

Research Note

Effectiveness of Bacteriophages in Reducing *Escherichia coli* O157:H7 on Fresh-Cut Cantaloupes and Lettuce†

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ABSTRACT

Consumption of produce contaminated with *Escherichia coli* O157:H7 has resulted in cases of foodborne illness. We determined the efficacy of a mixture of three *E. coli* O157:H7-specific bacteriophages (ECP-100) in reducing the number of viable *E. coli* O157:H7 on contaminated fresh-cut iceberg lettuce and cantaloupe. *E. coli* O157:H7 was spot inoculated on lettuce pieces (9 cm²) with a population of 3.76 log CFU/cm², allowed to dry, and then sprayed with a control (phosphate-buffered saline) or ECP-100 to deliver 7.98 log PFU/cm² to lettuce stored for 2 days at 4°C. Cut pieces of cantaloupe were spot inoculated with *E. coli* O157:H7 (4.55 log CFU/ml) and treated with the control or ECP-100 (6.69 log PFU/ml), and then stored at 4 or 20°C for up to 7 days. On days 0, 2, 5, and 7, cantaloupe samples were homogenized, and populations of *E. coli* O157:H7 were enumerated. Populations of *E. coli* O157:H7 on lettuce treated with ECP-100 on 0, 1, and 2 days (0.72, <0.22, and 0.58 log CFU/cm² of lettuce) and stored at 4°C were significantly ($P < 0.05$) lower than those treated with the control (2.64, 1.79, and 2.22 log CFU/cm²), respectively. Populations on cut cantaloupes treated with ECP-100 on days 2, 5, and 7 (0.77, 1.28, and 0.96 log CFU/ml) and stored at 4°C were significantly lower than those cut cantaloupes treated with the control (3.34, 3.23, and 4.09 log CFU/ml), respectively. This study is the first to show the effectiveness of bacteriophages to reduce *E. coli* O157:H7 on fresh-cut lettuce and cantaloupes.

Two outbreaks of *Escherichia coli* O157:H7 illness in 2006 associated with the consumption of bagged baby spinach and bagged shredded lettuce were collectively responsible for sickening 254 people and causing three deaths (3, 4). Higher rates of hospitalization of patients (52 to 75%) and incidences of hemolytic uremic syndrome (11 to 16%) from these two outbreaks were observed when compared with hospitalization rates (29%) and hemolytic uremic syndrome incidence (10%) associated with previous *E. coli* O157:H7 outbreaks (22, 29). Bagged spinach and shredded lettuce are vulnerable to bacterial contamination, due to the lack of a kill step during their harvesting, processing, and packaging.

Introduction of *E. coli* O157:H7 to preharvest environments for leafy greens can occur in a variety of ways including animal incursions, point sources (animal-rearing facilities), and other elements that may contaminate irrigation water or fields in which produce is grown (12). The persistence of *E. coli* O157:H7 can be enhanced on the surfaces of lettuce leaves by mechanical damage (9) and nitrogen levels of lettuce leaves (8). Bacterial foodborne pathogens like *E. coli* O157:H7 and *Listeria monocytogenes*

have shown a preference to attach in greater numbers to cut edges than to whole-leaf lettuce (7, 28).

Wash water containing sodium or calcium hypochlorite for shredded lettuce or baby spinach is primarily used to prevent water from cross-contaminating leafy greens before packaging, and is not sufficient to kill bacterial pathogens that can attach to the surface of leafy green tissue (6). Moreover, hypochlorite is ineffective in inactivating *E. coli* O157:H7 attached to cut edges or stomata of lettuce leaves (28). Lettuce can also be contaminated after harvesting and transport by a variety of methods. In a recent study (18), melting ice contaminated with 10⁷ CFU/ml *E. coli* O157:H7 transferred 3.5 to 3.8 log CFU/cm² to romaine lettuce leaves that had been previously washed in 200 µg/ml sodium hypochlorite.

