Developing and Optimizing Bacteriophage Treatment to Control Enterohemorrhagic *Escherichia coli* on Fresh Produce

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1 **ABSTRACT**

2 Bacteriophages are potentially useful in controlling foodborne pathogens on minimally
3 processed products since phage application is a non-destructive treatment. The purpose of this
4 study was to evaluate the efficacy of a newly isolated environmental bacteriophage against
5 enterohemorrhagic *Escherichia coli* on fresh produce, and optimize the treatment with
6 consideration for potential application. Seven anti *E. coli* O157:H7 EDL933 bacteriophages were
7 isolated from various sources; the most promising was isolated from municipal wastewater. This
8 isolate (designated as *E. coli* phage OSY-SP) was propagated with the host, in a growth medium,
9 to a titer of 10^8 PFU/ml. Before inoculation into fresh produce, *E. coli* phage OSY-SP was
10 incubated with the host bacterium, spent medium was filter-sterilized, and the resulting crude
11 lysate was used as a source of phage inocula for preliminary experiments. For optimized testing,
12 phage in the crude lysate was purified by ultra-centrifugation and resuspension in phosphate-
13 buffered saline. Efficacy of phage treatments was determined as a function of fresh produce type
14 (cut green pepper or spinach leaves), treatment time (2 or 5 minutes rinsing), and temperature of
15 holding treated produce (4°C, 25°, or a combination of both temperatures). Cut green pepper was
16 treated with UV light, to eliminate background microbiota, then spot-inoculated with *E. coli*
17 O157:H7 EDL933 on cut edges, and the inoculum was allowed to dry. Because of its
18 susceptibility to damage, baby spinach leaves were not subjected to a decontamination treatment.
19 These leaves were inoculated with the green fluorescent protein-labeled *E. coli* O157:H7 B6-914
20 to facilitate inoculum enumeration in the presence of background microbiota. Phage suspension
21 was applied to the inoculated fresh produce that was subsequently held for three days under
22 variable storage conditions. The optimized phage treatment decreased the populations of
23 pathogenic *E. coli* by 2.4-3.0 log CFU/g on cut green pepper (5-min rinse) and 3.4-3.5 log CFU/g
24 on spinach leaves (2-min rinse), during 72 h storage. The majority of this decline was caused by

25 the antimicrobial action of the phage. These findings suggest the utility of bacteriophage to
26 selectively control pathogens on fresh produce.

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28 **Keywords:** Biocontrol, bacteriophage, food safety, enterohemorrhagic *Escherichia coli*, fresh
29 produce

1. INTRODUCTION

Biocontrol strategies offer a viable alternative to conventional antimicrobial treatments against bacterial pathogens. Processors often rely on preservatives or heat-based interventions to assure the safety of food. Ensuring the safety of fresh produce is inherently challenging; by definition, the product undergoes minimal processing only before consumption. Hence, it was not surprising that one-third of the major foodborne disease outbreaks, occurring in the U.S. between 2003 and 2007, were associated with contaminated fresh produce (Scharff, 2011).

Fresh produce receives minimal treatment between harvest and the time it reaches the consumer. Once harvested, produce is cooled to remove field heat, slowing enzymatic reactions and microbial growth. Maintenance of the cold chain reduces quality changes resulting from enzymatic and microbial activity (Jedermann et al. 2014). Under these conditions, the use of phage to control pathogenic contaminants on fresh produce could be challenging. Phage-mediated lysis is known to depend on host cell proliferation (Drulis-Kawa et al. 2012). The limited ability of pathogenic mesophiles to replicate under refrigeration conditions, while itself beneficial, may limit the utility of phage biocontrol strategies (Garcia et al. 2006). Many researchers have reported the significance of target bacterium replication, in terms of its impact on phage efficacy (e.g., Abedon et al. 2011; Reaney and Marsh 1972). However, Sharma (2013) suggested the utility of lysis-from-without inactivation methods as useful in the biocontrol of foodborne pathogens in fresh produce, which are often stored under refrigeration for quality reasons.

Following cooling, produce may be subjected to additional processing such as cutting or shredding to become a ready-to-eat product (Kader, 2002). These manipulations increase lesions

and cut-edge surface area that create environments more conducive to pathogen proliferation than intact surfaces. It has been noted that the efficacy of phage treatments is highly dependent on the attributes of the food matrix (Bigwood et al. 2007; Choinska-Pulit et al. 2015; Geer 1988; Hudson et al. 2004).

