Control of Enterohemorrhagic *Escherichia coli* Using Bacteriophages

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DEDICATION

I dedicate this work to Andrea Jean Krause, who put up with me during this long process.

ABSTRACT

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 has been recognized as a major foodborne pathogen responsible for frequent gastroenteritis outbreaks. Phages can be used as a natural antimicrobial method to reduce bacterial pathogens from the food supply.

The objective of the first study was to isolate, identify and characterize a diverse collection of lytic bacteriophages capable of infecting EHEC serotypes O26, O111, and O157. Phages were isolated from dairy and feedlot manure using EHEC O157, O26, and O111 strains as hosts. Plaques were purified and screened against additional strains (14, O157; 10, O26; 10, O111) using the efficiency of plating method (EOP). Phage CEV2 and five other phages previously isolated were able to lyse all 14 O157 EHEC strains with EOP values consistently above 0.001. Two phages isolated from fecal slurry from dairy and feedlot cattle were highly effective against strains of *E. coli* O157, through EOP tests, and against O26 through spot tests, but not O111. Bacterial challenges against high titers of four *E. coli* O157 strains suggested that a mixture of the 8 most effective phages was just as effective as or more than each individual phage. This collection of phages can be grouped and potentially used as an antimicrobial cocktail to inactivate O157 and O26 serotypes.

The objective of the second study was to determine the effect of the bacteriophage cocktail, BEC8, on the viability of a mixture of EHEC O157:H7 strains applied on surfaces of materials representative of food processing plants. Sterile stainless steel chips (SSC), ceramic tile chips (CTC), and high density polyethylene chips (HDPEC) were

used. The EHEC O157:H7 strains used were EK27, ATCC 43895, and 472. Exponentially growing cells from tryptic soy (TS) broth cultures were spot inoculated on surfaces and dried. EHEC cells were placed at high, medium, and low inoculum levels (10^6, 10^5, and 10^4 CFU/mL, respectively). Appropriate controls and BEC8 (approx. 10^6 PFU/mL) were applied on treated surfaces. The surfaces were incubated at 4, 12, room temperature (RT), and 37^{o}C. EHEC survival was determined using standard plate count on TS agar. No survivors were detected after BEC8 treatment at a low inoculum level at the following incubation conditions: 37^{o}C for 10 min and RT after 1 h on SSC and CTC; 12^{o}C after 10 min on SSC, 1 h for CTC, and 24 h for HDPEC. These results indicated that the phage cocktail was effective within an hour against low levels of the EHEC mixture at RT on all 3 hard surfaces.

The objective of the third study was to determine the effect of the bacteriophage cocktail, BEC8, on its own and in combination with the essential oil *trans*-cinnameldehyde (TC) on the viability of a mixture of EHEC O157:H7 strains applied on baby romaine lettuce and baby spinach leaves. The EHEC O157:H7 strains used were nalidixic acid resistance mutants of EK27, ATCC 43895, and 472. The methods used were similar to the second study. The leaves were incubated at 4, 8, RT, and 37^{o}C in Petri dishes with moistened filter papers. EHEC survival was determined using standard plate count on nalidixic acid containing Sorbitol MacConkey agar. No survivors were detected when treated with BEC8 or TC separately at low inoculum level after 24 h at RT on lettuce and spinach. However, when the EHEC inoculum size and/or incubation temperature increased, the efficacy of BEC8 and TC decreased. When the two treatments

were combined, no survivors were detected after 10 min at all temperatures on lettuce and spinach. These results indicated that the phage cocktail and TC combination was highly effective against EHEC on leafy greens.

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CHAPTER 1

A REVIEW ON ENTEROHEMORRHAGIC ESCHERICHIA COLI: THE 20TH CENTURY'S EMERGING FOODBORNE PATHOGEN

Enterohaemorrhagic *E. coli* (EHEC) have been recognized as a cause of serious illness and mortality in outbreaks of food-borne disease that involve a large variety of foods. In general, most pathogenic strains behave biochemically and ecologically like any other non-pathogenic *E. coli*, making their detection among commensal *E. coli* an important problem, especially among EHEC. *E. coli* infections in humans are transmitted directly from animals, by person-to-person contact, or through contaminated foods. Multiple massive outbreaks associated with the consumption of fresh vegetables have occurred. There have been numerous studies on pre- and post-harvest intervention methods but the problem is still at large. In the U.S. and in other countries, the presence of this pathogen in foods is highly regulated and there have been rapid scientific advances in understanding the growth and survival of the pathogen in various foods. The present review highlights the current understanding of EHEC from the perspectives of food microbiology, molecular microbiology, biochemistry, epidemiology, and agricultural practices with an emphasis on leafy green vegetables. This thesis stresses the importance of developing natural or organic novel control strategies.

1.1. Enterohemorrhagic *Escherichia coli*

1.1.1. Introduction

Enterohaemorrhagic *E. coli* (EHEC) have been recognized as a cause of serious illness and mortality in outbreaks of food-borne illness that involve a large variety of foods (29). Most *E. coli* can be a harmless member of the normal microflora in humans and other animals. However, virulence genes acquired through various means have bestowed different types of pathogenicity to strains of *E. coli*. There are a number of different enteropathogenic groups of *E. coli* that have been shown to cause various types of gastrointestinal infections. Six main pathotypes of *E. coli* can be distinguished: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), diffusely-adhering *E. coli* (DAEC), enteroaggregative *E. coli* (EAEC), and EHEC. All these pathotypes of *E. coli* use multi-step systems of pathogenesis, comprised in general of colonization of the mucosal site, evasion of the host defenses, and multiplication and host damage (189). After the first outbreak in 1982, *E. coli* O157:H7 has become the most widely known EHEC strain (298).

In general, many pathogenic strains behave biochemically and ecologically like any other non-pathogenic *E. coli*, making their detection among commensal *E. coli* an important problem, especially among EHEC (33). Serotype O157 has been found to be unable to ferment the carbohydrate sorbitol (298), a phenotypic characteristic that is useful in the organism's detection. Compared to other pathogenic *E. coli*, this serotype causes hemorrhagic colitis (HC) and other severe symptoms, such as hemolytic uremic syndrome. Other serotypes, such as O26, O111, and sorbitol-fermenting O157:NM have

also been associated with hemorrhagic colitis and subsequently classified as EHEC (16). The ability to produce Shiga toxins is the common characteristics of all EHEC that are often referred to as Shiga toxin-producing *E. coli* (STEC).

In this review, we examine the versatility of *E. coli* O157:H7 in causing disease through various sources and document the incidence of infections. Potential methods for the control of *E. coli* O157:H7 on a pre- and post-harvest level are also discussed. The significance of *E. coli* O157:H7 as a human pathogen stresses the importance of establishing effective strategies to minimize the quantity of *E. coli* O157:H7 on the farm.

1.1.2. History

E. coli has been recognized as an important human pathogen since its discovery in 1885 by Dr. Theodor Escherich through his work on bacteria in infant stools. The finding of *Shigella dysenteriae* as an agent of epidemic bacterial dysentery by Kioshi Shiga was reported in 1898 (318). In 1955, hemolytic uremic syndrome (HUS) was first described and defined (138), while in 1972, Keusch et al (195) showed that Shiga toxins contribute to bloody diarrhea. In 1977, Konowalchuck et al (200) found that certain pathogenic *E. coli* strains produce a toxin capable of killing Vero cells and in 1982, there were two outbreaks of a severe bloody diarrheal syndrome in Oregon and Michigan associated with the consumption of fast food hamburgers (299). In 1983, O'Brien et al. (269) reported that an *E. coli* O157:H7 strain that was involved in an outbreak of HC in the U.S. produced a Shiga toxin and in 1985, Karmali et al (193) suggested that STEC were epidemiologically associated with HUS.

1.1.3 Epidemiology

1.1.3.1 Outbreaks and incidence

STEC and specifically *E. coli* O157:H7, are considered as emerging foodborne pathogens that occur worldwide, but are most common in some parts of the UK, US, and Canada (11, 257, 344). In the US, *E. coli* O157:H7 is estimated to cause 73,480 illnesses annually, with 2168 hospitalizations and 61 deaths (242), while the pathogen's associated economic costs have been estimated to be $405 million USD annually (129). In recent years, EHEC has been linked to outbreaks linked to fresh produce (29, 91), with *E. coli* O157:H7 being one of the leading causes of produce related outbreaks, accounting for 20% of the outbreaks in which the etiological agent was identified (275). Although some of the first outbreaks of *E. coli* O157:H7 were linked to inadequately cooked hamburgers, many outbreaks that followed have been associated with the consumption of raw vegetables, including a massive 1996 outbreak in Japan in which nearly 8,000 people were infected by contaminated radish sprouts (190).

Examples of large outbreaks associated with the consumption of fresh vegetables include the radish sprout outbreak in Japan (246), bagged spinach and lettuce in the United States (75), and fresh lettuce in Sweden (327). 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 (117). In England and Wales, salad, vegetables, and fruit caused 6.4% and 10.1% of all outbreaks with a known food vehicle in the periods of 1993–1998 and 1999–2000, respectively (51). The incidence of foodborne illness associated with the

consumption of minimally processed ready-to-eat salad vegetables has been consistently increasing (35, 188, 344). Between the years of 1990 and 2001, contaminated fresh produce was associated with a total of 148 outbreaks comprising approximately 9% of all foodborne outbreaks (323). Fresh fruits and vegetables are more often being identified as a source of foodborne outbreaks around the world (227). In the US, the percentage of outbreaks associated with fresh produce increased from <1% in the 1970s to 6% in the 1990s (320). The median size of outbreaks associated with fresh produce has doubled and the proportion of outbreak-associated cases related to fresh produce increased from <1% to 12% of illnesses. In Australia, fresh produce was responsible for 4% of all foodborne outbreaks reported between 2001 and 2005 (196).

In 2005 a large outbreak of STEC from lettuce occurred in Sweden and water from a nearby stream was confirmed positive for Shiga toxin 2 by PCR (328). Between August and October of 2006, the widely reported multistate outbreak of EHEC occurred in the US due to contaminated bagged spinach (71). In December of 2006, a STEC outbreak occurred in the Northeastern US, affecting individuals in New Jersey, New York, and Pennsylvania. The source of the outbreak was traced to Iceberg lettuce used at Taco Bell restaurants (118). Also in 2006, Utah and New Mexico health departments investigated a multistate cluster of STEC O157 associated with consuming bagged spinach (345). Between September and October of 2007, there was an outbreak of STEC O157 in the Netherlands and Iceland. The most probable cause of the outbreak was contaminated lettuce but samples of the product that were tested were all negative for the pathogen (132).

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 (7, 71, 160, 185, 232, 345, 353, 370). The most recent outbreaks of foodborne disease caused by bagged lettuce and spinach expose the limited effect of current conventional washes. The produce industry as a whole would benefit from research on alternative effective antimicrobial treatments (71, 232).

1.1.3.2. Transmission vehicles

The primary habitat of *E. coli* is the intestinal tract of warm-blooded animals including humans (29). *E. coli* infections in humans are transmitted directly from animals, by person-to-person contact, or through contaminated foods. Enteric pathogens are distributed from livestock to food crops and can occur in various ways such as application of manures, irrigation with contaminated water, dispersal by air, and dispersal via biological vectors, such as wildlife and insects (178). There is widespread fecal contamination of the environment due to farm and wild animals providing a continual source of EHEC in the environment (29). Ground beef is still the most frequently implicated source of *E. coli* O157:H7 outbreaks, accounting for approximately 75% of cases (363). Dairy products and undercooked minced beef can be directly contaminated by cattle feces during either milking or slaughtering processes (128).