One modality capable of reducing pathogenic bacterial loads on fresh produce is the targeted use of bacteriophages specific for foodborne pathogens. Bacteriophages (phages) are viruses that invade bacterial cells and subsequently cause lysis. Phages were previously used as therapeutic agents for human bacterial infections before the widespread dissemination of antibiotics (25). They are ubiquitous in large numbers in nature, making up to 10% of the mass of bacterial cells in aquatic environments (10). Bacteriophages are common commensals of various foods, e.g., they have been isolated from chicken and pork sausages, ground beef, freshwater and saltwater fish, raw skim milk, cheese, vari-

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† Mention of trade names or commercial products does not imply recommendation or endorsement to the exclusion of other products by the U.S. Department of Agriculture.

ous deli meats, mushrooms, lettuce, refrigerated biscuit dough, and frozen chicken potpies (14, 16, 17, 26). Bacteriophages specific for *L. monocytogenes* have been approved as “generally recognized as safe” and specifically for use in deli meats (2, 5). Phages can play a significant role in regulating the microbial balance of the foods, which may be used to improve the safety of food products contaminated with various foodborne bacteria, including *E. coli* O157:H7. In a study, a cocktail of three *E. coli* O157:H7-specific bacteriophages effectively reduced populations of *E. coli* O157:H7 on the surfaces of seven of nine beefsteaks examined (23). In another study, *E. coli* O157:H7 phage preparation was reported to significantly reduce the levels of the bacterium on various foods (tomatoes, spinach, broccoli, and ground beef) and hard surfaces (1, 24). During the studies reported in this article, we examined the effectiveness of bacteriophages specific for *E. coli* O157:H7 in reducing populations on fresh-cut lettuce and cantaloupes stored at 4°C.

MATERIALS AND METHODS

Strains and bacteriophage preparations used. *E. coli* O157:H7 B6914 gfp 86, obtained from the U.S. Department of Agriculture, Agricultural Research Service, Microbial Food Safety Laboratory, Wyndmoor, PA (13), was streaked from frozen stock onto tryptic soy agar (TSA; Difco, Becton Dickinson, Sparks, MD) supplemented with 100 µg/ml ampicillin (TSAA) (Sigma-Aldrich, St. Louis, MO) and incubated at 37°C for 24 h. An isolated colony was then inoculated into tryptic soy broth (Difco, Becton Dickinson) supplemented with ampicillin and incubated with agitation (175 rpm) for 24 h at 37°C. Strains were then serially diluted in 0.1% peptone water, and an appropriate dilution was applied to cut pieces of either cantaloupe or lettuce. ECP-100 is a bacteriophage cocktail, consisting of three *E. coli* O157:H7-specific lytic bacteriophages (ECML-4, ECML-117, and ECML-134) in the *Myoviridae* family isolated from fresh- and saltwater environments (1), and was obtained from Intralytix, Inc. (Baltimore, MD). Phages were mixed in sterile phosphate-buffered saline (PBS; pH 7.4) and were diluted 1:100 to 8.3 log PFU/ml in PBS.

Treatment of inoculated fresh-cut cantaloupe with ECP-100. Cantaloupes (*Cucumis melo* var. *cantalupensis*) were purchased at a grocery store in Beltsville, MD. Rinds of the whole cantaloupes were then sprayed with 80% ethanol. The cantaloupe was cut into two halves, using a sterile knife, and seeds from each side were removed with a sterile-gloved hand. A deli slicer (model 827, Berkel, Inc., La Porte, IN) was then used to cut each cantaloupe half into 1-cm-thick slices. Each cantaloupe slice was then cut by a sterile knife into 14 individual ~25-mm wedges. Using a sterilized metal cork borer (0.5 cm in diameter), a well was made in each cantaloupe wedge. After the wells were made, 25 µl of 6.15 log CFU/ml *E. coli* O157:H7 gfp 86 was added to each well. For cantaloupe samples treated with ECP-100, 25 µl of 8.3 log PFU/ml of ECP-100 was applied by using a pipette. The controls did not receive ECP-100 treatments. The phage-treated and non-treated samples were then placed in commercial, 530-ml, domed plastic fruit bowls (no. 518, Rock-Tenn Co., Chicago Plastics, Franklin Park, IL) with flip lids, and stored at 4 or 20°C for up to 7 days.

Treatment of inoculated fresh-cut lettuce with ECP-100. Iceberg lettuce (*Lactuca sativa*) was purchased at a grocery store