Fresh produce is often treated with an initial washing step in a dip tank or on a conveyor. This aids in the removal of soil and other debris, as well as reducing microbial load (Kader, 2002). The water used for washing is likely to contain chlorine, or other commercial antimicrobial or surfactant products. The surfactant helps remove solid dirt and debris from the surface of the product while antimicrobial agents prevent cross-contamination (Raiden et al. 2003). However, several European countries have restriction on the use of such sanitizers. For example, Germany, Switzerland, the Netherlands, Denmark, and Belgium prohibit the use of chlorinated wash waters (Uyttendaele et al. 2014). Use of phage is a viable intervention approach that could also be integrated in these fresh produce processes. However, the phage treatment should be adapted to take into account the inactivation mechanism. The “lysis-from-within” mechanism, which occurs following assembly and release of progeny phages from the host, may be limited by the reduced proliferation of bacteria under refrigerated conditions (Maurice et al. 2013). Increasing the phage-bacteria ratio in the washing step may increase bacterial death by lysis-from-without (Goode et al. 2002; Kudva et al. 1999). Using concentrated phage suspensions can improve the treatment’s efficacy because the absorption and penetration of the cell envelope by multiple phage particles can be sufficient to cause cell death (Abedon, 2011; Wiggins et al. 1985).

Commercialization of phage preparation is limited by several factors. Similar to other intervention strategies, phage treatment is not likely to be effective against pathogens on all

products, or even on a given product that has undergone physical modifications (e.g., whole vs. cut). Although a phage preparation may be effective as a spray (or wash) on whole iceberg lettuce, it could be much less effective on whole romaine lettuce, because of the botanical differences between these two products. Phage treatment may also be more promising on bagged fresh-cut than it is on whole produce due to the increased accessibility to product surface. While consumers are likely to accept the concept of using natural antimicrobial agents in food, introduction of viruses as natural predators to harmful bacteria may require consumer education (Greer, 2005; Rimal et al. 2001; Wilcock et al. 2004). Additionally, phage treatment optimization is necessary to make this application a viable tool in ensuring food safety. The goals of this study were to isolate and select a phage that effectively inactivates pathogenic *Escherichia coli*, and optimize the bacteriophage treatment to control the targeted pathogen on different fresh produce.

2. MATERIALS AND METHODS

2.1 Bacterial culture. *E. coli* O157:H7 was used as the target bacterium in this study. Stock culture, stored at -80°C, was transferred to Luria Bertani (LB) broth (Becton, Dickinson and Co., Franklin Lakes, NJ) and incubated overnight at 35°C. The culture was transferred again in LB broth and incubated at 35°C for 12 h with shaking for use in bacteriophage isolation and purification experiments. For green pepper treatments, *E. coli* O157:H7 EDL933 (Ohio State University, food safety laboratory culture collection) was used as the inoculum, whereas the fluorescently-labeled *E. coli* O157:H7 B6-914 (J. LeJeune laboratory, The Ohio State University) was used in experiments for baby spinach. *E. coli* cells were harvested by centrifugation (10,000 x g, 3 min), re-suspended in phosphate-buffered saline (PBS, pH 7.0) to contain ~10⁹ CFU/ml for

use in food challenge experiments. However, to increase the initial bacterial load on spinach samples, the cell concentration in the suspension was adjusted to $\sim 10^{10}$ CFU/ml.

2.2 Purification of phage from the environment. Samples (~ 10 g each) of manure from cattle, sheep, and horse farms (The Ohio State University, Columbus, OH) and sewage effluent (Columbus, Ohio) were screened for the presence of phage active against *E. coli* O157:H7 EDL933 as described previously (Garcia-Aljaro et al. 2008). Manure enrichments were prepared by mixing a sample with 90 ml LB broth supplemented with 2 mM calcium chloride and incubating the mixture at $\sim 25^{\circ}\text{C}$ for ~ 1 h with shaking. Dilutions (1:10) prepared from enriched sewage and manure were centrifuged ($10,000 \times g$, 10 min). The supernatants (50 ml) were sterilized using a $0.22 \mu\text{m}$ pore size syringe-driven filter unit (Millipore, Billerica, MA). Filter-sterilized manure samples were inoculated with the target bacterium and incubated at 35°C for 48 h with shaking. Filtered sewage samples were mixed with equal volumes of 2X LB broth, supplemented with 2 mM CaCl_2 , and inoculated with the target bacterium, followed by incubation under the conditions described previously. Enriched samples were again centrifuged and filter-sterilized to prepare the “crude phage filtrate.”