Talley et al (339) investigated the role of insects in produce contamination. In their study, house flies were confined on manure or agar medium containing *E. coli* O157:H7 tagged with green fluorescent protein (GFP) followed by testing their ability to

transfer the pathogen to spinach plants. GFP-tagged bacteria were detected on surfaces of 50 to 100% of leaves examined by fluorescence microscopy and in 100% of samples tested by PCR. Evidently, flies are capable of contaminating leafy greens and confirm the importance of the role of insects in contamination of fresh produce, even if it occurs on a relatively small scale.

Raw fruit and vegetables, which are indirectly contaminated via irrigation water or through soil treated with farm effluents, are an important vehicle of EHEC contamination (160, 246). A recent study on the persistence of *E. coli* O157 in irrigation waters that could potentially be transmitted to fresh produce was conducted in the Kubanni River region of Nigeria (81). The prevalence of the pathogen in the river was studied over a 10-month period. The detection rate for *E. coli* O157 was 2.1% and fecal coliform counts exceeded acceptable limits. The investigators concluded that the Kubanni River represented a public health risk and unfit for fresh produce irrigation.

The factors responsible for the emergence of the problem are listed on Table 1.1. A study by Voetsch et al (362) determined that most STEC O157 infections in 1999-2000, associated with eating pink undercooked hamburgers, drinking untreated surface water, and contact with cattle. Eating produce was inversely associated with infection. Furthermore, direct or indirect contact with cattle waste was identified as a leading source of sporadic STEC O157 infections. Mukherjee et al (252), conducted microbiological analyses of fruits and vegetables on Minnesota and Wisconsin farms in conjunction with collecting a farmer survey on farm management practices that may affect the risk of *E. coli* contamination in fresh produce. They found that using animal wastes for fertilization

of produce plants posed an increased risk of *E. coli* contamination of organic and semi-organic produce. In addition, improper composting of untreated animal manure also increased the risk for contamination of organic produce. It should be noted that organic growers who used cattle manure for fertilization had significantly greater risk compared to those who used other types of manure-based fertilizer. Furthermore, leaf age has also been shown to be a risk factor for O157 contamination (54). Younger leaves may be associated with a greater risk of contamination with *E. coli* O157:H7.

However, commercially grown fresh produce that has been microbiologically tested for pathogenic *E. coli* generally give rise to negative findings, suggesting that contamination with pathogenic *E. coli* is a relatively rare occurrence (92). A study by Johnston et al. (185) reported that there was no *E. coli* O157:H7 detected in 466 produce and environmental swabs collected in eight packing sheds in the southern United States. Leaf lettuce collected directly from Norwegian farms with organic production practices that included the use of manure fertilizers was also free of *E. coli* O157:H7 (224). In addition, *E. coli* O157:H7 and Shiga toxins 1 and 2 were not detected within *E. coli* isolates recovered from organically or conventionally grown produce in Minnesota (254). Similarly no *E. coli* O157:H7 were reported in an expanded investigation that included farms with varied agronomic practices in both Minnesota and Wisconsin (255).

Table 1.1. Factors involved in the emergence of produce-linked outbreaks of food-borne pathogens (51, 343).

Changes in the produce industry
Intensification and centralization of production
Wider distribution of produce over longer distances
Introduction of minimally processed produce
Increased importation of fresh produce
Changes in consumer habits
Increased consumption of meals outside the home
Increased popularity of salad bars
Increased consumption of fresh fruits and vegetables, and fresh fruit juices
Increased size of at-risk population
Enhanced epidemiological surveillance
Improved methods to identify and track pathogens
Emerging pathogens with low infectious dose

1.1.4. Microorganism characteristics

1.1.4.1. Unique traits

The genus *Escherichia coli* is comprised of Gram-negative, facultative anaerobic bacilli, common inhabitant of the gastrointestinal tract of mammals, and belong to the *Enterobacteriaceae* family. They are bile tolerant, non-fastidious organisms that are easily cultured on routine laboratory media. They ferment lactose and grow best under mesophilic temperatures with an optimum at 37^{o}C. Most *E. coli* have the β-glucoronidase enzyme that breaks down complex carbohydrates. This enzyme is used in a fluorogenic assay that takes advantage of the breakdown of 4-methyl-umbeliferone glucoronide (MUG) by β-glucoronidase producing a fluorescent compound. However, *E. coli* O157:H7 does not have β-glucoronidase. Furthermore, *E. coli* O157:H7 cannot ferment sorbitol within 24 hrs, while 90% of *E. coli* strains can.

The infectious dose of EHEC is very low, between 1 and 100 CFU, which is a much lower dose than for most other pathogens of the intestines (282). One of the main characteristics of EHEC that are required to cause disease in humans is their ability to attach to intestinal cells and to colonize the human gut (369).

Since the 1980's, EHEC strains have been established as food-borne pathogens associated with an array of human infections including hemorrhagic colitis (HC), milder forms of diarrheal illness, and as the major etiologic agent responsible for a potentially fatal infection, hemolytic-uremic syndrome (HUS). In general, infection with EHEC O157:H7 is self limiting, but depending on the virulence of the infecting strain, the extent of the disease may vary. Hemorrhagic colitis (HC) is the principal disease associated with EHEC and is characterized by severe abdominal cramping and bloody diarrhea. Hemolytic uremic syndrome (HUS) may eventually develop in response to EHEC infection and HC. HUS is characterized by microangiopathic hemolytic anemia, thrombocytopenia, and renal insufficiency or failure and occasional azotemia (144, 183, 282). Approximately 8% of those infected with EHEC O157:H7 will develop HUS (241, 341).). Diarrhea-associated HUS is largely responsible for morbidity and mortality due to EHEC, resulting in death in up to 5% of infected individuals and frequent permanent renal injury at a rate of 25% of patients (137). The endothelial cell damage leads to swollen, detached endothelial cells, which in turn exposes the basement membrane. This leads to platelet activation and local intravascular thrombosis or thrombotic microangiopathy, blood clot formation within the vasculature, and ultimately, a reduction in platelet counts. Hemolytic anemia is an abnormal breakdown of erythrocytes. This

results from clots and possible side-effects from leukocytes on the erythrocyte cell membranes.

Children less than 5 years of age have a higher incidence of HUS. They express higher levels of the Gb3 receptor present on the renal endothelial cells and form an attachment to Shiga toxin that may be circulating due to infection. Renal injury occurs from leukocyte infiltrates and clots that may lead to acute renal failure and azotemia. Azotemia is characterized by the increase of nitrogenous compounds due to poor filtering by the kidneys (171, 341, 342). There have been no specific therapeutic regimens developed for *E. coli* O157:H7, but supportive care guidelines have been improved so that mortality rates are low (342).

1.1.4.2. Non-O157 EHEC

Non-O157 serotypes have been reported before 1982 and continued to occur on a regular basis in human and animal cases and outbreaks, but very few clinical laboratories actively screen for them, due to lack of reliable detection methods (33). These serotypes lack any obvious phenotypic characteristic that would allow for differentiation from commensal *E. coli* and therefore, no standard reliable method exists to isolate, characterize, and identify these pathogens. Unfortunately, they are only found and reported by the most proactive laboratories (33). Even though *E. coli* O157 is reported as the most common EHEC strain in many countries of the world, serotypes O26 and O111 are also recognized as serious threats to public health and have been recovered from patients (16, 32). The lack of isolation methods for non-O157 EHEC has inadvertently

led to a lack of awareness for these microorganisms. Based on physiological features, non-O157 EHECs do not posses distinct growth patterns and metabolic characteristics compared to *E. coli* O157:H7 and therefore require confirmation of additional genetic virulence factors (182). However, these methods have the disadvantage of not being able to recover and characterize the identified strains, which is ultimately crucial in tracking non-O157 disease epidemiology and developing strategies for containment (184). In general, isolation methods for non-O157 EHEC still rely on plating on sorbitol MacConkey (SMAC) agar, followed by individual PCR or colony hybridization confirmation for Shiga toxin genes.

The six most common non-O157 STEC, associated with disease, in the U.S. have been identified by the CDC as O26, O45, O103, O111, O121, and O145 (56). Currently, limited data exists for STEC prevalence in ground beef. A recent study reported the prevalence of STEC on final carcasses was found to be 3%, while the prevalence of *stx1* and *stx2* genes in ground beef was 26% (44). The prevalence of STEC on final carcasses was found to be 3%, while the prevalence of *stx1* and *stx2* genes in ground beef was 26%. The most common STEC was serotype O113. However, other common serotypes, such as O26, O45, O111, and O145 were not isolated from any beef sources. The Minnesota Department of Health conducted a study in which the characteristics of non-O157 and O157 STEC infections identified through sentinel surveillance were compared (152). Overall, the non-O157 STEC isolates were recovered from stool specimens obtained from ill patients slightly more frequently than O157. Non-O157 cases were identified

with the same frequency in the urban and rural populations. O157 infections were found to be more severe, but non-O157 infections caused significant morbidity as well.

1.1.4.3. Stress responses

Acid resistance is defined as the ability of bacteria to survive low pH and weak acids (28). Many intestinal bacteria have the ability to survive extreme acid conditions to colonize the host's intestine and survive the low pH gastric environment (28). As many as four stress response systems capable of protecting stationary-phase cells against acidic environments have been identified in *Escherichia coli* (220, 221). When bacteria reach stationary phase they undergo genetic or morphologic changes allowing them to be more resistant to stresses such as osmotic, heat, cold, or acid shock, compared to log-phase cells (78). It has been shown that σ^s is the master regulator of the stationary phase response and is the product of the *rpoS* gene of *E. coli* (207). Stress resistance is the result of the expression of proteins on entry into stationary phase that help protect housekeeping and metabolic enzymes from denaturation (20). DNA replication is repressed in stationary phase by H-NS proteins, compacting DNA and increasing resistance to various stresses (373).

Five kinds of decarboxylases have been identified in bacteria in general: glutamate-, arginine-, lysine-, ornithine-, and histamine-decarboxylase (136). Most enteric bacteria are likely to possess at least one of the aforementioned decarboxylases. They function by converting their respective amino acid into carbon dioxide and an

amine product while increase the intracellular pH (136). Glutamate, arginine, and lysine decarboxylase have been shown to protect bacteria from pH values between 2 and 3 (28).

The oxidative acid resistance mechanism is observed only if cells are grown in mild acidic complex media in the absence of glucose, and once activated, cells can survive a pH of 2.5 or greater in minimal glucose medium (73). The *rpoS* is involved in controlling gene expression while transitioning from log-phase to stationary-phase, while other factors such as cyclic AMP, and cAMP receptor protein influence the regulation of this system (73). One of the AR systems requires glutamate for protection at pH 2.5 or lower, and involves two glutamate decarboxylase isozymes and the putative glutamate: γ-aminobutyric acid (GABA) antiporter encoded by *gadAB* and *gadC*, respectively (121). The genes encoding GadA and GadB are found at different sites in the chromosome, 78 and 33 min, respectively, and they share a 98% homologous sequence and encode glutamate decarboxylase isozymes (90).

