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Reduction of *Escherichia coli* O157:H7 viability on leafy green vegetables by treatment with a bacteriophage mixture and *trans*-cinnamaldehyde

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ABSTRACT

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 has been recognized as a major foodborne pathogen responsible for frequent gastroenteritis outbreaks. Phages and essential oils can be used as a natural antimicrobial method to reduce bacterial pathogens from the food supply. The objective of this study was to determine the effect of a bacteriophage cocktail, BEC8, alone and in combination with the essential oil *trans*-cinnameldehyde (TC) on the viability of a mixture of EHEC O157:H7 strains applied on whole baby romaine lettuce and baby spinach leaves. The EHEC O157:H7 strains used were Nal^R mutants of EK27, ATCC 43895, and 472. Exponentially growing cells from tryptic soy (TS) broth cultures were spot inoculated on leaves and dried. EHEC cells were placed at low, medium, and high inoculum levels (10^4, 10^5, and 10^6 CFU/mL, respectively). Appropriate controls, BEC8 (approx. 10^6 PFU/leaf), and TC (0.5% v/v) were applied on treated leaves. The leaves were incubated at 4, 8, 23, and 37 °C in Petri dishes with moistened filter papers. EHEC survival was determined using standard plate count on nalidixic acid (50 μg/mL) Sorbitol MacConkey agar. No survivors were detected when both leaves were treated with BEC8 or TC individually at low inoculum levels after 24 h at 23 and 37 °C. When the EHEC inoculum size increased and/or incubation temperature decreased, the efficacy of BEC8 and TC decreased. However, when the two treatments were combined, no survivors were detected after 10 min at all temperatures and inoculum levels on both leafy greens. These results indicated that the BEC8/TC combination was highly effective against EHEC on both leafy greens. This combination could potentially be used as an antimicrobial to inactivate EHEC O157:H7 and reduce their incidence in the food chain.

1. Introduction

Fresh vegetables are increasingly being identified as a source of foodborne outbreaks around the world (Lynch et al., 2009). There have been many outbreaks associated with the consumption of fresh vegetables linked to Shiga toxin-producing *E. coli* (STEC). Some examples include the radish sprout outbreak in Japan (Michino et al., 1999), fresh lettuce in Sweden (Söderström et al., 2005), and bagged spinach and lettuce in the US (CDC, 2006). In December of 2006, an STEC outbreak occurred in the Northeastern US; affecting residents of New Jersey, New York, and Pennsylvania. The source of the outbreak was traced to iceberg lettuce used at Mexican-style fast food restaurants (FDA, 2006). Also in 2006, Utah and New Mexico health departments investigated a multistate cluster of STEC O157 associated with consuming bagged spinach (FDA, 2007).

In Europe, approximately 14,000 cases in over 24 countries have occurred from 2000 to 2005, of which 62% were caused by the O157 serogroup (Fisher and Meakins, 2006). In England and Wales, salad, vegetables, and fruits caused 6.4% and 10.1% of all outbreaks with a known food vehicle in the periods of 1993–1998 and 1999–2000, respectively (Brandl, 2006). In Australia, fresh produce has been responsible for 4% of all foodborne outbreaks reported between 2001 and 2005 (Kirk et al., 2008). The incidence of foodborne illness associated with the consumption of minimally processed ready-to-eat salad vegetables has been consistently increasing (Beuchat, 1998; Kaneko et al., 1999; Tauxe, 1997). In the US, the percentage of outbreaks associated with fresh produce increased from less than 1% in the 1970s to 6% in the 1990s (Sivapalasingam et al., 2004). The median size of outbreaks associated with fresh produce has doubled and the proportion of outbreak-associated cases related to fresh produce increased from less than 1%–12% of illnesses. The increase of foodborne outbreaks due to the consumption of fresh vegetables has stressed the importance of developing antimicrobial strategies to reduce their microbial load (Food and Drug Administration, 2007; Johnston et al., 2006;

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Hilborn et al., 1999; Ackers et al., 1998; Calvin, 2007; Maki, 2006; Uhlich et al., 2008; Welinder-Olsson et al., 2004).