2.3 Isolation of phage candidates. The presence of phage was confirmed by spotting 5 μl aliquots of the crude phage filtrate onto a lawn of the target bacterium on LB agar supplemented with CaCl_2 . Following overnight incubation at 35°C , the presence of zones of inhibition (i.e., plaques) indicated the presence of lytic bacteriophages. The crude phage filtrate was further purified to obtain isolates of a single phage as follows. Serial dilutions of the filtrate were briefly incubated (45 min) at $\sim 25^{\circ}\text{C}$ with the target bacterium; incubated culture was added to LB soft agar (0.75% agar w/w) supplemented with CaCl_2 and overlaid on a plate of LB agar. The agar plates were incubated at 35°C for 24 h. Isolated plaque forming units (PFU) were marked, and

agar of a single PFU was excised and re-incubated in growth medium with the target host before pour-plating again to obtain isolated plaques. The plaques were excised once more and the process was repeated a second time to achieve isolation of a single candidate phage.

2.4 Purified phage preparations. “Crude lysate” for the selected phage was prepared by incubation of the excised plaques with the host, *E. coli* O157:H7 EDL933, in LB broth supplemented with CaCl₂ (2 mM) at 35°C for 48 h with shaking. Following incubation, the culture was centrifuged (10,000 x g, 10 min), filter-sterilized and the resulting “crude lysate” was stored at 4°C until used in the preliminary studies. For the food challenge experiments, purified phage suspension was prepared from the crude phage lysates by ultracentrifugation (28,000 x g, 3 h) to sediment the phage particles, followed by resuspension of the pellet in PBS. Phage preparations were titered prior to use (as described later) and were determined to contain ~10⁸ PFU/ml.

2.5 Phage titration. Phage titration method was adapted from Wommack et al. (2009). Serial dilutions of phage lysate, or purified phage, were prepared and aliquots were incubated with overnight culture of the host bacterium at ~25°C for 45 min. The incubated aliquots were added to molten LB soft agar supplemented with CaCl₂ and overlaid on LB agar plates. These plates were incubated at 35°C for 24 h and the resulting PFU/ml was recorded as the phage titer.

2.6 Target sensitivity to the phage. Bacteriophage candidates were individually incubated with the target bacterium in LB broth supplemented with CaCl₂, 0.1% peptone water (Becton, Dickinson and Co.) with CaCl₂ (2 mM), or phosphate-buffered saline (pH 7.0) with CaCl₂ (2 mM). Cultures were incubated at 35°C for 8 h with shaking. 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. The phage causing the

greatest inactivation of the target bacterium was selected for further investigation (Watanabe et al. 1970).

2.7 Cross-reactivity. In addition to the target bacterium used for phage isolation, activity of phages against closely-related strains was examined. Activity of the phage isolates was first assessed by spotting the crude lysate (10^7 to 10^8 PFU/ml) on a lawn of the potential host bacteria. *E. coli* serotypes or strains (n=14) were transferred from a stock culture (frozen at -80°C) into LB broth and incubated at 35°C for 12 h with shaking. Phage isolates were spotted on lawns of various *E. coli* serotypes/strains. Observed inhibition of growth, as marked by clearing where the lysate was spotted, was denoted as susceptibility of the bacteria to phage lysis. Each susceptible strain was then used as the host bacterium to determine titer, as previously described. The degree of reactions (lysis) was characterized as described by Peters et al. (2010).

2.8 Genome size estimation. Pulsed field gel electrophoresis (PFGE) was used to estimate the genome sizes for bacteriophage isolates according to published protocols (Gutierrez et al. 2011; Lingohr et al. 2009). Phage-containing 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.

2.9 Imaging. The *E. coli* phage was imaged using a published procedure (Ackerman, 2009). Briefly, 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). Stained phage was loaded on copper microscopic grids (Electron Microscopy Sciences, Hatfield, PA) and transmission electron microscopy (TEM) was conducted (OSU Medical Center, The Ohio State University, Columbus, OH). The electron

microscope was calibrated using carbon line grating replica (Electron Microscope Sciences, Hatfield, PA).