The glutamic acid decarboxylase (GAD) enzymes are pyridoxyl phosphate-containing enzymes that replace the alpha-carboxyl groups of their amino acid substrates with a proton that is taken up from the cytoplasm, while carbon dioxide and GABA are produced as the end products (120). Studies conducted by Foster (120), show that once an AR system has been induced, *E. coli* O157:H7 can remain acid-resistant for at least a month during refrigeration. The ability of *E. coli* to survive acid exposure depends on the growth stage that they reach before acid challenge (208). In addition, after exposure to acidic condition, the survival rate is significantly lower for log-phase cells compared to stationary-phase cells under the same conditions (120, 208). This is because AR systems

are controlled in part by the RpoS that is activated in stationary phase and in turn activates and represses proteins required for the response to various stresses (208, 245). It has been shown that *rpoS* mutants are more acid sensitive and show an inability to utilize arginine and glutamate AR systems (220). Stationary phase cells may be more AR compared to exponentially growing cells, but it is commonly accepted that they can become AR.

Some genes expressed in the stationary phase can cause the same gene expression as when *E. coli* is pre-exposed to physical stress and can ultimately lead to protection from other stresses also known as cross protection (78, 181). The *gadA*, *gadBC*, and the regulatory *gadE* gene are induced by low pH in exponential phase cells grown in minimal glucose media (228). Under normal conditions, when *E. coli* is growing in rich media, the expression of most decarboxylase systems is repressed by the cAMP receptor protein (CRP), and is relieved in minimal media for glutamate decarboxylase (98). Acidic anaerobic media have been found to induce AR in *E. coli* cells, while a number of genetic systems are known to be co-induced by acid and anaerobiosis (322). Adding $CdCl_2$ and ethanol to log-phase *E. coli* W3110 has been shown to inhibit growth by 50% and shift the growth curve to stationary phase (357), during which cells are most AR. The induction of *gadA* through exposure to N-acyl-L-homoserine lactone increased acid tolerance (pH 4) of *E. coli* in log-phase (162). Neutralized medium filtrates from *E. coli* grown to stationary phase at pH 5 induce acid tolerance in log phase cells growing at pH 7 (303). It has been suggested that *E. coli* expels one or more compounds during growth

to the stationary phase that can confer a protection against acid shock in the log-phase (236).

The gadAB/C genes are induced in response to stationary phase signals, while known regulators that affect their expression include RpoS, GadW, and GadX (90). The level of involvement of each regulator apparently depends on the growth phase and the medium. Any acid resistance induction that can withstand low pH levels in the exponential phase in minimal glucose media has been demonstrated to depend on the activation of GadAB/C that results primarily from the induction of GadE (228).

1.1.4.4. Virulence factors

The pathogenicity of STEC is determined by several virulence factors that are encoded by chromosomal pathogenicity islands, phage chromosomes integrated in the bacterial genome, as well as plasmids. Virulence factors that appear necessary for virulence of EHEC O157 are the Shiga toxins, the locus for enterocyte effacement (LEE) and the large plasmid pO157

1.1.4.4.1. Shiga toxins

Shiga toxins are members of a toxin family that share many common features. The shiga toxins identified in EHEC are classified into two distinct subgroups; Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2) (268). The toxins are produced by the pathogen in the colon and cause local damage as well as having the ability to travel through the bloodstream to the kidney where it is thought to play a role in causing HUS (189).

Common characteristics of Shiga toxins include the fact that both toxins are polymeric consisting of an A subunit and pentameric B subunits. Both are encoded in an operon with the A subunit gene proximal to the B subunit gene. Furthermore, they are both phage and chromosomally encoded (109, 110). The B subunit mediates binding to receptors in eukaryotic cell membranes, while the A units have N-glycosidase activity, which lead to cell death by inhibition of protein synthesis at the level of 28S ribosomal RNA. Stx2 is 1,000 times more cytotoxic than Stx1 (109, 110). The toxin phenotypes are variable among non-O157 STEC, while there is considerable epidemiological evidence that suggests that STEC isolates producing Stx2 are more commonly associated with more serious disease compared to isolates producing only Stx1 (22).

1.1.4.4.2. Attaching and effacing adherence

A whole cluster of virulence factors encoded by a chromosomal region called the locus of enterocyte effacement (LEE) is present in many STEC strains and are responsible for attaching and effacing lesions. The LEE encodes for a type II secretion factor, an adhesion called intimin (*eaeA*) and for the translocated receptor of intimin (257). Intimin is a 94- to 97-kDa outer membrane protein that has been identified in EHEC (9). Intimin is the only bacterial adherence factor identified in EHEC for intestinal colonization in an animal model (191). The ability of STEC to produce attaching effacing lesions is sufficient to cause non-bloody diarrhea but shiga toxin is essential for the development of bloody diarrhea, HC and HUS (257). The host epithelial receptor for

intimin, also known as the translocated intimin receptor (Tir), is encoded by LEE and is secreted for efficient delivery into the host cell (94).

Other structures that assist EHEC in adhering to host cells are fimbriae and fimbrial adhesins, thread-like structures that extend out from the bacterial surface. Type 1 fimbriae are the first adhesins described in *E. coli* (102) and are the most common adhesins produced. These adhesins promote the adherence of the pathogen to mannose-containing glycoproteins found on the surfaces of eukaryotic cells. P fimbriae recognize a disaccharide of uroepithelial cells and human erythrocytes (210), while S fimbriae mediate adherence to endothelial cells (281). F1845 is one of four adhesins that belong to the Dr family of adhesins (266) and the only one associated with diarrheagenic *E. coli.*

1.1.4.4.3. The pO157 plasmid

Plasmid pO157 is approximately 90 kb in size and has been found in almost all EHEC O157 isolates. The plasmid contains EHEC-hemolysin that is cytotoxic for human and bovine cell lines. EHEC strains also produce two different types of enterohemolysins. Plasmid encoding enterohemolysin genes are found on the 60-MDa EHEC plasmid, which when expressed can cause a hemolytic phenotype on washed sheep blood agar with small zones of lysis (308). There is also a bacteriophage associated enterohemolysin that has been identified as serologically and genetically distinct from the plasmid-encoded hemolysin and only the plasmid-encoding hemolysin are typically associated with EHEC pathogenicity (308). Another pO157-encoded virulence factor is EHEC KatP catalase-peroxidase, which is produced in addition to the 2 chromosomally encoded

catalases or hydroperoxidases of *E. coli* (57). Catalases assist the bacterium against oxidative stress, while peroxidases are hem-binding enzymes that carry out a variety of functions (223).

1.1.4.4.4. EHEC virulence profile

EHEC clinical isolates from HUS patients have been found to have a distinct virulence profile. Strains capable of producing both shiga toxins have been found to be highly associated with bloody diarrhea and HUS, while strains with only Stx1 are rarely found in HUS patients (209). In addition, clinical strains associated with HUS have also been found to be more enterohemolytic and are more likely to possess intimin (209). The virulence of STEC strains like *E. coli* O157:H7 is not only dependent on virulence factors, but also partially due to the pathogen's ability to survive environmental stress conditions, such as low pH levels found in the gastrointestinal tract, contributes to its very low infectious dose of 50-100 cells or lower (16). It has been shown that the combined presence of *stx2* and *eae* genes is an important predictor of HUS (112). From a clinical standpoint, when a patient has been infected with *E. coli* and has developed HUS symptoms, then STEC tend to be classified as EHEC (244). A summary of the virulence found in EHEC/STEC isolates can be found in Table 1.2.

Table 1.2. Possible and known virulence factors in EHEC/STEC isolates of human and cattle origin (369).

Virulence factors	gene	P*/C**	Proven/possible importance
Shiga toxin 1	*ST1*	C	Inhibits protein synthesis, causing cell death
Shiga toxin 2	*ST2*	C	
Intimin	*eaeA*	C	Mediates intimate adherence to epithelial cells
EHEC-hemolysin	*E-hly*	P	Hemolytic to washed sheep RBC
Type II secretion	*etpD*	P	Secretes extracellular proteins
Catalase-peroxidase	*katP*	P	Defense against oxidative stress etc.
Serine protease	*espP*	P	Cleaves coagulation factor V etc.
Type 1 fimbriae	*fimA*	C	Adhesins that enable adherence to host cells
P fimbriae	*papC*	C	
S fimbriae	*sfaD/sfaE*	C	
F 1845	*daaE*	C	
Aerobactin	*iutA*	C	Mediates bacterial iron acquisition
α-hemolysin	*hlyA*	C	Osmotic lysis of target cells

*P: plasmid, ** C: chromosomal.

1.1.5. Isolation

According to the USDA's Food Safety and Inspection Service (FSIS), ground beef is considered adulterated if as little as 1 CFU of EHEC O157:H7 is detected in 25 g of ground beef. *E. coli* O157:H7 is the only EHEC for which an official isolation method is in place. There are three type of methods used for STEC detection and most of them depend on shiga toxin gene detection. Cell culture cytotoxicity assays are conducted with Vero cells, allowing for screening of clinical samples, but may result in false negatives when food samples are processed. Immunological detection methods are dependant on antibodies that bind to antigens such as shiga toxins or O somatic antigens of cells. These

methods can produce presumptive results in a short period of time. DNA-based methods, such as polymerase chain reaction (PCR) amplification, can be used to screen different types of STEC with the primer targeting shiga toxin genes (294, 380). Currently, a number of multiplex-PCR assays have been developed for the various virulence genes enterohemolysin (*hly*), intimin (*eae*), and the two shiga toxins (*stx1, stx2*) (113, 283). Since these three virulence factors can be detected simultaneously, this allows for specific detection of EHEC.

Feces generally have low numbers of *E. coli* O157:H7, and enrichment steps are required to increase the levels of the organism to allow for detection. Enteric bacteria have similar physiological characteristics and therefore enrichment may cause the outgrowth of competitive microflora as well. To improve selective growth of *E. coli* O157:H7, selective media have been developed through the addition of antibiotics (158). There are three types of enrichment media that are often used for recovering *E. coli* O157:H7: buffered peptone water supplemented with 8 mg/L vancomycin, 10 mg/L cefsulodin, and 0.05 mg/L cefixime, modified EC broth (mEC with novobiocin), or mTSB with 20 mg/L novobiocin or 10 mg/L acriflavin (273).

Strains of *E. coli* O157 are relatively easy to isolate because of their unique biochemical characteristics. STEC O157 is unable to ferment the carbohydrate sorbitol, which led to the development of the sorbitol-MacConkey agar (SMAC) (235) used for its isolation. More specific media have also been developed, such as Rainbow Agar, CHROMagar ®, and O157:H7 ID agar that are able to recover STEC O157 along with sorbitol-fermenting O157 and non-O157 strains (33). Other methods have also been

developed to differentiate and identify STEC O157, but can be also used in various ways to identify non-O157 strains. Non-O157 serotypes can be differentiated from commensal *E. coli* by using specialized molecular techniques such as multiplex PCR (113). Immunomagnetic beads have been designed for the capture of the O antigen of O157 and some have been developed for the most commonly reported non-O157 strains such as O111 and O26 (Oxoid, Inc.). SMAC containing cefixime and tellurite (CT-SMAC) provide highly selective recovery of *E. coli* O157:H7 from other *E. coli* and enteric bacteria. Currently, CT-SMAC is widely used to isolate *E. coli* O157:H7 followed by PCR or latex agglutination confirmation. However, the use of CT-SMAC is not recommended for detection of non-O157 EHEC because most non-O157 EHEC that produce shiga toxins behave physiologically the same as other commensal *E. coli* strains (18, 182). Recently, a 16-plex PCR was developed, taking advantage of the multitude virulence factors found in diarrheagenic *E. coli*, including EHEC, EIEC, EAEC, EPEC, and ETEC (14). The virulence factors used for EHEC included *stx1*, *stx2*, *eae*, *escV*, *ent*, and *hly*, while the specificity of the PCR was 100% when tested with 289 control strains.