Bacteriophages are obligate parasites of bacteria capable of killing specific species and offer a natural method to control contamination of foods (Callaway et al., 2008). *E. coli* phages can be isolated from sewage, waste water, polluted rivers and fecal samples of humans or animals (Brussow, 2005). Using specific phages to eliminate or reduce the levels of contaminated bacteria on fresh-cut fruits and vegetables is under investigation for *E. coli* O157:H7 by various investigators. As part of an ongoing study, Sharma et al. (Sharma et al., 2009) tested the effectiveness of a mixture of bacteriophages in reducing *E. coli* O157:H7 gfp 86 on cut pieces of iceberg lettuce and cantaloupe. They found that the bacteriophage treatment reduced the pathogen immediately upon application to lettuce and the bacteriophage treatments had significant lower counts of the pathogen for both the lettuce and the cantaloupe compared to the negative control. Advantages of using phages over traditional antimicrobial systems for foods have been reviewed at the pre-harvest (Barrow and Soothill, 1997; Joerger, 2003) and post-harvest level (Leverentz et al., 2001, 2003).

Phages are highly specific and their use in agriculture is not likely to select for phage resistance in untargeted bacterial species. Furthermore, bacterial resistance mechanisms against phages and antibiotics differ, thus the possible emergence of resistance against phages will not affect the susceptibility of bacteria to antibiotics used for humans. In addition, phage preparations can readily be modified in response to changes in bacterial pathogen populations or susceptibility, while antibiotics have a long and expensive development cycle (Sulakvelidze and Barrow, 2005). In addition, there has been recent exploration in different phage delivery systems. Puapermpoonsiri et al. (2009) showed that phages specific for *Staphylococcus aureus* or *Pseudomonas aeruginosa* could be encapsulated into biodegradable polyester microspheres via a modified water/oil/water double emulsion–solvent extraction protocol resulting in only a partial loss of lytic activity. Despite the poor shelf-life of the formulation, the work is proof-of-concept for the formulation and controlled delivery of bacteriophages, as acceptable for the treatment of bacterial lung infections.

Plant-derived essential oils (EO) can be used as flavoring agents in foods and beverages and have potential as natural agents for food preservation due to their content of antimicrobial compounds (Helander et al., 1998). A study by Helander et al. (1998) used different essential oils to inhibit *E. coli* O157:H7 and *Salmonella* and they found that *trans*-cinnamaldehyde gains access to the periplasm and to the deeper parts of the cell, yet does not result in the disintegration of the outer membrane or deplete the intracellular ATP pool. Another study found that the minimum inhibitory concentration of cinnamaldehyde against *E. coli* was 500 μg/mL and its high antimicrobial activity was attributed to its aldehyde group, while a conjugated double bond, a long CH chain outside the ring, and the hydroxyl group may also be responsible (Chang et al., 2001). Baskaran et al. (2010) investigated the antimicrobial effect of low concentrations of *trans*-cinnamaldehyde on *E. coli* O157:H7 in apple juice and apple cider. They found that at 4 °C, 0.125 and 0.075% v/v cinnamaldehyde decreased the pathogen counts in the juice and cider to undetectable levels on days 3 and 5, respectively. These results showed that low concentrations of cinnamaldehyde could be used as an effective antimicrobial to inactivate *E. coli* O157:H7 in apple juice and apple cider.

