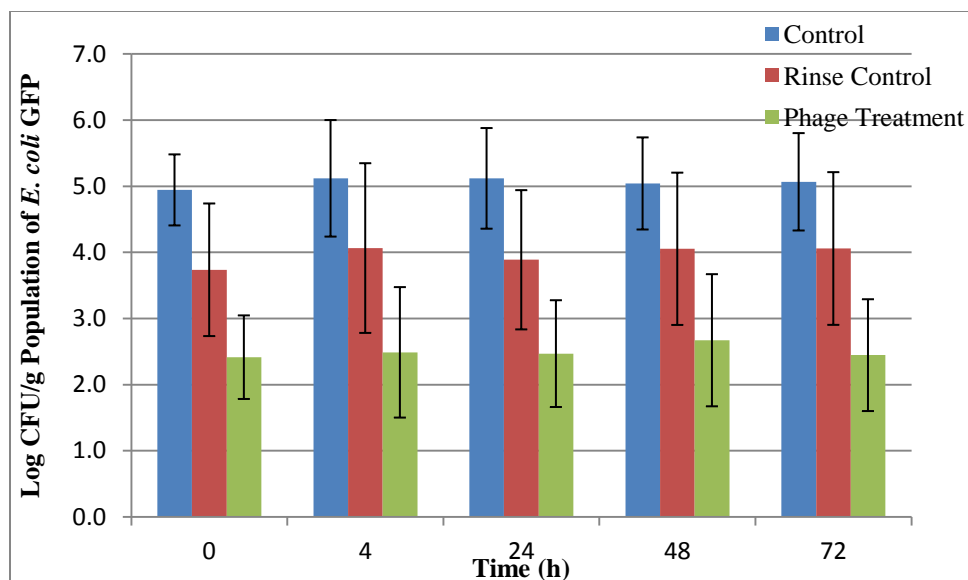
**Figure 3.14:** *Escherichia coli* phage titer on baby spinach leaves following a 2 min dip in purified lysate and incubation at 4°C for 3 trials