2.10 Fresh produce. Green bell peppers were obtained from a local grocery store (Columbus, OH) and held at 4°C until use. Peppers were cut into slices (~2 in x 3 in) and transferred to Petri dishes. Sliced pepper was treated with UV in a biosafety cabinet for 1 h on each side of the slices to eliminate background microbiota (Han et al. 2001). The population of background microorganisms on pepper slices was determined using the method described later; the population was $\sim 10^4$ CFU/g before and ≤ 10 CFU/g after UV treatment. Pepper slices were then aseptically cut into smaller pieces (~1 g each) immediately prior to inoculation, and 5 pepper pieces were used for each sampling point. Organic baby spinach was obtained from a local grocer (Columbus, OH) and stored at 4°C. Spinach was divided into 50 g samples and no attempt was made to eliminate background microbiota to avoid product damage.

2.11 Inoculation of fresh produce. Samples of fresh produce were spot-inoculated with 10- μ l aliquots of the 12-h bacterial culture in a biosafety cabinet. Green pepper pieces were inoculated on cut-edge surfaces to facilitate absorption while spinach leaves were spot-inoculated on leaf surfaces with 10 x 10- μ l spots per 50 g. Inocula were allowed to dry for 1 h, at ambient temperature, prior to treatment.

2.12 Treatments. The phage-containing crude lysate was used in the preliminary optimization tests. *E. coli*-contaminated green pepper pieces were dipped in a beaker containing crude lysate (25°C) with mixing for 2 or 5 min. Control treatment involved dipping *E. coli*-inoculated green pepper in phage-free LB broth. Treated pepper was stored at 4°C or 25°C, for both the 2- and 5-min rinse treatments. *E. coli*-contaminated spinach leaves were treated with crude phage lysate for 2-min with agitation; the 5-min treatment was damaging to the fragile leaves, thus it was not

used further. Spinach leaves were then incubated at 4°C or 25°C. Control treatment for spinach leaves was prepared as described for the green pepper control.

Optimized conditions were determined based on the preliminary findings. For the final experiments, purified phage in phosphate-buffered saline solution was used to treat the fresh produce, while the control treatment involved dipping in PBS alone. *E. coli*-contaminated pepper was rinsed in phage suspension (0.5 liter) for 5-min and stored at either 4°C for 72 h or a combination of 25°C for 4 h and 4°C for the remainder of the 72-h incubation time. Inoculated spinach was rinsed for 2-min in the phage suspension (1 liter) and stored under the same conditions used to treat pepper. Surviving *E. coli* populations were enumerated at 4, 24, 48, and 72 h following the phage treatment. Phage titer also was determined at each of these time points.

2.13 Recovery of bacteria. Pepper samples were homogenized with peptone water in a stomacher and homogenates were diluted and spread-plated on LB agar (Becton, Dickinson and Co.). All colonies recovered from green pepper pieces were enumerated. Spinach samples were similarly prepared, but were plated on LB agar supplemented with 100 µg/ml each ampicillin and cycloheximide (Sigma-Aldrich, St. Louis, Mo). After incubation, *E. coli* O157:H7 colonies which produced green fluorescence under UV light were counted. Replica plating indicated no additional growth limitation to target bacteria grown on ampicillin-containing plates. All plates were incubated at 35°C for 24 h.

2.14 Recovery of phage. Homogenized produce samples were serially diluted and titered for phage as described previously. Homogenized spinach samples were filter-sterilized using a 0.22 µm pore size syringe-driven filter unit (Millipore, Billerica, MA) before phage titer was determined.

2.15 Data analysis. Optimized experiments were run in triplicate for each treatment condition and standard deviations for each time point were calculated. Comparisons between treatments, with or without phage rinse, were made using the Statistical Analysis Software (SAS), General Linear Method (GLM) procedure (SAS9.2, SAS Institute Inc., Carey, NC). Data were analyzed using the model:

$$\text{Log reduction} = \mu + \text{phage treatment} + \text{incubation temperature} + \text{storage time} + \text{error}$$

where $p < 0.05$ was considered significant. Log reduction = $\text{Log } N_0/N$ CFU/g, where N_0 is the initial population and N is the population at a given sampling time interval.

Statistical analysis revealed that time was not a significant factor (i.e., populations did not change significantly over time during storage). Therefore, results were presented as single values representing *E. coli* population reductions resulting from a given treatment. To measure treatment efficacy, reduction in *E. coli* population (on log CFU/g basis) was calculated as the difference between treated and untreated *E. coli* populations at the end of 72 h storage.