1.1.6. Ecology and evolution

1.1.6.1. Microbial ecology

1.1.6.1.1. Animals

Past studies have shown that as many as 30% of cattle can be asymptomatic carriers of *E. coli* O157:H7 (297, 330). Furthermore, manure from cattle feedlots and production facilities may contain viable *E. coli* O157:H7 and can be subsequently washed

into the water supply. This may be consumed directly by cows in drinking water, or be used as irrigation water on crops, or transmitted by other animal vectors (161, 212, 307). To this end, interventions that help reduce *E. coli* O157:H7 populations in food animals before they enter the food chain have great potential to reduce human illnesses (67). Studies have shown that EHEC O157:H7 are carried through the bovine gastrointestinal tract, and recently have been shown to be highly colonized and associated with the terminal rectal mucosa (256, 258). There is an increase in the amount of EHEC O157:H7 shed in cattle feces during the summer months, which correlates with increases in human illness. In fact, in 2006 the CDC reported that 43.47% of all STEC infections occurred in July, August, and September (241). Furthermore, carriage and shedding of EHEC has been reported to be more frequent with heifers and calves that generally show longer periods of shedding than older cattle (16).

A survey conducted by Cobbold et al (82) compared non-O157 Shiga toxigenic *E. coli* recovered from bovine, human, raw milk, and beef. The distribution of Shiga toxin genes among isolates indicated that *stx1* was predominant in milk, *stx2* on carcasses, and the combination of both *stx1* and *stx2* in beef. They found that the virulence factors *eae* and *hly*, were found at 23 and 15% of isolates, respectively. These findings highlight the importance of non-O157 as a threat to health since they are commonly found in food, and warrants critical assessment.

1.1.6.1.2. Environment

Some cattle are referred to as "super-shedders" and excrete EHEC for extended periods of time at levels as high as 10^4 CFU/g of feces (238). The shedding of EHEC in the environment by cattle increases the probability of other cattle harboring the pathogen through contamination of feed, water, fecal contamination of their hides, and other routes. These factors contribute to the almost ubiquitous presence of EHEC in any environment near cattle. Fresh fruit and vegetables may become contaminated with EHEC at various stages of their production. Contact with the soil, use of improperly composted manure, contaminated irrigation water, poor personnel hygiene, poor sanitation of equipment and wild animals could contribute to the dissemination of EHEC onto the field (36, 125, 349).

Contamination of vegetables grown in soils that have been enriched with contaminated manure depends on how well the pathogen survives in manure and manure-amended soils (124). The survival of enteric pathogens decreases once excreted from the animal gut (354). *E. coli* O157 has been reported to be able to survive for long periods of time in manure (126, 312) and manure-amended soil (127). Contamination of crops can occur through one of three ways: uptake via the root system, splash dispersal from the soil surface, or directly by irrigation water (124). Several studies demonstrated the association of *E. coli* O157:H7 and *Salmonella* with vegetable surfaces when grown in soils enriched with contaminated manure (172, 173), while the potential internal presence of pathogens is of concern since these cells will most likely not be removed by post-harvest or consumer sanitation actions, thereby posing a serious public health threat.

Recent work by plant pathologists and food microbiologists suggests that the connections between foodborne pathogens and fresh produce are more complicated than

simple passive transfer (352). The exact mechanism of contamination in the majority of produce-related outbreaks is usually unexplained, but research following the outbreaks suggests a highly complex ecology of the environment. Outbreak-related and other strains of *E. coli* O157:H7 have been routinely isolated from water sources in and around the area found to be the likely source of several lettuce-related *E. coli* O157:H7 outbreaks (84).

Harvesting and processing of fresh produce can cause plant tissue damage (23). A recent study attempted to assess the role of plant tissue damage in relation to contamination of leafy greens with *E. coli* O157:H7 (53). The effect of mechanical, physiological, and plant disease-induced lesions on the growth of the pathogen on postharvest romaine lettuce was investigated. Only 4 h after inoculation, concentrations of *E. coli* O157:H7 increased 4.0-, 4.5-, and 11.0-fold on lettuce leaves that were mechanically bruised, cut into large pieces, and shredded into multiple pieces, respectively. *E. coli* O157:H7 concentrations increased only twofold on leaves that were left intact after harvest. In addition, the concentration of *E. coli* O157:H7 was 27 times greater on young leaves affected by soft rot due to infection by *Erwinia chrysanthemi* compared to healthy middle-aged leaves. It would be suggested that growers postpone contaminated water irrigation of lettuce crops with suspected injuries for a minimum of 2 days, or use the highest microbiological quality of water available (23).

EHEC O157 and non-O157 have been shown to adhere to the leaf surface of spinach and lettuce through the use of EspA filaments, which play a major role in colonization of human and bovine hosts (104, 316). A study by Shaw et al showed that

O157 and non-O157 EHEC use a filamentous type III secretion system (fT3SS) for colonization of lettuce leaves (316). In contrast to the colonization of the mammalian host, EHEC adhesion to leaves is independent of effector protein translocation. EHEC do not cause disease once introduced into the inter-mesophyl cellular space but instead act as opportunistic epiphytes using the plant as a transmission vector (316).

1.1.6.1.3. Organic practices

Recently, there has been a decrease in consumer confidence in foods in general and especially in conventionally produced foods that may use pesticides, antibiotics, and other chemicals in food production (100, 319). Studies have shown that many consumers choose to purchase organic foods due to the perceived health and nutrition benefits of organic products. One of these studies suggests that the main reasons consumers purchase organic foods were to avoid pesticides (70%), for freshness (68%), for health and nutrition (67%), and to avoid genetically modified foods (55%) (376). Consumers appear to be willing to pay the typical 10% to 40% price premium that organic products claim.

Organic production is defined as an ecological production management system that promotes and enhances biodiversity, biological cycles, as well as soil biological activity (234, 310). U.S. regulations require that organic foods are grown without the use of synthetic pesticides, growth hormones, antibiotics, modern genetic engineering techniques, including genetically modified crops, chemical fertilizers, or sewage sludge. Synthetic materials are not allowed in organic production unless the materials are on the National List of Allowed and Prohibited Substances (376). Organic farming takes

advantage of animal and crop wastes, botanical, biological, or nonsynthetic pest controls, and allowed synthetic materials that can be broken down rapidly by oxygen and sunlight (170). Organic farming also promotes the use of specific methods to minimize air, soil, and water pollution. It can take several years to convert a conventional farming field to an organic one since the land must not have prohibited substances for 3 years before the harvest of an organic crop. According to the international federation of organic agriculture movement (IFOAM), livestock must be fed 80% organic feed for 9 months to convert the farm to organic, followed by 3 months of 100% organic feed, but they could receive vitamin and mineral supplements (170). Preventive management practices such as vaccinations can be administered when absolutely necessary to keep animals healthy, but those animal products cannot be sold as organic. Antibiotics cannot be used on products to be sold as organic.

In the U.S., the U.S. Department of Agriculture (USDA) initiated the Organic Foods Production Act (OFPA) as a component of the 1990 Farm Bill. There were 3 main goals that the OFPA was intended to achieve and included developing standards for marketing of organically produced products, assuring consumers that organic products meet a consistent standard, and facilitating interstate commerce (376). The OFPA promoted the establishment of the 15-member National Organic Standards Board (NOSB), established with the intent of making recommendations to the National Organic Program about substances that should or should not be allowed in organic production or handling, to help in developing standards for substances intended for organic production, and to counsel the Secretary of Agriculture on other aspects of the OFPA. The 15

members of the NOSB are appointed by the Secretary of Agriculture and represent all aspects of the organic food spectrum. The OFPA also established the National List of Allowed and Prohibited Substances, which is an inventory of synthetic substances and ingredients that are allowed, as well as natural substances and ingredients that are prohibited from organic production. The OFPA endorsed the establish National Organic Program Standards by the USDA. These standards specify methods, practices, and substances that are allowed for use in any capacity of producing organic foods.

Consumers are increasingly aware of the nutritional benefits of fresh fruits and vegetables. The USDA has stressed the importance of an adequate intake of fresh produce in our daily diet. Overall, the market for organic foods, and organic fresh produce in particular, has expanded at an annual rate of 20% (99). According to the USDA Organic Rule that was implemented in 2002, organic producers are required to conform to specific farm management practices that include treatment and application of animal-manure-based fertilizers (253).

Numerous reports have documented that animals such as cattle, sheep, pig and chicken are major reservoirs of foodborne pathogens such as *E. coli* O157:H7 and *Salmonella* . Animal manure can contribute several pathogens to soil, and some may die off in time, but others can persist for long periods of time. The use of untreated manure on produce crops carries a higher risk of contamination compared with treated manure, which has significantly reduced levels of pathogens (285). Using manure can lead to the transmission of pathogenic bacteria to fruits and vegetables (49). This has been demonstrated in a study by Solomon et al in lettuce when manure, inoculated with

laboratory cultures of *E. coli* O157:H7 was used for fertilization (329). Composting can generate high temperatures ranging from 55 to 65°C and under appropriate conditions of aeration, moisture, particle size and carbon-to-nitrogen ratio for long enough, it can inactivate foodborne pathogens (151).

Utilizing treated and untreated manure as a source of crop fertilizer is widespread in both organic and conventional agriculture (230). However, conventional farmers have a large variety of effective synthetic fertilizers at their disposal, along with manure, while organic farmers do not. In general, the importance of manure as a source of plant nutrients is far greater in organic production than in conventional systems (10). To this end, manure application in organic farmland is much more intensive and widespread. It has been suggested that organically grown vegetables are 8 times more likely to be contaminated with *E. coli* O157:H7 (21), but no data were ever presented to responsibly support such a statement. These kinds of claims remain unconfirmed, and two relevant investigations did not report any occurrence of *E. coli* O157:H7 in both organic and conventional vegetables (254, 274). It must be noted that both studies found that leafy green vegetables in general had the highest prevalence of non-pathogenic *E. coli*.

1.1.6.2. Molecular evolution

Evolutionary analysis through genome sequencing has shown that O157:H7 strains are genetically closely related to enteropathogenic *E. coli* O55:H7 strains (115). A model has been developed that shows that O157:H7 has evolved to its current form through a series of transitional steps from a nontoxigenic ancestor (115). The most

common ancestor of O157:H7 and O55:H7 strains contained the locus of enterocyte effacement (LEE) that allowed the strain to cause diarrhea through an attachment-effacement mechanism (374). Furthermore, this common ancestor had the ability to ferment sorbitol and express β-glucoronidase, thus resembling conventional *E. coli* (374). One of the first evolutionary steps included the acquisition of Stx2 hypothetically through transduction, followed by the large virulence plasmid (pO157), leading to the change of somatic antigen from O55 to O157 (374). The genes encoding Stx are encoded in the genome of heterogeneous lambdoid prophages. Stx-phages represent highly mobile genetic elements that play an important role in the expression of Stx, horizontal gene transfer, and genome diversification (155). From that early STEC two distinct lines emerged. The first line lost motility through a mutation in the flagellar operon, which lead to the sorbitol fermenting O157 clone that has been found in HUS cases in Germany (192, 250). The second line lost the ability to ferment sorbitol and acquired Stx1. Consequently, the *uidA* gene was inactivated through mutation, giving rise to the non-sorbitol-fermenting, β-glucoronidase-negative phenotype of the frequently occurring serotype O157:H7.