in Beltsville, MD. The outside of the package was rinsed with 70% ethanol before removal of the head. Outer leaves of the lettuce were removed, and inner leaves were cut with a sterile, stainless steel scalpel. A sterile, stainless steel coupon was used to cut lettuce into pieces of 3 by 3 cm, on a sterile metal tray. Pieces of lettuce were stored on a moistened piece of filter paper and sealed in a petri plate (15 by 150 mm) before being spot inoculated with *E. coli* O157:H7. Each piece of lettuce was inoculated with 8 to 10 droplets consisting of a total volume of 50 µl of 4.7 log CFU/ml of *E. coli* O157:H7 gfp 86. Lettuce was allowed to dry for 1 h at 25°C in a sterile biological cabinet. After 1 h, a handheld airbrush (model 200, Badger Air-Brush Co. Franklin Park, IL) was filled with 20 ml of ECP-100 (8.3 PFU/ml), and sprayed onto fresh-cut pieces of lettuce. ECP-100 or control (PBS) was applied by using a sprayer that delivered 2.4 ml to an area of 500 cm², which resulted in a delivered volume of 5.98 log PFU/cm² of ECP-100. Twenty-four pieces of lettuce were sprayed with either the control or ECP-100 per replicate in this manner. Treated pieces of lettuce were stored for up to 2 days at 4°C on a piece of filter paper, wetted with sterile water, and sealed in a petri dish.

Microbiological analysis of *E. coli* O157:H7 populations in cantaloupe and on cut lettuce. On days 0, 2, 5, and 7, inoculated wells on wedges of cantaloupe were removed from phage-treated or nontreated cantaloupe by using a sterile cork borer (1 cm in diameter). Cores of cantaloupe from the cork borer were placed individually into a sterile 80-ml stomacher bag (Fisher Scientific, Newark, DE), and 4.5 ml of 0.1% peptone was added to each sample. Samples were then homogenized in a laboratory blender (Bagmixer 100, Intersciences, St. Nom, France) for 2 min. Homogenates were then poured into a sterile 10-ml syringe containing glass wool, and the liquid homogenate was pushed through by using a sterile plunger, and collected in a sterile 15-ml conical tube (VWR Scientific, West Chester, PA). Homogenates or serial dilutions of homogenates were spiral plated (WASP2, Don Whitley Scientific, Frederick, MD) onto sorbitol MacConkey agar (SMAC; Difco, Becton Dickinson) containing 100 µg/ml ampicillin (SMACA). Plates were incubated at 37°C for 24 h before enumeration.

On days 0, 1, and 2, pieces of lettuce inoculated with *E. coli* O157:H7 and treated with either the control or ECP-100 were added to 15 ml of 0.1% peptone in a 50-ml conical tube (VWR Scientific) and homogenized with a Polytron Pt 2100 laboratory blender (Kinematica AG, Switzerland) for 30 s. The blender was rinsed with 70% ethanol and sterile deionized water to prevent cross-contamination between each sample. Appropriate dilutions of homogenates (0.1 ml, in duplicate) were plated on SMACA. For each sample of lettuce treated with the control, 0.1 ml of homogenate, in duplicate, was plated on SMACA. For pieces of lettuce treated with ECP-100, 1 ml of homogenate was plated over five plates of SMACA (200 µl per plate). Plates were incubated at 37°C for 24 h before enumeration. For enrichments of both ECP-100-treated pieces of cantaloupe and lettuce, 1 ml of homogenates was enriched in 9 ml of mEHEC broth (BioControl, Bellevue, WA) supplemented with 100 µg/ml ampicillin and incubated at 37°C for 24 h. After incubation, 10 µl of enrichment was isolated on SMACA, and colonies were confirmed as fluorescent *E. coli* O157:H7 gfp 86 under UV light at 395 nm, as well as by latex agglutination (Remel, Inc., Lenexa, KS).

To separate unattached (free) bacteriophages from target *E. coli* O157:H7 cells in the lettuce homogenates, 1.5 ml of homogenate of an inoculated piece of lettuce treated with the bacteriophage or the control was centrifuged for 1.5 min at 10,000 × g at 25°C. Supernatants were separated from cell pellets, which were

TABLE 1. Survival of *Escherichia coli* O157:H7 populations on fresh-cut cantaloupe stored at 4°C and treated with the control or the O157-specific bacteriophage ECP-100

Treatment	Population of <i>E. coli</i> O157:H7 (log CFU/ml) ^a			
	Day 0	Day 2	Day 5	Day 7
Control	3.74 A ^b	3.34 A	3.23 A	3.46 A
ECP-100	3.53 A	0.77 B	1.28 B	0.96 B

^a The detection limit for *E. coli* O157:H7 on cantaloupe was 1 log CFU/ml.