3. RESULTS

3.1 Isolation and selection of an effective *Escherichia coli* phage. Screening environmental samples (soil, wastewater, livestock manure) yielded seven promising phage isolates active against *E. coli*. These active phages were isolated from sewage and manure. Titers of isolated phages ranged from 10^4 to 10^8 PFU/ml following 48-h incubation with host bacteria. The highest-titer isolate was further assessed for potential use in food safety applications. Selected phage was incubated at 35°C with the host bacterium and produced an average titer of 2.5×10^8 PFU/ml; this was designated as *E. coli* phage OSY-SP. The genome size of this phage was estimated to be ~150 Kb using pulsed field gel electrophoresis. Bacteriophage genomes have been found ranging from 15 to 500 Kb (Gutierrez et al. 2011). Imaging by TEM showed it was a

tailed phage (Table 1) belonging to the family *Myoviridae* (Choinska-Pulit et al. 2015).

3.2 Characterization of the selected phage. The antibacterial efficacy of the *E. coli* phage OSY-SP was evaluated against 14 *E. coli* strains incubated at 35°C. Eight *E. coli* strains showed considerable sensitivity to the phage, and the corresponding phage titer ranged from 6.9 to 8.9 log PFU/ml (Table 1). Sensitivity was observed in both pathogenic and nonpathogenic *E. coli* strains. Three *E. coli* strains appeared resistant to infection; whereas three others had limited sensitivity but phage titer against these strains was not successfully measured. Of the seven *E. coli* O157:H7 strains tested, five showed relatively high sensitivity to this phage isolate.

Inhibition and inactivation of *E. coli* by the phage were tested in LB broth, peptone water, and phosphate-buffered saline (Fig. 1). Phage lytic activity increased when the host cells were dividing rapidly, but refrigerated fresh produce is not expected to support rapid bacterial growth. Therefore, a preliminary assessment of the effectiveness of the candidate phage against *E. coli* in media which limit the host growth is important in selecting an industry-relevant biocontrol agent. Additionally, re-growth of the host over the course of incubation would not be expected in a nutrient-poor medium. Consistent with these assumptions, *E. coli* in LB broth showed a typical growth curve, whereas growth was suppressed in the presence of the phage (Fig. 1A). Difference in the populations of treated and untreated *E. coli* was > 4 log within the first three hours of incubation. However, the population of *E. coli* recovered to its starting levels by the end of the 8-h incubation time. When *E. coli* was suspended in 0.1% peptone water, instead of LB broth, growth of untreated populations was impacted negatively and considerable inactivation was observed in the phage-treated culture (Fig. 1B). *E. coli* phages decreased the target population by about 3 log within the first hour of incubation and the difference in populations of untreated and phage-treated cultures was 5.6 log after 8 h of incubation. When *E. coli* cells were suspended in

phosphate-buffered saline solution, very little or no growth was observed and presence of phage decreased the population of the bacterium by 3.5 log, on average (Fig. 1C).

3.3 Preliminary phage testing on inoculated fresh produce. Cut green pepper and baby spinach leaves represent the fresh-cut and leafy-green categories of fresh produce, respectively. Background microbiota were relatively easy to eliminate by UV treatment of cut green pepper, hence this product was inoculated with a typical enterohemorrhagic strain, *E. coli* O157:H7 EDL 933, and the population was monitored by plating homogenized samples on LB agar medium. On the contrary, it was not feasible to eliminate background microbiota on spinach leaves without damaging the product. Therefore, this fresh produce was used without prior decontamination and was inoculated with the marker-containing strain, *E. coli* O157:H7 B6-914. The strain expresses green fluorescent protein (GFP) and antibiotic resistance, hence it was possible to enumerate the inoculated strain on spinach leaves using ampicillin and cycloheximide-containing selective agar medium and differentially count the green-fluorescent colonies under UV light. The crude lysate (10^8 PFU/ml) was used in phage treatments in this set of experiments. A short phage application time (2 min) was employed in order to minimize damage to spinach leaves. Population decrease due to mechanical action of rinsing was assessed by use of two controls: (i) products inoculated with *E. coli* O157:H7 with no further treatment (control-1), and (ii) products inoculated with *E. coli* O157:H7 and rinsed in sterile LB broth (control-2).

It took 4 h to prepare the fresh produce for testing; this covers the time between product inoculation until placement in storage. Once fresh produce was treated and placed in the correct storage condition, samples were immediately analyzed and this was considered the zero sampling time. Initial differences (at time zero) in *E. coli* populations between phage treatment and control-1 are due to the combined effects of phage-induced lysis of the target bacterium and the

rinsing action of the lysate solution used for washing the fresh produce. However, initial differences between phage treatment and control-2 are due to the phage action only.