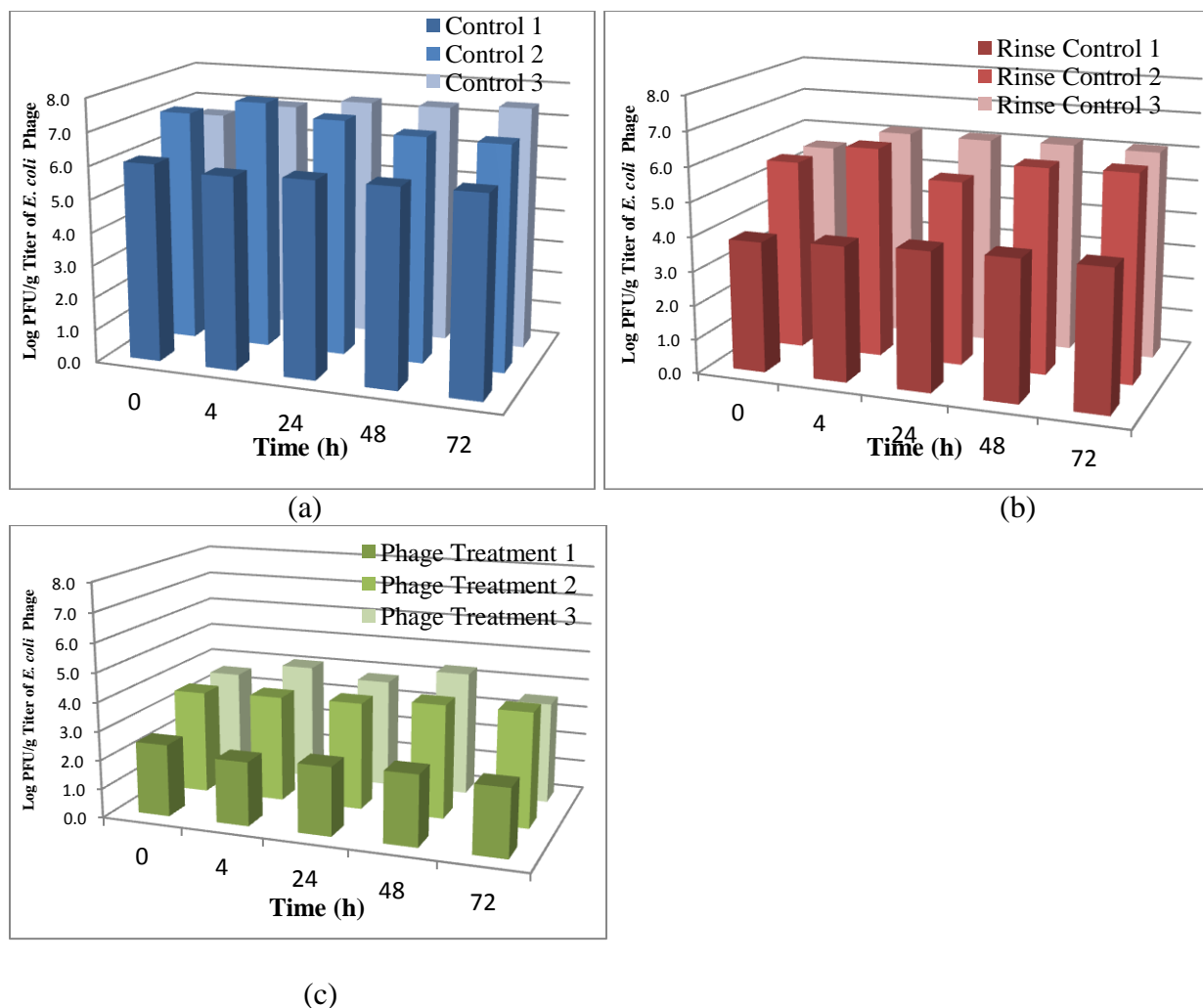
**Figure 3.15:** *Escherichia coli* phage titer on baby spinach leaves following a 2 min dip in purified lysate and incubation at 4°C for 4 hours followed by incubation a 25°C for 3 trials

Baby spinach leaves treated and stored at 25°C for 4 h then transferred to refrigeration had similar population values for control, rinse control, and phage treated samples. The starting population for control samples was ~5 log CFU/g spinach (Figure 3.16). Rinsing samples reduced target population by about 1 log to ~4 log CFU/g spinach. The average *E. coli* population on phage treated samples was ~2.5 log CFU/g spinach. There was no significant difference between the two refrigeration conditions in the spinach studies. Additionally, the target population did not change significantly over time for either incubation temperature for treatments applied to spinach.



**Figure 3.16:** *Escherichia coli* GFP population on baby spinach leaves following a 2 min dip in purified lysate and incubation at 25°C for 4 hours followed by incubation at 4°C

Optimized spinach studies had more variability from trial to trial across all treatment conditions as indicated by the large error (Figure 3.16). The variability in initial population may account for this variability since within a trial log reductions resulting from various treatments were fairly consistent (Figure 3.17). As shown in the figure below, the same trial with highest initial population on the control (Figure 3.17 (a)) as indicated by the lightest blue bar is also the same trial with the highest population on the rinse control (Figure 3.17 (b)) and phage treated (Figure 3.17 (c)) baby spinach leaves as indicated by the lightest bars on those respective graphs. Among all three trials, the differences between populations for spinach leaves were consistent while the initial population levels varied considerably.