There have been several outbreaks involving O157 contamination of fresh produce, such as lettuce and spinach that were associated with more severe disease. These outbreaks lead to higher frequencies of hemolytic uremic syndrome and hospitalization, and suggested that increased virulence has evolved (233). This hypothesis was tested through phylogenetic analyses that identified 39 SNP genotypes and were separated into nine distinct clades. There were significant differences observed between

the different clades regarding their frequency and distribution of Shiga toxin genes as well as the type and severity of clinical disease they caused. It was found that patients with HUS were more likely to be infected with clade 8 strains (233). The frequency of clade 8 strains has increased from 10% in 2002 to 46% in 2006 despite a decrease in total O157 cases identified in this time period (233).

A study by Ogura et al (272) compared the genomes of EHEC serotypes O157, O26, O111, and O103. The investigators demonstrated how *E. coli* strains of different phylogenies can independently evolve into EHEC strains. Specifically, this study determined that the EHEC genomes contained many lambdoid phages, integrative elements, and virulence plasmids that contained the same or similar virulence genes but apparently had distinct evolutionary histories. This indicated that independent acquisition of these mobile genetic elements had driven the evolution of each particular EHEC. Of interest is the evolution of the type III secretion system (T3SS). The study found that the T3SS of EHEC is composed of genes that were introduced by 3 different types of genetic elements: a) an integrative element referred to as the locus of enterocyte effacement, which encodes a central part of the T3SS, b) SpLE3-like integrative elements, and c) lambdoid phages carrying numerous T3SS effector genes and other T3SS-related genes. In addition to gains and losses of phage elements, O157:H7 genomes are rapidly diverging and radiating into new niches as the pathogen disseminates (374).

E coli O157:H7 strains can be classified into different genotypes based on the presence of specific Shiga toxin-encoding bacteriophage insertion sites. Some genotypes are highly associated with human clinical cases, while others are more frequently isolated

from bovines (31). A recent study compared the expression patterns of clinical genotype 1 strains with those of bovine-biased genotype 5 strains using microarrays (356). What they found was that virulence factors, such as LEE genes, enterohemolysin, and pO157 genes, were highly expressed in clinical-biased genotypes. Furthermore, genes essential for acid resistance and stress fitness were upregulated in bovine-biased genotypes.

1.1.7. Methods of control

1.1.7.1. Post-harvest interventions

Some varieties of fresh-cut fruits and vegetables are no longer considered low risk in terms of food safety (40). One criticism of many of the investigations for sanitizing treatments for fresh produce is that they are used in extreme doses, excessive washing times, and sometimes unauthorized substances (139).

1.1.7.1.1. Temperature

Thermal processing is one of the most common interventions applied to foods to inactivate EHEC (111). The heat sensitivity of the pathogen has been extensively studied and reviewed. In recent years, mild heat treatments and high temperature short time treatments have been evaluated to inactivate STEC on raw produce and meat. However, a new study raised an important question. Pasteurization temperatures have been validated for STEC, but not for free Shiga toxin (295). The investigators measured Shiga toxin's inhibition effect on Vero cell dehydrogenase activity and protein synthesis after treatment with pasteurization temperatures. Shiga toxin 2 was found to be heat-stable and that

pasteurization of milk, as suggested by the U.S. Food and Drug Administration, (63^{o}C for 30 min, or 72^{o}C for 15 s or 89^{o}C for 1 s), did not reduce the biological activity of Stx2. However, treatment at 100^{o}C for 5 min inactivated the toxin.

A recent study investigated the effect of temperature on the survival and growth of EHEC on pre-washed, chopped Romaine lettuce (CRL) and grated iceberg lettuce (GIL) (333). It was found that on CRL held at 4^{o}C, the EHEC population decreased by 2 log CFU/g after five days. At 20^{o}C, the population increased by one log within 24 hours and remained constant thereafter. In GIL, the EHEC population increased by >1 log CFU/g after 24 hours and continued to increase throughout the five day study period. Storage at 20^{o}C for 48 hours resulted in deterioration of quality which was more in GIL.

1.1.7.1.2. High pressure

The application of High Pressure Processing (HHP) to enhance the safety of seeds or sprouts has been studied in the past (15, 286) and various degrees of efficacy have been shown. A recent study showed that the application of HHP on sprouts inoculated with EHEC O157:H7 can lead to more than a 5 log CFU/g reduction after 15 min at 650 MPa at 20^{o}C (259).

1.1.7.1.3. Ultrasound

Power ultrasound, as used for cleaning in the electronics industry, has a potential to be used as an application to decontaminate fresh produce (313). In a recent study conducted by Zhou et al. (384) ultrasound was used in combination with chlorine,

acidified sodium chlorite, peroxyacetic acid, acidic electrolyzed water, and on its own to inactivate EHEC O157:H7 spot inoculated on spinach leaves. Ultrasonication significantly enhanced the reduction of the pathogen for all treatments by 0.7 to 1.1 log cycles over that of each sanitizer on its own. The best combination of treatments was ultrasonication and acidified sodium chlorite (200 mg/L), resulting in a 4 log CFU inactivation.

1.1.7.1.4. Ionizing irradiation

Food irradiation uses high-energy Gamma rays, electron beams, or X-rays; all are penetrating processes and are used commercially to eliminate pathogens from meat products (326). Irradiation may be better than most technologies in penetrating fresh produce and it could be a powerful tool if used correctly in different produce items and among different varieties. Irradiation is able to effectively eliminate *E. coli* O157:H7 from lettuce (262). Niemira compared the antimicrobial efficacy of sodium hypochlorite washes and ionizing radiation for the elimination of leaf-internalized *E. coli* O157:H7 (261). The study found that 300 and 600 ppm sodium hypochlorite resulted in less than 1 log reduction in the pathogen. However, when ionizing radiation was used at 1.5 kGy, the pathogen was reduced by 4 log CFU in Romaine lettuce and 3 log CFU in spinach. Another study showed that irradiation up to 1.0 kGy can result in 3 to 4 log CFU reduction of internalized *E. coli* on lettuce leaves (140).

X-ray is a non-thermal technology that has potential for reducing pathogens on spinach leaves. A recent study found that more than a 5 log CFU reduction/leaf can be achieved with 2.0 kGy X-ray for *E. coli* O157:H7 (231).

1.1.7.1.5. Ozone

Ozone destroys microorganisms through progressive oxidation of critical cellular components, with the cell surface suggested as the primary target of the process. Chlorine, one of the most commonly used disinfecting agents, destroys certain intracellular enzyme systems, while ozone causes widespread oxidation of internal cellular proteins ultimately leading to rapid cell death (198). When apples inoculated with *E. coli* O157:H7 are treated with bubbling ozone during washing or through dipping, there was a 3.7 log CFU and 2.6 log CFU decrease, respectively (3).

1.1.7.1.6. UV light

Ultraviolet light (UV) is a type of non-ionizing radiation with wavelengths from 100 to 400 nm. Radiation has been used both to delay ripening associated processes and to reduce microorganism growth (358). When pulsed UV-light was applied to strawberries there were maximum reductions of *E. coli* O157:H7 of 2.1 log CFU/g at 25.7 J/cm^2, on raspberries 3.9 log CFU/g at 72 J/cm^2, and on blueberries 2.9 log CFU/g at 1.27 J/cm^2. There was no observable damage to the fruits at these UV doses (41, 42).

1.1.7.1.7. Radio frequency

A study by Nelson et al (260) evaluated Radio Frequencies (RF) as a method for reducing *Salmonella, E. coli O157:H7* and *Listeria monocytogenes* contamination in alfalfa seeds. Short RF exposures produced reductions in the target organisms without adverse effect on seed germination. However, it was found that there was an adverse effect on germination by extending RF exposure to produce the desired level of microbial reduction.

1.1.7.1.8. Chemical antimicrobials

Sanitizers that can be used to wash fruits and vegetables are regulated by the U.S. Food and Drug Administration in accordance with the Federal Food, Drug and Cosmetic Act as outlined in the Code of Federal Regulations, Title 21, Ch. 1, Section 173.315. One of the few treatments commonly used by large distributors of fresh produce is washing, and this procedure is often enhanced by including a sanitizing agent in the washing water. Use of a disinfectant can enhance efficiency of removal up to 100 fold, but chemical treatments administered to whole and cut produce typically will not reduce populations of pathogens by more than 2 to 3 log CFU/g (35). Chlorine is used under widely varying postharvest procedures (38). Little specific information is available on chlorine dosages and contact times to achieve maximum inactivation of produce-associated microbes. In general, the chlorine dosages (50 to 200 ppm) and contact times (1 to 2 min) used by produce processors generally result in 1 to 2 log (90–99%) bacterial inactivation (74).

Chlorine is also used in the form of sodium hypochlorite; less commonly used are citric and ascorbic acid (1%), and there have been a number of recent commercially

available products based on synthetic sanitizers (123). Past studies have made a strong case for the shortcomings of chlorine as an effective antimicrobial. It has been shown that sometimes it is as effective as, or marginally more effective than, deionized water in removing *E. coli* O157:H7 from the lettuce leaf and is unable to kill pathogens within damaged portions of the leaf or pathogens that have infiltrated into the leaf tissue (37, 219, 338) 33). Past research suggests that the surface structure of lettuce provides protects *E. coli* O157:H7 from inactivation by chlorine (338).

1.1.7.1.9. Cinnamaldehyde

There has been an increased interest in the development and application of new effective and nontoxic antimicrobial compounds. Plant essential oils have been found to have antimicrobial activity against a multitude of pathogens and show promise as an alternative to the currently used sanitizers (130). 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 (153). Cinnamon oil is commonly used in the food industry because of its special aroma. Furthermore, its antimicrobial activity has also attracted great attention from many researchers.

Cinnamaldehyde is present in numerous commercial foods at concentrations of up to 0.03% (131) and is listed as generally accepted as safe by the Flavor and Extract Manufacturers Association (8). *Cinnamomum cassia* bark oil is used in food and beverages and has high value from a commerce perspective. The main components of *C. cassia* bark oil are cinnamaldehyde and coumarin (164). These two compounds are used

as food additives. It has also been demonstrated that *Cinnamomum zeylanicum* oil can inhibit meat spoilage organisms (278).

When assessing the antimicrobial action of essential oil components, the lipophilic character of their hydrocarbon skeleton and the hydrophilic character of their functional groups are of the main importance. The activity rank of essential oil components is as follows: phenols > aldehydes > ketones > alcohols > ethers > hydrocarbons. Helander et al (153) used different essential oils to inhibit *E. coli* O157:H7 and *Salmonella.* 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 depleted 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 (76). In addition, the carbonyl group is thought to bind to proteins, preventing the action of amino acid decarboxylases in *E. aerogenes* (372). Di Pasqua et al (96) used fatty acid extraction and gas chromatographic analysis to assess changes in membrane fatty acid composition of *E. coli* treated with *trans*-cinnamaldehyde. Substantial changes were observed on the long chain unsaturated fatty acids when the *E. coli* strains grew in the presence of limonene and cinnamaldehyde.

Essential oils such as *trans*-cinnamaldehyde (187, 368, 379) have been successfully applied to suppress the activity of phytopathogens. Yang et al (379) evaluated the effects of supplementing the diet of feedlot cattle with cinnamaldehyde on

intake, growth performance, carcass characteristics, and blood metabolites. They found that including cinnamaldehyde in the diet of feedlot cattle, particularly early in the feeding period, may help promote intake and reduce the effects of stress. Charles et al (77) investigated the potential of low concentrations of *trans*-cinnamaldehyde to inactivate *E. coli* O157:H7 in cattle drinking-water. All *trans*-cinnamaldehyde concentrations used effectively inactivated *E. coli* O157:H7 in water and the magnitude of killing significantly increased as *trans*-cinnamaldehyde concentrations increased and storage temperature increased. The presence of feed or feces in water significantly decreased the antibacterial effect of *trans*-cinnamaldehyde on *E. coli* O157:H7.