Results of the preliminary experiments are summarized here, and average changes in *E. coli* population during storage are presented in Supplementary Figures S1-S7. An example of these data, for results of a single run, is shown in Fig 2. The initial anti *E. coli* action of the phage (i.e., at time zero), expressed as average reduction in log CFU/g, was 0.9 for cut green pepper and 1.7 for spinach leaves. The cut surfaces on the green pepper pieces may have trapped inoculated *E. coli* cells and made them less accessible to applied phage, hence the lesser population reduction on green pepper than on spinach leaves. After 72 h of storage at 4°C, the average difference in *E. coli* population attributed to phage action was 0.9 log for cut green pepper and 2.0 log for spinach leaves. Based on these data, presence of phage maintained a relatively constant difference in *E. coli* population between phage-treated and LB broth-dipped produce during the storage time.

Populations of *E. coli* on produce stored at 4°C remained relatively unchanged during storage, but they increased on products stored at 25°C, regardless of phage treatment. The increase was greater on spinach leaves than on cut pepper. The nutrients in the crude lysate may have supported some of the growth of *E. coli* observed on the fresh produce held at 25°C. These treatments, nevertheless, may represent an abuse scenario where heavily-soiled, microbially-contaminated product is held without refrigeration.

The initial reduction in *E. coli* populations, caused by the rinsing action of the crude lysate, was greater in spinach leaves than in cut pepper; the corresponding average log reductions for these two produce types were 2.7 and 1.0. Therefore, dipping fresh produce in the phage medium was more effective at removing the cells attached to the leaf surface of spinach as opposed to the

cells which may have been internalized into the tissues of the cut green pepper.

Increasing the rinsing time of cut green pepper in the crude lysate from 2 to 5 min augmented the population reduction achieved in phage treatment. The 5-min dip reduced *E. coli* populations initially by 2 to 2.5 log, and this reduction differential was sustained during the 72 h storage at 4°C (data not shown). As no noticeable damage was observed on the cut pepper by the 5-min rinse in phage suspension, this treatment was selected for further testing under the optimized studies.

3.4 Optimized phage treatment. Purified phage, suspended in phosphate-buffered saline, was used in these experiments. The same suspension medium was used to rinse the inoculated produce of control-2. Considering the growth of *E. coli* on produce at 25°C (e.g., Fig. 2), it was determined that the storage of phage-treated fresh produce at 4°C would be the most appropriate for optimization studies. However, a distinct advantage was observed in the first 24 h of storage at 25°C; i.e., considerable differences between *E. coli* populations on phage-treated and untreated produce were apparent. Therefore, the optimization experiments included a new storage condition, in which an initial holding period for 4 h at 25°C preceded the transfer of produce to the 4°C refrigeration, where it was held for the remainder of the 72 h storage. It was hypothesized that the initial storage at 25°C for 4 h is sufficient to increase phage titer through replication in the host without increasing the population of the pathogen. Considering the potential pathogen internalization through cut surfaces on green pepper pieces, a 5-min rinse in phage suspension was selected over a 2-min rinse. Baby spinach has delicate leaves that were adversely affected by the 5-min rinse; therefore, the 2-min rinse was selected for this phase of the study.

An example of the optimized study results, for one treatment condition, is shown in Fig. 3,

but pooled data are shown in Fig. 4. For all treatment conditions, incubation time was not found to be significant ($p > 0.05$), i.e., populations did not significantly change over the course of the 72 h period. Therefore, population reductions for each treatment are reported as a single value, the difference between the treated and untreated population levels at the end of 72 h storage (Fig. 4).

Phage treatment of contaminated baby spinach leaves reduced *E. coli* populations by 3.4-3.5 log under either storage condition (Fig. 4). Rinsing alone (i.e., without phage) caused 1.4 log reduction, while the additional 2 log reduction can be attributed to the antibacterial activity of the phage treatment. Phage treatment reduced the population of *E. coli* on cut green pepper, stored at 4°C, by 2.4 log (Fig. 4). The reduction in *E. coli* population on cut green pepper stored at 25°C/4°C for 72 h increased modestly to 3.0 log. Rinsing with phage-free medium decreased *E. coli* populations on cut green pepper by 0.8 log; thus presence of phage resulted in 1.6-2.2 log reduction. Holding produce at the 25°C/4°C storage condition, compared to 4°C, did not seem to improve the phage action considerably.