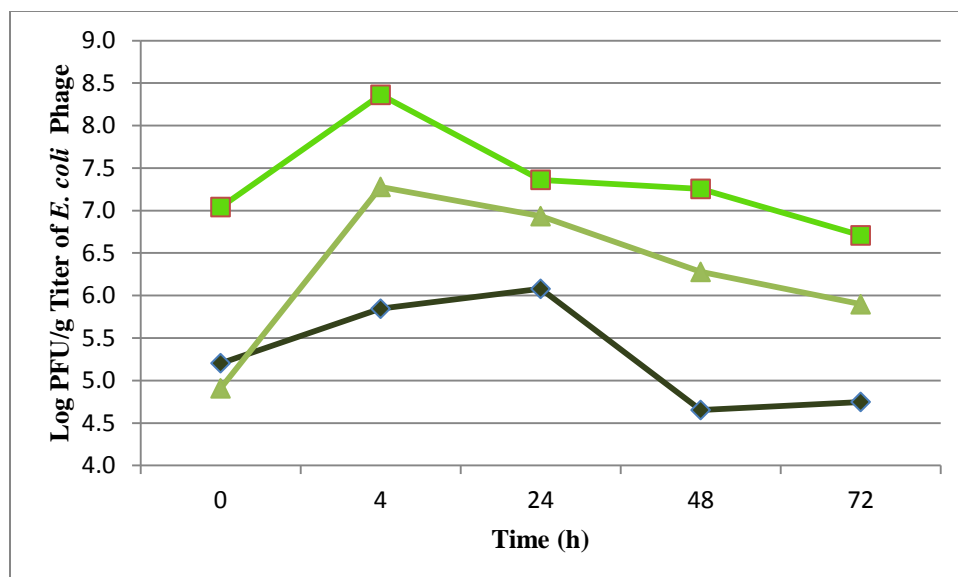


**Figure 3.17:** *Escherichia coli* GFP population on baby spinach leaves incubated at 25°C for 4 hours followed by incubation a 4°C for: (a) control samples for 3 independent trials (b) rinse control samples for 3 independent trials (c) treated samples for 3 independent trials\*

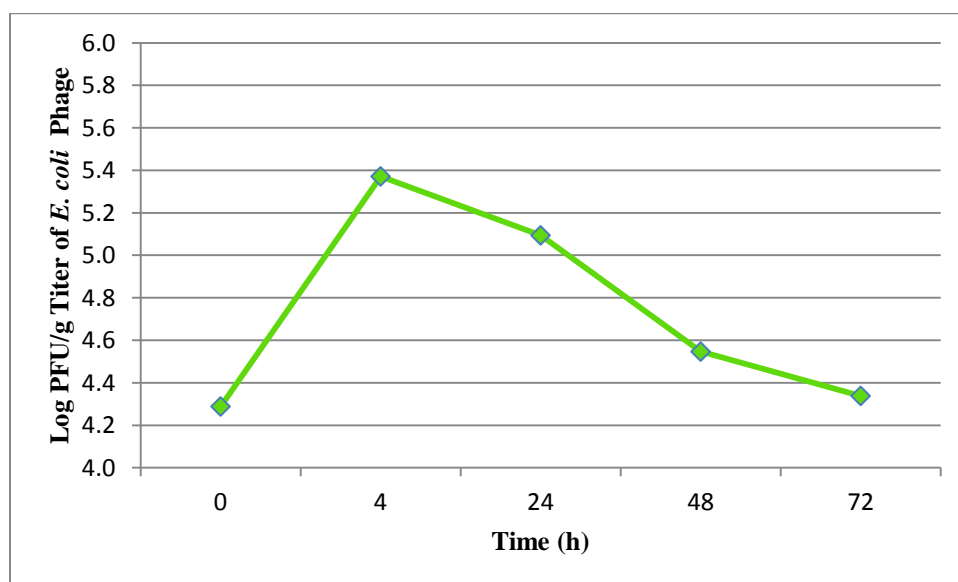
\*Samples were treated with a 2 min dip in purified lysate

Phage titers for baby spinach leaves incubated at 25°C then transferred to 4°C for the remainder of storage were also measured for three trials (Figure 3.18). Again, initial population varied greatly but the average of these trials showed the general trend of an initial increase followed by a decline throughout the storage period (Figure 3.19). This pattern was observed among all storage conditions for both green pepper and baby spinach studies.