It may be possible to use EOs in foods not previously associated with a herby or spicy flavor if the presence of one or more synergists can produce the desired antibacterial effect at a concentration which does not produce undesirable changes in the flavor or aroma (60). Baskaran et al (27) investigated the antimicrobial effect of low concentrations of trans-cinnamaldehyde on *Escherichia 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.

A study by Juneja and Friedman (187) tested the heat resistance of a four strain mixture of *E. coli* O157:H7 in raw ground beef in the presence of cinnamaldehyde. They found that contaminated sous vide processed ground beef should be heated to an internal

temperature of 60^{o}C for at least 30.3 min to achieve a 4-D reduction. Cinnamaldehyde and thymol are effective against six *Salmonella* serotypes on alfalfa seeds when applied in hot air at 50°C as fumigation. Increasing the temperature to 70°C reduced the effectiveness of the treatment (368). An active component of allspice, eugenol has been found to have a suppressive effect on the production of intracellular and extracellular shiga toxins by stationary phase *E. coli* O157:H7 (337). Antimicrobials in the vapor phase might be more effective in inactivating *E coli* O157:H7 cells attached to leafy greens than aqueous antimicrobials.

EO can also be used against hospital acquired infection in humans, specifically uropathogenic *E. coli* attached to urinary catheters as biofilms. Recently, Amalaradjou et al (12) treated polystyrene plates and urinary catheters inoculated with uropathogenic *E. coli* (5 to 6.0 log CFU) with difference concentrations of *trans*-cinnamaldehyde at 37°C. They found that all concentrations of the antimicrobial resulted in effectively preventing the pathogen from forming a biofilm on plates and catheters, while producing no cytotoxic effects on human bladder epitehelial cells.

1.1.7.1.10. Electrochemically activated water (EAW)

EAW water has been reported to have strong bactericidal effects on most pathogenic bacteria that are important to food safety (165). EAW water is produced by passing a diluted salt solution through an electrolytic cell that contains an anode and cathode separated by a membrane. By subjecting the electrodes to direct current voltages, negatively charged ions such as chloride and hydroxide in the diluted salt solution move

to the anode and become oxygen gas, chlorine gas, hypochlorite ion, hypochlorous acid and hydrochloric acid, while positively charged ions move to the cathode to take up electrons becoming hydrogen gas and sodium hydroxide (163). The main advantage of EAW is its safety. Koseki et al (201) used mildly heated (50°C) EAW water to treat lettuce for 5 min, followed by cold (4°C) EAW water to treat for 1 or 5 min. They found the treatment could reduce both *E. coli* O157:H7 and *Salmonella* at a level of 3–4 log CFU/g. Pangloli et al (279) evaluated the efficacy of electrolyzed water in killing *E. coli* O157:H7 on iceberg lettuce through the use of washing and/or chilling treatments simulating those followed in food service kitchens. They found that the greatest reduction levels on lettuce were achieved by sequentially washing with 14-A acidic electrolyzed water for 15 or 30 s followed by chilling in 16-A acidic electrolyzed water for 15 min. This procedure reduced the pathogen by 2.8 and 3.0 log CFU per leaf, respectively. A study by Keskinen et al (194) compared the efficacy of chlorine (20 to 200 ppm), acidic electrolyzed water (50 ppm chlorine, pH 2.6), acidified sodium chlorite (20 to 200 ppm chlorite ion concentration, Sanova®), and aqueous chlorine dioxide (20 to 200 ppm chlorite ion concentration, Trinova®) washes in reducing populations of *E. coli* O157:H7 on lettuce. They found that the chlorite ion solutions were the most effective against *E. coli* O157:H7 populations on Iceberg lettuce, with log reductions as high as 1.25 log CFU/g and 1.05 log CFU/g for TriNova® and Sanova® wash treatments, respectively. In contrast to previous studies, the acidic electrolyzed water as well as the rest of the treatments resulted in reductions of less than 1 log CFU/g on Iceberg lettuce. Chlorine

(200 ppm), TriNova®, Sanova®, and acidic electrolyzed water were all equally effective against *E. coli* O157:H7 on Romaine, with log reductions of approx. 1 log CFU/g.

1.1.7.1.11. Bacteriophages

Bacteriophages are viruses that prey on bacteria, offering a natural non-antibiotic method to reduce pathogens from the food supply (65). *E. coli* phages can be isolated from sewage, hospital waste water, polluted rivers and fecal samples of humans or animals (58). There are several steps through the life cycle of a bacteriophage that are universal to viruses: adsorption, separation of nucleic acids from protein coat, expression and replication of the nucleic acids, virion assembly, release and transmission (101). The sequence of phage infection and propagation includes the following events: (367): initially, they are adsorbed to a defined cell surface structure which can be reversible and could possibly allow the phage to opt out of infection. Next, an irreversible binding between a phage structure and the receptor occurs. After adsorption, the cell wall can be penetrated by special phage enzymes found in the tail or the capsid and the nucleic acid is transported into the cell, yet the capsid remains outside the cell. After injection, the genetic material can be either integrated into the host genome or stay in the cytoplasm.

After the initial viral invasion, the following step is a common stage for all viruses which involves the genome existing within the host but still outside the host genome. This is the step in which gene expression, genome replication and morphogenesis occurs. The capsids and tails and the packing of the genomes into the capsids takes place at this point. The latent period is defined as the phase of the infection,

in which there are no extracellular or free phages and describes the phage life cycle from adsorption to the cell lysis. The part of the latent period before capsids and genomes are formed into mature phages comprises the eclipse period. During the rise period mature phages are released into the environment due to cell lysis and detection of free phages is possible. The burst size is defined as the number of virions released per cell into the extracellular environment. Tailed phages accomplish cell lysis through a dual lysis system comprised of peptidoglycan hydrolases, also known as endolysins, that attack the murein portion of the cell wall, and holins, which damage the plasma membrane, thus allowing endolysin to break down the peptidoglycan (4). The extracellular stage ends with death of a phage or a new infection.

Viruses can undergo several different life cycles such as: lytic, lysogenic, pseudolysogenic and chronic infections (5). During the lytic cycle, the virulent phage is able to redirect the host metabolism towards its own benefit and the production of new phages, which are subsequently released during lysis of the cell. During the lysogenic cycle, the genome of the temperate phage in essence remains within the host in a dormant stage, also known as a prophage, and replicates along with the host, until the phage lytic cycle is somehow induced at some point. A chronic infection can take place when a cell is infected and progeny are continuously released into the environment from the host cell through budding or extrusion but not leading to cell lysis. In continual infections, also known as pseudolysogeny or the phage-carrier state, phages can multiply in a fraction of the population.

A variety of morphological characteristics within viral species has been described in past studies (5, 50). Typical phages have a head and a tail bound together by a connector. Other types of phages are also known, such as cubic, spindle, lemon-shaped, filamentous or pleomorphic viruses. There are many different structures such as head appendages, collar and tail fibers or spikes and exist in a large variety. Furthermore, the capsid diameter and genome size of phages can vary by more than one order of magnitude. Tailed phages can be traced back to the time before the separation of life into the three domains of Bacteria, Archaea and Eukarya, and are at least 3.5 to 3.7 billion years old (4). Species that are part of the order Caudovirales have a double-stranded DNA as genetic material and are separated into three families depending on the morphology of their tail and biochemical and molecular characteristics (4, 154). Phages that have long flexible tails are called Siphoviridae, contractile tails are called Myoviridae, and very short tails are called Podoviridae.

The metabolic state of the bacterial host is crucial for bacteriophage infection and propagation, since adsorption, replication, lytic activity and survival of the phage are affected (114, 375) }. A number of culture experiments have demonstrated that optimal proliferation and yield of phages are observed at ideal growth conditions of the host (215). Host generation times can influence phage latent periods (147, 247) and low nutrient availability may result in increased latent periods and reduced burst sizes (247, 292) suggest that phage propagation depends on host metabolism significantly. Stationary phase cells may allow phage maturation to proceed, but cell lysis can be stopped (247).

It is highly recommended to create cocktails of several phages to obtain sufficient breadth of host range and to reduce the probability of phage resistance (58). Phages utilized for pathogen control in foods and food production systems usually originate from environmental samples and other nonfood sources such as municipal waste water, feces, sewage, soil, farms and processing facility effluents (83, 103, 270, 280). Phages have also been suggested as a possible treatment strategy for dealing various bacterial infections and have been demonstrated to be effective against urinary tract infections in mice (263) and respiratory infections in chickens (167). In a large study, stool samples from 600 healthy patients and 140 patients suffering from traveler's diarrhea were investigated for the presence of coliphages on 10 different *E. coli* strains (135). From healthy subjects, 34% of the stool samples contained phages, but only 1% showed high amounts. Furthermore, most of them were temperate phages. However, 70% of the stools from diarrhea patients contained phages, of which 18% were in high concentrations. Coliphages can be used as a surrogate measure for fecal contamination of recreational waters or other waters of public health interest (108).

Using specific phages to eliminate or reduce the levels of contaminated bacteria on fresh-cut fruits and vegetables is also under investigation for *E. coli* O157:H7 (203). As part of an ongoing study, Sharma et al. (315) tested the effectiveness of a mixture of bacteriophages in reducing *E. coli* O157:H7 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. A study by Niu et al (265) evaluated the host range and lytic capability of four phages against *E. coli* O157 from cattle and humans. They found that the phages were effective against the majority of the bovine and human STEC O157 isolates and suggested that lytic capability and host range should be considered when selecting a therapeutic phage for on-farm control of STEC O157. Furthermore, they advocated for the use of phage cocktails as an effective mitigating approach for STEC O157 due to the observation that some STEC O157 isolates exhibited resistance to some but not all phages.

Bacteria frequently live in biofilms, which are surface-associated communities encased in a hydrated extracellular polymeric substances (EPS) matrix that is composed of polysaccharides, proteins, nucleic acids, and lipids and helps maintain a complex heterogeneous structure (88, 377). Bacterial biofilms have been implicated as a source of infection and contamination in medical, industrial, and food processing settings because of their resistance to antimicrobial agents and host defenses (377). There is a growing need for novel and effective treatments for biofilms due to their increasingly apparent antibiotic resistance and the fact that antibiotics can even induce biofilm formation (331).

Bacteriophages have been proposed as a method for controlling biofilms in several studies. Sharma et al (314) used an alkaline cleaner and a bacteriophage to treat *E. coli* O157:H7 in biofilms on stainless steel and found that even though populations of cells that were attached on coupons were reduced by the phage, the cells enmeshed in biofilms were protected. Corbin et al (86) found that biofilms under carbon limitation can act as natural reservoirs for bacteriophage and that the phages can have some influence

on biofilm morphology. In a study by Tait et al (336), bacteriophages specific for *Enterobacter* strains were isolated from primary effluent sewage. Combinations of three phages were required to completely eradicate biofilms of *Enterobacter cloace*. However, when trying to eliminate a susceptible bacterial population within a dual species biofilm, the attempt was unsuccessful. This suggested that phages would be a poor tool by themselves for controlling biofilm formations, but a combined treatment with a disinfectant may be successful.