Phage titers on produce during storage were measured in triplicate. These trials showed the general trend of an initial increase followed by a decline throughout the storage period (Fig. 5). However, large variability among trials made differences in phage titers during produce storage statistically insignificant ($p > 0.05$). Additionally, phage titers on produce held initially at 25°C for 4 h and that held at 4°C for the whole storage period were not statistically significant ($p > 0.05$) at all sampling points.

4. DISCUSSION

Phage candidates active against enteric pathogens were readily isolated from environmental samples, and preliminary selections were made based on titer levels. Generally, a

higher titer is preferable since it indicates more replication of the phage or larger burst size and, as a consequence, more phage particles produced to perpetuate its lytic cycle. It may also indicate a high affinity of the phage for the target receptor displayed by the host. The concentration of phages in the crude lysate is dependent on many factors, but higher titer values suggest the sensitivity of the target to phage infection (Wiggins et al. 1985).

Titer is only one of the criteria for predicting phage efficacy in pathogen control. A limitation of using titer as a proxy for activity was illustrated by the results of the cross reactivity analysis (Table 1). Although 11 of 14 *E. coli* strains tested showed sensitivity to the candidate phage when the lysate was spotted on a lawn of target bacteria, three of these strains failed to produce successful phage titer because the resulting plaques were too faint to enumerate. Cross reactivity results also suggested that it would be desirable that food processors use a cocktail of phages active against a broader spectrum of pathogenic *E. coli* strains. A cocktail of phages not only increases the activity spectrum for the treatment (Leverentz et al. 2001), it also decreases the probability of resistance mutations developing within the target population (Hudson et al. 2004; O'Flynn et al. 2004; Stern and Sorek 2011).

Research on a single phage isolate is required for characterization of its potential efficacy. Previous findings have demonstrated that the use of a single phage can reduce target bacterium populations by approximately 1.0 to 2.0 log (Hudson et al. 2004; Sharma 2013). The *E. coli* phage isolated from sewage effluent was selected based on titer level, cross reactivity spectrum, and inhibition of target's growth in liquid culture. The selected phage was able to reduce pathogenic *E. coli* in a medium that supported its growth, as well as in media which were less conducive to bacterial growth (Fig. 1). This suggested that the selected *E. coli* phage would be successful in reducing target populations in a relatively unfavorable environment. Bigwood et al.

(2008) have shown that high phage-bacterial ratio is more effective in reducing both high and low levels of target pathogen. In the challenge studies described here, high levels of *E. coli* were applied to inoculated produce. However, in many instances of natural contamination, pathogens are present at relatively low levels in food (Bhagwat, 2003). This may prove challenging to commercialization as Kasman et al. (2002) found that in systems with low levels of bacterial targets, additional barriers to efficacy exist and, subsequently, that cell concentration and absorption time should be considered in determining a sufficient concentration of phage to apply in biocontrol strategies.

Preliminary results suggested that the combination of incubation conditions (initially at 25°C, followed by refrigeration at 4°C) for phage-treated produce would allow for growth of the target bacterium, which would facilitate the lytic activity of the phage while preventing later outgrowth of the host and other plant associated biota. However, optimized studies did not support this hypothesis (Fig. 4). No significant difference was observed between produce stored solely under refrigeration conditions compared to produce that was initially incubated at 25°C for 4 h. The discrepancy between the preliminary and optimized experimental findings may be explained by the differences in treatment and produce inoculation methods. Preliminary tests included an LB broth rinse as a control. Additionally, *E. coli* inocula were suspended in LB broth. This is in contrast to optimized studies where rinse treatments were done in PBS and bacterial inoculum was similarly suspended in PBS. The presence of residual media on the produce may have facilitated outgrowth of the host to a greater extent than what could be attributed to incubation conditions alone (Kocharunchitt et al. 2008).

Results of this study show that the phage treatment was effective under refrigerated storage conditions and does not require outgrowth of the host; this is in agreement with previous

investigations (e.g., Wiggins et al. 1985). The reduction in bacterial population may be due in part to lysis-from-without. Alternatively, bacteria may initially still be replicating new phage, even if the bacterial replication rate does not result in considerable population growth. On the contrary, cell death may result once phage-infected bacteria are plated onto growth media at which point replication is initiated and lysis-from-within occurs. Because of the lower attachment rate of some phages, including T4-like *E. coli* phages, it is impractical to rely only on lysis-from-within for the control of mesophilic pathogens in products which are stored in refrigerated conditions to preserve their quality (Hudson et al. 2004). Additional work is required to assess efficacy under conditions of lower bacterial host concentrations often present in natural contamination (Hagens and Loessner 2010; Kasman et al. 2002).