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**Figure 3.18:** *Escherichia coli* phage titer on baby spinach leaves following a 2 min dip in purified lysate and incubation at 25°C for 4 hours followed by incubation a 4°C for 3 trials



**Figure 3.19:** Average *Escherichia coli* phage titer on baby spinach leaves following a 2 min dip in purified lysate and incubation at 25°C for 4 hours followed by incubation a 4°C for 3 trials

Optimized spinach studies showed a significant decrease between GFP-labeled *E. coli* populations on control baby spinach samples and target populations on baby spinach leaves dipped in buffer, indicating that the action of the washing alone accounted for a significant

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reduction in bacterial load. Additionally, baby spinach leaves dipped in purified phage lysate had significantly lower *E. coli* populations when compared to rinse control samples, indicating that the action of the phage significantly reduced the amount of target on the produce. However, there was not a significant difference between the two incubation temperatures for spinach studies and populations did not significantly change over time for any of the treatment conditions.

### 3.5 Conclusion

Preliminary studies assessing the application of phage treatments for the control of *E. coli* O157:H7 were used to determine which dip times and storage conditions were most effective for the control of the target microorganism. The initial work with green peppers indicated that a 5 min dip in phage lysate increased the initial reduction of the target microorganism and limited the re-growth of the target over the storage period. Not surprisingly, storing the produce at 4°C also limited the re-growth of the target when compared to re-growth resulting from storage at 25°C for the entire holding period. However, produce stored at 25°C had a greater initial reduction of *E. coli* and the difference sustained between the controls and the phage treated samples was greater as well. For this reason, two storage conditions were selected for optimized testing. Both storage at 4°C and an initial storage at 25°C for 4 h then transfer to 4°C for the remainder of the three day storage period were used for green pepper and spinach studies.

Pepper pieces treated with a 5 min dip in purified phage lysate and incubated under refrigeration conditions had a target population that was ~2.5 log lower than the control pepper pieces and ~1.5 log lower than the rinse control pepper pieces which was significantly different from both control treatments. Pepper pieces treated with a 5 min dip in purified phage lysate and incubated for 4 h at 25°C and then for the remainder of the three days at 4°C had a target population that was ~2.7 log lower than the control pepper pieces and ~2 log lower than the rinse [Type text]

control. Although the reductions for pepper pieces incubated under the combination storage condition were greater than those achieved from refrigeration incubation, the total population for pepper pieces stored in the combination of temperatures was significantly greater than that of the population on refrigerated samples. This suggests that immediate refrigeration following a 5 min dip in phage lysate is the most promising treatment condition.

For spinach samples, there was not a significant difference between populations on spinach leaves incubated at 4°C compared to spinach leaves incubated for 4 h at 25°C and then for the remainder of the three days at 4°C. Both had control populations of ~5 log CFU/g spinach. The action of the washing accounted for ~1 log reduction so that the rinse control population was ~4 log CFU/g spinach. The phage treated samples had an *E. coli* population that was ~2.5 CFU/g spinach making them 2.5 log lower than the control and 1.5 log lower than the rinse control populations.

The results from these studies indicate that a dip in lysate of a single phage can reduce *E. coli* by approximately 2.5 log compared to untreated green pepper pieces and baby spinach leaves and 1.5 log compared to rinsed green pepper pieces and baby spinach leaves. This reduction may be increased by the use of a phage cocktail, which could add a synergistic effect and broadens the spectrum of activity for the lysate dip. Produce treated with this phage lysate should be refrigerated following treatment to improve efficacy. Phage titer across all conditions increased initially then steadily declined as the storage period progressed. Further research is needed to assess the application of the phage for control of enterohemorrhagic *E. coli* in other food products, the effect of multiple phages in a cocktail on food safety applications, and the potential for a phage dip in combination with another hurdle technology.

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