Research by Lu and Collins (226) showed that the use of engineered enzymatic bacteriophages was effective in dispersing biofilms of *E. coli* at 4.5 orders of magnitude. The investigators engineered a bacteriophage to express a biofilm-degrading enzyme that was capable of attacking the bacterial cells in the biofilm and the biofilm matrix itself. The enzyme, Dispersin B (DspB), is produced by *Actinobacillus actinoinyceteincomitans*, and is able to hydrolyze β-1,6-*N*-acetyl-D-glucosamine, which is an adhesin needed for biofilm formation and integrity in *Staphylococcus* and *E. coli* (169). Though not a "natural" antimicrobial, engineered bacteriophages that produce polysaccharide depolymerases can reduce bacterial biofilms by attacking both the biofilm collectively and the bacteria individually. Reports of natural lytic phages with phage-borne polysaccharide depolymerases have shown that phage-induced lysis and extracellular polymeric substances (EPS) degradation can be used in combination in natural systems to reduce biofilms (169).

Advantages of using phages over traditional antimicrobial systems for foods have been reviewed on the pre-harvest (26, 180) and post-harvest level (216, 218). 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 (334). In addition, recently there has been exploration in different phage delivery systems. Puapermpoonsiri et al (293) showed that phages specific for *Staphylococcus aureus* or *Pseudomonas aeruginosa* could be encapsulated into biodegradable polyester microspheres via a modified w/o/w 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.

Using combined treatments is consistent with the hurdle concept (211), which 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. 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.

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 (382) and sprouting mung beans and alfalfa seeds (381). 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 *Enterobacter asburiae* could be enhance with phages when used on tomatoes. Leverentz et al (216) applied phages in combination with nisin against *Listeria monocytogenes* on fresh-cut honeydew melons and fresh-cut apples. They found that the phages on their own inactivated *L. monocytogenes* by 2.0 to 4.6 log CFU over the control, while nisin on its own resulted in a 3.2 log CFU reduction on melons. However, when the two treatments were used in combination, there was a 5.7 log CFU inactivation. On the other hand, the synergy between the phages and nisin that was exhibited on melons was not demonstrated on fresh-cut apples. Roy et al (304) used a combination of *L. monocytogenes* specific phages and QUATAL, a quaternary ammonium compound to disinfect *L. monocytogenes* from stainless-steel and polypropylene surfaces. A synergistic activity was observed when the phages were suspended in QUATAL and found that the phages were not affected by the compound at 50 ppm and a contact time of 4 h.

In another example of using a phage with an additional antimicrobial, Huff et al (166) used a bacteriophage and Baytril (enrofloxacin) to treat colibacillosis in broiler chicken. Mortality in the birds was 3% when treated with enrofloxacin and 15% when treated with the phage alone. However, the birds that received both treatments had total protection. a recent study by Los et al (225) suggested that when isolated phages from the environment; the use of sublethal concentrations of antibiotics could result in an increase in plaque diameters and isolation of phages which could have been overlooked due to formation of small plaques or no plaques at all. The success of the method can be

attributed to a partial imbalance in the regulation of lysis inhibition in the host with an impaired, but not fully suppressed, protein synthesis system. The synergy between these two treatments suggested that phages combined with an antibiotic or other antimicrobial, preferably a natural one, have significant value.

1.1.7.2. Pre-harvest interventions

Human illnesses caused by the most common food-borne pathogens cost the US economy alone more than $7 billion each year (62). Successful strategies to control the prevalence of *E. coli* O157:H7 in ruminants can potentially reduce the threat this pathogen poses. Intervention/supplementation strategies can be grouped into three approaches: competitive enhancement strategies, direct anti-pathogen strategies, and animal management strategies, of which some are available now and some will be available in the future (67). It should be noted that currently, no reliable intervention or animal vaccine is commercially available. Probiotics used to create an intestinal environment that can inhibit *E. coli* O157:H7 have been tested, but without consistent success (214, 271). Hay feeding has been shown to reduce colonization of *E. coli* O157:H7 and decrease prevalence from 52 to 18%, but this effect has not been consistently observed by other researchers (66). An estimated 50 to 70% of antibiotics used in the US are given to farm animals (148) for three main reasons: 1) prophylactically, to prevent disease in flocks and herds, 2) to treat sick livestock, and 3) to improve digestion and utilization of feed, often resulting to improved weight gain (334). The use of antibiotics in livestock has become a major source of concern due to the

possibility of contributing declining efficacy of antibiotics used to treat bacterial infections to humans (324). Bacteria have many complex mechanisms to resist antibiotics, and the widespread use of antibiotics in both human medicine and animal agriculture has led to the broad dissemination of antibiotic resistance genes (61, 288). Due to the concern over antibiotic resistance, it is likely that the prophylactic use of antibiotics to promote growth in food animals will become even more highly regulated, or perhaps even prohibited (67). Banning or reducing the application of antibiotics may pose a risk in the safety of foods and the treatment of sick animals, unless an effective, safe, and environmentally friendly alternative is developed such as the use of bacteriophage-based antibacterial products (334).

Using bacteriophages to reduce contamination of foods with various pathogens requires in-depth understanding of the epidemiology of the pathogen against which the phage preparation will be used (332). There are three areas of application of phage technology: 1) phages may be used to reduce intestinal colonization of live animals that carry pathogens, 2) phages may be applied directly onto raw foods or onto environmental surfaces in raw food processing facilities, 3) phages may be applied directly on ready to eat (RTE) food (334).

Phages have been evaluated to control pathogens in a variety of foods of animal origin. Phages of *E. coli* O157:H7 have been characterized and their antibacterial activities have been determined in vitro in broths (203, 270, 300). Bacteriophage CEV1 is a phage that specifically infects *E. coli* O157:H7 and was isolated from sheep resistant to colonization by the pathogen (296). Sheep that received a single oral dose of the phage

showed a 2-log-unit reduction in intestinal *E. coli* O157:H7 levels within two days compared to levels in controls. A study conducted by the same research group (277) showed that the prevalence of O157:H7-infecting phages in livestock may be grossly underestimated if an enrichment method is not used. In this study, fecal samples from commercial beef feedlot were screened to detect O157:H7-infecting phages and after an initial screen which produced no recovery of phages an enrichment protocol was used. This resulted in detection of phages for O157:H7 or non-pathogenic *E. coli* in the majority (97%) of the samples. Jensen et al. (179) suggested that a multiple-host enrichment protocol may be more effective for the isolation of broad-host-range bacteriophages by avoiding the selection bias that single-host methods typically have.

Tanji et al (340) used three phages to rapidly evacuate *E. coli* O157:H7 in artificially inoculated mice, but the difference of *E. coli* concentration in the feces of mice in the group with phage became slight after the 9-day test period compared to the control group. Tanji et al. (340) demonstrated the effective use of phage cocktails to avoid the emergence of phage-resistant cells. Barrow et al. (25) used an *E. coli*-specific bacteriophage to prevent septicemia in chickens. The control group had a mortality rate of 100% after inoculation with 10^6 CFU, while a single injection of the phage preparation prior to the bacterial challenge prevented morbidity and death. The higher the dose of the phage, the more effective the protection it provided. Similarly, colostrums-deprived calves were challenged with the same strain of *E. coli* leading to septicemic disease, but when calves were injected with the phage preparation, they remained healthy. Despite the statistical limitations of the study, the phages did have a positive therapeutic effect. Huff

et al. (168) studied the ability of phage therapy to prevent fatal *E. coli* respiratory infections in broiler chickens by challenging the groups of 3-day-old chickens with mixtures of the pathogen and phages. They also made 1-week-old chickens drink water with a certain pathogen/phage mix. In the first experiment, mortality rates decreased significantly, from 80% to 5%, while in the second they did not.

A cocktail of phages reduced O157:H7 populations in the feces of sheep by 24 hours after treatment (65). Furthermore, a 1:1 ratio of plaque forming units over colony forming units was found to be more effective than higher ratios of 10:1 or 100:1. Another cocktail of three bacteriophages was used by O'Flynn et al (270) for biocontrol of *E. coli* O157:H7. Bacteriophage-insensitive mutants (BIMs) were recovered at a very low frequency (10^{-6}) and reverted to phage sensitivity after 50 generations. In a meat trial experiment, the phage cocktail completely eliminated *E. coli* O157:H7 from the beef meat surface in seven of nine cases (270). Sheng et al (317) have argued that efforts to consistently clear *E. coli* O157:H7 from cattle may be unrealistic and their study showed that phage therapy would be effective at reducing the levels of intestinal *E. coli* O157:H7 in ruminants, but also highlighted the difficulties in developing an effective phage intervention. In their study, Sheng et al. (317) found that phage therapy reduced the average number of *E. coli* O157:H7 CFU among phage-treated streers compared to the control group, but did not eliminate the bacteria from the majority of steers.

A study by Rozema et al (305) compared the effects of oral and rectal administration of O157-specific phages aimed at reducing the fecal shedding of *STEC* O157. They found that orally treated steers produced the fewest STEC O157 culture-

positive samples compared with rectally treated steers and a combination of orally and rectally phage treated steers. However, this number was barely lower than that for the untreated steers. It is worth noting that phages was isolated from untreated steers, indicating that these specific steers had acquired phages from the environment and shed them at a level similar to that of rectally treated steers. Constant phage therapy has been shown to be an effective method for reducing the shedding of *E. coli* O157:H7 in cattle, as long as the host bacterium is not resistant to phages (305).

Ionophores, such as monensin and lasalocid, are regularly included in the majority of feedlot and dairy rations and are intended to inhibit gram-positive bacteria, resulting in an improved feed to gain ratio and production efficiency (70). It was hypothesized that due to their gram-negative membrane physiology, EHEC would not be affected by these feed additive antimicrobials, giving them a competitive advantage with regard to their role in colonization and shedding (64). However, it was found that Ionophoric feed additives (monensin, lasalocid, laidlomycin and bambermycin) had no effect on *E. coli O*157:H7 in vitro (106). Several studies have investigated the relationship between these feed additive antimicrobials and many have suggested there is a potential interaction between diet type and antimicrobial treatment, but no definitive proof of this linkage has been demonstrated (87, 156, 240).

Neomycin is another antimicrobial that has proven to be effective against EHEC both in ruminants' guts and hides, but it is also use in human medicine and there is concern for antimicrobial resistance (64). A nonantibiotic alternative for reducing the prevalence of *E. coli* O157:H7 includes the use of sodium chlorate by applying it to cattle

feed and water (68). Hide washing involves physical removal of contaminants from the hide and hooves from cattle and can significantly reduce carcass contamination (47). Other approaches include using ozonated or electrolyzed water (46).

Vaccination is used to prevent pathogen colonization and fecal excretion in ruminants and it is based on inducing the animal's immune system to protect itself from antigens expressed by *E. coli* O157:H7 (213). Priming the mucosal immune system to have a protective response against an organism that is usually commensal is a difficult task, but researchers have created vaccines targeted against cellular components and proteins that help the organism adhere to the intestinal mucosa of calves (213). These include type III proteins, Tir, intimin and the O157 lipopolysaccharide (34, 199, 290). In a recent clinical vaccine trial, commercially fed cattle were used to test the effect of a two-dose regimen of a vaccine against type III secreted proteins of *E. coli* O157:H7 (325). The study found that pens of vaccinated cattle were less likely to test positive for *E. coli* O157:H7. Another study tested the efficacy of a siderophore receptor and porin proteins-based vaccine on *E. coli* O157:H7 in feedlot cattle (348). The investigators found that the prevalence of *E. coli* O157:H7 was lower in vaccinated compared to control animals and vaccination was associated with a 98.2% reduction in *E. coli* O157:H7 concentration in fecal samples.