Comparing phage efficacy between the two produce types is difficult due to the difference in inoculation method and bacterial strains utilized. *E. coli* O157:H7 EDL933 was used to inoculate green pepper pieces following a decontamination step which reduced the aerobic mesophilic counts on the surface of the green pepper below the detection limit (10 CFU/g). The surface of baby spinach leaves was not decontaminated because of its inherent texture and fragility, so a fluorescently-labeled *E. coli* O157:H7 was used to enumerate the surviving population. Although efficacy of the phage was strongly affected by produce type ($p < 0.0001$), for which the difference in strain was likely not solely responsible. This difference may also be explained by the inoculation methods for 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 the leaf surface. Additionally, spinach leaves also harbored normal levels of commensal biota, which may impact not only the attachment and growth of the inoculated pathogen, but also compete

with the targeted bacteria as alternative phage receptors.

In conclusion, phage treatment was successful at significantly reducing target populations in the tested fresh produce. The addition of phage to the rinse buffer accomplished an additional population reduction of up to 2 log, compared to rinsing alone. Given that a rinse step is already in place for industrial processing of produce, no modification to infrastructure or processing environment would be expected to allow implementation of the phage treatment proposed here, but identification of specific challenges would require larger scale experimentation and involvement of industry partners. Currently, members of this research team are investigating the potential for a phage rinse in combination with other hurdle technologies.

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Table 1. Anti- *Escherichia coli* spectrum and transmission electron microscope imaging of the newly-isolated bacteriophage

Serotype/Strain	Titer ^a	Inhibition ^b	
054 220	6.9	+++	
455	7.0	+++	
ATCC 25922	-	-	
B8508	Not measurable	++	
K12	8.3	++	
O157:H1	Not measurable	S	
O157:H7 32A	-	-	
O157:H7 32C	8.0	+++	
O157:H7 32DB	8.8	+++	
O157:H7 EC88	8.5	+++	
O157:H7 EDL933	8.4	+++	
O157:H7 GFP labeled	8.9	+++	
157:H7 WT	-	-	
O157:H12	Not measurable	+	

^aTiter values, recorded as Log (PFU/ml), for the phage isolate when the corresponding serotype/strain was used as a host; results are averages of triplicate assays. The designation “not measurable” indicates that the plaques were not defined enough for enumeration.

^bInhibition values recorded as: +++, confluent lysis; ++, semi-confluent lysis; +, individual plaques; S, shadow lysis; -, no lysis.

Figure 1: Changes in *Escherichia coli* populations in the presence of bacteriophage in different media. (A) LB broth; (B) peptone; (C) phosphate-buffered saline. Symbols: Untreated (▲); Phage-treated (◆).

Figure 2: Changes in *Escherichia coli* B6-914 population on baby spinach leaves after a 2-min rinse in crude lysate and incubation for 72 hours at 25°C (an example of preliminary data). Initial *E. coli* population before the application of any treatment (i.e., time zero) for all treatments was 6.6 Log CFU/g. Symbols: Untreated (■); Rinse with medium only (◆); Rinse with phage-containing crude extract (●).

Figure 3: Initial reduction in *Escherichia coli* O157:H7 B6-914 population on baby spinach leaves after washing in a purified phage suspension, and subsequent changes in population during storage of inoculated product at 25°C for 4 hours, followed by storage at 4°C for the remaining 68 hours. Initial *E. coli* population for all spinach treatments was 5.6 Log CFU/g. Zero time represents the commencement of storage for treated produce. Symbols: Control 1: Untreated (■); Control 2: Rinse with medium only (◆); Treatment: Rinse with phage suspension (●).

Figure 4: Reduction in populations of *Escherichia coli* O157:H7 (Log N_0/N CFU/g) on fresh produce caused by phage treatment and incubation at 4°C for 72 h, or at 25°C for 4 h followed by 4°C for 68 h. N_0 is the initial population and N is the population after 72 h storage. *Indicates significant difference $P < 0.001$

Figure 5: *Escherichia coli* phage titer (Log PFU/g) on baby spinach leaves (Panel A) following a 2-min rinse, and on green pepper pieces (Panel B) following a 5-min rinse in purified phage suspension and holding the product at 4°C for 72 h (solid lines) or at 25°C for 4 hours, then 4°C for 68 hours (dashed lines).

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5