^b Within the same day, different letters after the mean values of populations indicate significant ($P < 0.05$) differences between treatments.

then resuspended in 1.5 ml of sterile PBS and spiral plated on SMACA agar. *E. coli* O157:H7 populations were determined as described above. The centrifugation was to remove any unattached phages from the *E. coli* O157:H7 cells, so that the any reduction in the bacterial counts would be due to the effect of phages on the lettuce surfaces rather than to additional infections by unattached phages during the overnight incubation of samples on the SMACA plates. In order to determine the effectiveness of phage removal by the centrifugation step, supernatants (0.9 ml) were decanted from the pellets by using a pipette, and the levels of “removed” phages were determined by counting plaques after incubation (37°C for 24 h) on TSAA agar. Bacterial populations were determined on days 0 and 1 from centrifuged homogenates from inoculated lettuce stored at 4°C.

Statistical analysis. All experiments were repeated at least three times. Population of *E. coli* O157:H7 gfp 86 recovered from cantaloupe and lettuce homogenates treated with either the control or ECP-100 were subjected to an analysis of variance and least-significant difference test (Fisher’s), using SAS, version 9.1.2, software (SAS Institute, Inc., Cary, NC) for effects of the phage treatment and sampling days. In all cases, the level of statistical significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

Fresh-cut cantaloupes inoculated with *E. coli* O157:H7 and treated with the bacteriophage mixture (ECP-100) had significantly ($P < 0.05$) lower counts than had inoculated, untreated cut cantaloupes stored at 4°C (Table 1). Populations of *E. coli* O157:H7 recovered on SMACA were significantly lower on bacteriophage-treated pieces of cantaloupe than on nontreated cantaloupe on days 2, 5, and 7. The largest difference in populations of *E. coli* O157:H7 on untreated and ECP-100-treated cantaloupes was observed on day 2 (2.87 log CFU/ml). No statistical differences were observed in *E. coli* O157:H7 populations on day 0 between cut cantaloupes treated with ECP-100 and the control. Cantaloupes inoculated with *E. coli* O157:H7, treated with ECP-100, and subsequently stored at 20°C did not show the same levels of reductions in populations as at 4°C (Table 2). At 20°C, significant differences in populations on cut cantaloupes treated with ECP-100 and those treated with the control were only observed on day 5. In all instances, the ability of ECP-100 to reduce *E. coli* O157:H7 populations was more effective during refrigerated storage than during storage at 20°C.

TABLE 2. Survival of *Escherichia coli* O157:H7 populations on fresh-cut cantaloupe stored at 20°C and treated with the control or the O157-specific bacteriophage ECP-100

Treatment	Population of <i>E. coli</i> O157:H7 (log CFU/ml) ^a			
	Day 0	Day 2	Day 5	Day 7
Control	3.74 A ^b	7.53 A	7.83 A	8.36 A
ECP-100	3.53 A	6.17 A	6.59 B	6.99 A

^a The detection limit for *E. coli* O157:H7 on cantaloupe was 1 log CFU/ml.

^b Within the same day, different letters after the mean values of populations indicate significant ($P < 0.05$) differences between treatments.

Spraying with ECP-100 significantly reduced the number of viable of *E. coli* O157:H7 on fresh-cut lettuce (Table 3). Furthermore, the bactericidal effect occurred very quickly after spraying: the initial application of ECP-100 reduced *E. coli* O157:H7 counts by 1.92 log CFU/cm² on day 0. Lettuce was treated by spraying, whereas cantaloupe was treated by application with a pipette. Other workers reported that spraying *L. monocytogenes*-specific phages on fresh-cut honeydew melon produced a significantly greater reduction in *L. monocytogenes* counts than when contaminated melons were immersed in a phage mixture (20). During our studies, spraying the phages onto the contaminated lettuce may have improved contact between the phages and *E. coli* O157:H7 cells. These observations may have important practical implications during postharvest processing of fresh-cut lettuce. For example, phages may be sprayed on lettuce at a number of postharvest stages, e.g., after (i) coring during storage in the field, (ii) shredding but before placement in the flume water, or (iii) after drying and immediately before packaging and transport.

Lettuce homogenates were centrifuged in an attempt to separate unattached ECP-100 phages from recovered bacteria in order to determine whether the observed reduction in *E. coli* O157:H7 counts was due to the lytic activity of the phages on the lettuce surfaces rather than to infection by free phages that remained in homogenates present on SMACA agar. On day 0, the control and ECP-100-treated populations recovered from centrifuged homogenates were 2.43 and 1.14 log CFU/cm², respectively. On day 1, control

TABLE 3. Survival of *Escherichia coli* O157:H7 populations on fresh-cut lettuce stored at 4°C and treated with the control or the O157-specific bacteriophage ECP-100

Treatment	Population of <i>E. coli</i> O157:H7 (log CFU/cm ²) ^a		
	Day 0	Day 1	Day 2
Control	2.64 A ^b	1.79 A	2.22 A
ECP-100	0.72 B	<0.22 B	0.58 B

^a The detection limit for *E. coli* O157:H7 on lettuce was 0.22 log CFU/cm².