Biocontrol strategies offer a more practical and cost-effective approach for controlling pathogens in the environment (Ye et al., 2009, 2010). Furthermore, using combined treatments is consistent with the hurdle concept (Leistner, 1992), that states that effective control of foodborne pathogens can be achieved through the use of a combination of compatible control measures to ensure the safety of food. Both bacteriophages and essential oils such as *trans*-cinnamaldehyde (Yang et al., 2010; Juneja and Friedman, 2008; Weissinger et al., 2001) have been successfully applied to suppress the activity of phytopathogens. Ye et al have used a combination of *Enterobacter asburiae* JX1 and a cocktail of five lytic bacteriophages to evaluate their efficacy against *Salmonella* Javiana on tomatoes (Ye et al., 2009) and sprouting mung beans and alfalfa seeds (Ye et al., 2010). They found that the combination was successful for the sprouting mung beans and alfalfa seeds, however, there was no evidence to suggest that the antagonistic activity of *E. asburiae* could be enhanced with phages when used on tomatoes.

The phage treatment is a new and effective hurdle, which in combination with *trans*-cinnamaldehyde and/or other control measures may maximize protection from foodborne pathogens on vegetables. To overcome the limited efficacy of bacteriophages and essential oils as antimicrobial methods, this study investigates the effectiveness of their combined use against *E. coli* O157:H7 on leafy green vegetables. The objectives of this study were to determine the effect of i) a previously characterized collection of bacteriophages, BEC8, ii) *trans*-cinnamaldehyde and iii) their combination on the viability of a mixture of EHEC O157:H7 strains applied on leafy green vegetables.

2. Materials and methods

2.1. Bacteriophage and trans-cinnamaldehyde preparation

The bacteriophage mixture designated BEC8 included eight lytic, *E. coli* O157:H7-specific phage strains: 38, 39, 41, CEV2, AR1, 42, ECA1, and ECB7 (Viazis et al., 2009). All eight phages were members of the family Caudovirales, and are highly effective against strains of *E. coli* O157:H7 as shown previously through efficiency of plating (EOP) tests, spot testing, and activity against high bacterial titers. Phages were propagated by mixing 1 mL of each phage strain (approx. 10^8 PFU/mL), 10 mL TSB with 1 mM $CaCl_2$, and 100 μL of the mid-exponential phase (approx. 10^7 CFU/mL) host bacterial strain and incubating overnight while shaking at 37 °C in 50 mL centrifuge tubes. Equal amounts of $CHCl_3$ were added to the tubes, vortexed for 5 s, and centrifuged for 5 min at 14,500 × *g*. The supernatant was then filtered through a 0.45 μm pore size filter and stored in 50 mL centrifuge tubes at 4 °C until ready for use. Equal concentrations of individual phages were included in the mix. The phage cocktail was suspended in a solution of tryptic soy broth (TSB; Neogen, Inc., Lansing, MI). *Trans*-cinnamaldehyde (TC; >99% pure, molecular weight of 132.2, Sigma–Aldrich, St. Louis, MO) was suspended in TSB to form a 0.5% v/v emulsion and was stored at 4 °C until ready for use. To prepare the BEC8/TC combination, TC was diluted in the phage cocktail in TSB to give rise to a solution that contained approx. 10^7 PFU/mL BEC8 and 0.5% v/v TC.

2.2. Bacterial strains

The bacterial strains used to inoculate leafy green vegetables were nalidixic acid resistant mutants of *E. coli* O157:H7 ATCC 43895, an isolate from a 1982 hamburger outbreak, EK27 TWO8635, a clade 8 isolate received from Dr. Thomas Whittam (STEC Center, Michigan State University), and I-2005003658-472, a 2005 spinach outbreak isolate (Minnesota Department of Health, St. Paul, MN). The EHEC strains were selected for nalidixic acid resistance (Nal^R) by serially passaging the original isolates on sorbitol MacConkey (Neogen, Inc., Lansing, MI) plates supplemented with increasing concentrations of nalidixic acid (Sigma–Aldrich, St. Louis, MO) (NA-SMAC). Each strain underwent more than 10 serial passages on NA-SMAC before it was considered to be Nal^R at a concentration of