^b Within the same day, different letters after the mean values of populations indicate significant ($P < 0.05$) differences between treatments.

and ECP-100–treated populations recovered from homogenates were 2.76 and 0.95 log CFU/cm², respectively. Plaque titers of ECP-100 from supernatants and noncentrifuged ECP-100–treated homogenates were 6.55 and 6.39 log PFU/cm², respectively, on day 0. The small difference in plaque titers (0.16 log PFU/cm²) of supernatants compared with ECP-100–treated homogenates indicates that centrifugation removed the majority of unattached phages from *E. coli* O157:H7 cells. Once these free phages were removed, reductions in *E. coli* O157:H7 counts were still observed on ECP-100–treated lettuce at similar levels reported in Table 2. These results provide evidence that the free phages in ECP-100 did indeed kill *E. coli* O157:H7 on the lettuce surfaces rather than on the surfaces of agar plates.

Several parameters in the packaging and processing of bagged leafy greens may enhance the virulence and persistence of *E. coli* O157:H7. Populations of *E. coli* O157:H7 inoculated on lettuce and stored under modified atmosphere packaging conditions >15°C were more tolerant to gastric acidity than were cells inoculated on lettuce under modified atmosphere packaging conditions stored at <10°C in one study (11). Our work here shows that *E. coli* O157:H7 cells attached to the surface of lettuce can be rapidly lysed by O157-specific phages, and possibly prevent the bacteria from inducing acid resistance during transport and retail storage under potentially abusive temperature conditions. Vacuum cooling is a commonly utilized to remove field heat from leafy greens and involves water sprays. In a study, more *E. coli* O157:H7 cells infiltrated the lettuce when vacuum cooled for 5 min (6.42 log CFU/g) than when inoculated leaves were not vacuum cooled (5.29 log CFU/g) (21). If O157-specific phages were applied to lettuce before vacuum cooling, the rapid killing of *E. coli* O157:H7 on lettuce surfaces may prevent the potential infiltration of these cells into the tissue. However, further work is needed evaluate this hypothesis. In our study, the time between application of the O157-specific bacteriophage and microbiological analysis was less than 1 h, indicating the rapid nature of killing of *E. coli* O157:H7 cells on the surfaces of lettuce.

Several previous studies have evaluated the effectiveness of phage cocktails specific for *Salmonella* and *L. monocytogenes* on produce commodities. *Salmonella* Enteritidis populations were reduced by 3.5 and 2.5 log CFU on fresh-cut honeydew melons when stored at low temperatures (5 to 10°C) and room temperature (20°C), respectively (19). Phages specific for *L. monocytogenes* reduced populations by 2 to 4.6 log CFU per sample on fresh-cut honeydew melons when stored at 10°C (20). These results are consistent with our observed results that the ECP-100 mixture was more effective at a lower temperature (4°C) than at a higher temperature (20°C) in inactivating *E. coli* O157:H7 populations on cantaloupes, possibly due to the lack of growth of the bacterial population on produce. Our data are also in agreement with the recent report that ECP-100 significantly reduces experimental contamination of tomatoes, broccoli, spinach, and ground beef with *E. coli* O157:H7 (reductions ranged from 94% at 120 ± 4 h post-

treatment of tomato slices, to 100% at 24 ± 4 h posttreatment of spinach) (1). In the data reported in our study, the superior efficacy of ECP-100 at 4°C compared with 20°C on cantaloupes may be due to unexamined factors, including superior survival of the phages at that temperature (so that more phages are available to infect *E. coli* O157:H7 cells) or more prolific growth of *E. coli* at 20°C than at 4°C.

The results of our study, for the first time, show that *E. coli* O157:H7–specific bacteriophages are effective in killing the pathogen on fresh-cut cantaloupe and lettuce at refrigerated temperatures. Further studies are required to determine the effect of bacteriophages on the quality and appearance of produce commodities, and their bactericidal activity in combination with modified atmosphere packaging conditions commonly used for fresh-cut produce.

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