Feed management has been suggested as a viable method to affect conditions within ruminant gastrointestinal tracts and ultimately modify the survival of *E. coli* O157:H7 (213). There have been some conflicting studies on various feedstuffs and interpretations of results do not always agree between different research groups. For

example, early studies suggested that cottonseed and clover feeding could reduce fecal excretion of *E. coli* O157:H7 in dairy cattle (87), while later studies actually reported a positive association between the two feeds and the prevalence of the pathogen (306). Corn silage, barley, and beet pulp have been found to increase the prevalence of O157 in cattle (30, 87). There is a plethora of explanations on how a specific feed influences the gastrointestinal microflora such as altering volatile fatty acid concentrations, changing the pH conditions, and altering the composition of the resident bacteria (213).

A *Lactobacillus acidophilus* culture has demonstrated effectiveness at reducing *E. coli* O157:H7 in feedlot cattle by up to 50% (383). This particular product is currently available commercially in the U.S. and is being used in many large US feedlots (69). Molecules released by probiotic strain La-5 influence the transcription of EHEC genes involved in colonization of epithelial cells (243). Furthermore, these molecules are able to prevent the adherence of EHEC to epithelial cells and its capacity to concentrate F-actin at adhesion sites.

Natural microflora present on fresh produce may help reduce the pathogen load. A recent study isolated natural microflora from fresh-cut iceberg lettuce and baby spinach and found them to be antagonistic toward *Escherichia coli* O157:H7 (186). Samples were collected under conditions that mimicked actual practices between production and retail sale. The inhibitory activity by several isolates was due to either acid production or antimicrobial peptides. The most common isolates obtained from multiple processing and storage steps were members of the genera *Pantoea, Pseudomonas, Klebsiella, Enterobacter, Aeromonas,* and *Burkholderia*. Cooley et al (85) investigated the

interaction between *E. coli* O157:H7and epiphytic bacteria in lettuce extracts and on inoculated seedlings. Coinoculation with *Enterobacter asburiae* was found to reduce survival, while *Wausteria paucula* was found to increase it. These observations suggest that species specific competitive or commensal relationships likely occur in natural systems (92).

Lately, due to increased ethanol production, there has been an increased availability of distillers grains, an ethanol fermentation coproduct derived from corn and included in cattle diets as a protein and energy source (197). Recently, there have also been several reports showing evidence that by including distiller's grains solids (DGS) in cattle feed leads to an increase of the level of fecal shedding and prevalence of *E. coli* O157:H7 in cattle. Dewell et al (95) showed that feeding DGS was a risk factor for *E. coli* O157:H7 carriage in beef cattle. An increased fecal prevalence of this pathogen was also observed by another study when animals were fed 30 and 40% DGS in their ratios (287). Other work that was done included 379 naturally infected animals and showed that the prevalence of *E. coli* O157 positive pen samples was 2.5-fold larger in cattle fed 25% distiller's grains compared to control samples (176). The same investigators observed significantly higher levels of *E. coli* O157:H7 in feces and intestinal tissues of animals fed 25% DGS compared with animals fed steam flaked corn (177). However, in their latest study, Jacob et al. (175) did not report a significant difference in the prevalence of naturally infected animals fed DGS or dry-rolled corn.

Bolisevac et al (45) examined *E. coli* O157:H7 prevalence in feedlot steers that were fed diets with or without wet distillers' grains with soluble (WDGS). They found

that the average percentage of fecal *E. coli* O157:H7 during the finishing phase for WDGS fed cattle was 2.7% compared to 0.1% for corn fed control cattle. Also, there was no significant difference in the average percentage of *E. coli* O157:H7 hide samples between diets, but the WDGS fed cattle had higher levels. Overall, feeding 40% WDGS may increase the level and prevalence of *E. coli* O157:H7 in feedlot cattle. However, the magnitude of the difference detected in this study could possibly have been skewed by the low prevalence in control cattle. The overall mechanism responsible for the phenomenon of increased *E. coli* O157 prevalence with increased feeding of DGS in cattle is not known (174). There are two proposed general mechanisms for this trend; distillers grains alter the hindgut ecology of cattle, making the environment suitable for *E. coli* O157, or a component of distillers grains stimulates the growth of the pathogen O157 (176). Hindgut ecology has been shown to change when cattle are fed distillers grains and at the same time distillers grains have been shown to alter rumen microbial populations (133). Further research is required before the mechanism can be elucidated.

1.1.8. Outlook

Currently, *E. coli* O157:H7 outbreaks and product recalls as a result of *E. coli* O157:H7 contamination comprise one of the largest threats to the long-term sustainability of the fresh produce industry. Outbreaks such as the lettuce and spinach outbreaks of 2006 undermine consumer confidence and target the fresh produce industry as unable to protect their product and consumers and the beef cattle industry as the source of environmental contamination. To solve this problem, proactive steps must be taken to

develop effective strategies capable of reducing fecal shedding of the pathogen by feedlot cattle and to reduce its prevalence and persistence in the environment. Evaluation of pre-harvest control measures that effectively reduce fecal shedding of *E. coli* O157 by cattle and other ruminants is crucial prior to developing on-farm strategies. These steps could potentially reduce the number of *E. coli* O157:H7-positive animals and thus minimize foodborne illness associated with this pathogen. Effective approaches must start at the farm level before sending cattle to slaughter and follow through with proper measures during growth, harvesting and packing of fresh produce.

1.2. Statement of the problem

Since the 1980's, EHEC strains have been recognized as food-borne pathogens with serotype O157:H7 most the most frequent cause of outbreaks. EHEC cause human infections that involve HC, milder forms of diarrheal illness, and they are the major etiologic agent responsible for the HUS (144, 282). It has been shown that the combined presence of *stx2* and *eae* genes is an important predictor of HUS (112, 209). Clinical strains associated with HUS have also been found to be more enterohemolytic and are more likely to possess intimin (209). The high virulence of EHEC strains like *E. coli* O157:H7 is not only dependent on virulence factors, but is also partially due to the pathogen's ability to survive environmental stress conditions, such as resistance to low pH levels found in the gastrointestinal tract, contributing to its very low infectious dose of 50-100 cells or lower (16).

The incidence of foodborne illness associated with the consumption of minimally processed ready-to-eat salad vegetables has been consistently increasing (35, 188, 344). Between the years of 1990 and 2001, contaminated fresh produce was associated with a total of 148 outbreaks comprising approximately 9% of all foodborne outbreaks (323). In recent years, EHEC has been the culprit of raw food outbreaks world-wide and fresh produce in particular (29, 91), with *E. coli* O157:H7 being one of the leading causes of produce related outbreaks, accounting for 20% of the outbreaks in which the etiological agent was identified (275). The increase of foodborne outbreaks due to the consumption of fresh vegetables stresses the importance of developing antimicrobial strategies to reduce their microbial load (7, 71, 160, 185, 232, 345, 353, 370). The most recent outbreaks of foodborne disease caused by bagged lettuce and spinach have exposed the limited effect of current conventional washes and the industry as a whole would benefit from research on alternative effective antimicrobial treatments (71, 232). Unfortunately, due to the fresh characteristic of produce, the number of antimicrobial treatments is rather limited.

Sanitizers that can be used to wash fruits and vegetables are regulated by the F.D.A. One of the few treatments commonly used by large distributors of fresh produce is washing, and this procedure is often enhanced by including a sanitizing agent in the washing water. Use of a disinfectant can enhance efficiency of removal up to 100 fold, but chemical treatments administered to whole and cut produce typically will not reduce populations of pathogens by more than 2 to 3 $\log_{10}$ CFU/g (35). Chlorine is used under widely varying postharvest procedures (38). Little specific information is available on

chlorine dosages and contact times to achieve maximum inactivation of produce-associated microbes. In general, the chlorine dosages (50 to 200 ppm) and contact times (1 to 2 min) used by produce processors generally result in 1 to 2 log CFU (90–99%) bacterial inactivation (74). This sanitizer is also used in the form of sodium hypochlorite; less commonly used are citric and ascorbic acid (1%), and there have been a number of recent commercially available products based on synthetic sanitizers (123). Past research has indicated that organic fresh produce did not pose greater risk of contamination with foodborne pathogens and indicator organisms than conventional produce (254, 255). However, the greatest number of *E. coli*-positive samples in both organic and conventional produce has been found in leafy vegetables such as lettuce, spinach, kale and collard. These results stress the importance of targeting high risk vegetables for developing interventions. Mixtures of bacteriophages with a wide spectrum of activity could potentially serve as one of such antimicrobial treatments when applied to fresh produce.

Organic agriculture is a production system that in principle bans the use of synthetic materials in food manufacture (202). From 1992 to 1997, organic farmland in the U. S. doubled, while between 1990 and 2002, sales of organic foods increased from $1 to $9 billion (105). In addition, fresh fruits and vegetables accounted for approximately 40% of the total sales of organic food products in 2001 (321). Current regulations acknowledge the lack of available organic materials for many stages of production and have allowed the use of a limited number of non-organically produced substances (116, 289). Most synthetic sanitizers cannot be currently used in washing of

organic produce because they are in direct contact with the product. Organic producers of fresh fruits and vegetables have almost no available effective sanitizers to wash fresh produce. A concentration of 4 ppm of chlorine has little effect on microorganisms and other sanitizers such as hydrogen peroxide and lactic acid have also limited effect and could negatively impact the quality of the product. In the U.S., the National Organic Program has summarized the allowable materials in the National Organic List (NOL) according to their use in crops, livestock or processing.

Inclusion of new materials into the NOL is reviewed and approved by the National Organic Standards Board based on a set of criteria: 1) they must be compatible with organic practices, 2) cannot be produced from a natural source and there are no organic substitutes, 3) no adverse effect to the environment, 4) no effect on nutritional quality, 5) not used to improve quality, 6) GRAS substance, and 7) essential for organic products (13). Most conventional sanitizers are produced synthetically and have not been approved for inclusion into the NOL. Currently the only non-rinse food contact sanitizers listed for processing are hydrogen peroxide, ozone and peracetic acid. The NOL also includes sodium/calcium hypochlorite and chlorine dioxide, but the maximum allowed concentration of 4 ppm is not effective to sanitize food contact surfaces (123).

The limited choice of approved sanitizers for organic food processing has forced most certification agencies to allow the use of unlisted synthetic sanitizers as long as they are rinsed with water before the equipment or surface can be in contact with food. The practice of rinsing sanitizers, not only can pose serious safety risks since it leads to product contamination, but it seriously compromises the principles of organic production

systems. It is critical to identify and develop technologies that will not only be allowed by organic regulatory agencies, but that will be produced in accordance with organic regulations. One such potential solution is the use of bacteriophages.

Bacteriophages are viruses that prey on bacteria, offering a natural non-antibiotic method to reduce bacterial pathogens from the food supply (65). In particular, *E. coli* phages can be isolated from sewage, hospital waste water, polluted rivers and fecal samples of humans or animals (58). Phages utilized for pathogen control in foods and food production systems usually originate from environmental samples and other nonfood sources such as municipal waste water, feces, sewage, soil, farms and processing facility effluents (83, 103, 270, 280). On August 18, 2006, the FDA announced the approval of the use of bacteriophage as an antimicrobial food additive targeting *Listeria monocytogenes* on ready-to-eat (RTE) meat and poultry products (119).

Advantages of using phages over traditional antimicrobial systems for foods have been reviewed for pre-harvest (26, 180) and post-harvest stages (216, 218). 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. It is highly recommended to create cocktails of several phages to obtain sufficient breadth of host range and to reduce the probability of phage resistance (58).

Application of bacteriophage for control of pathogenic bacteria may also have some disadvantages. Phages can convert from lytic phages to temperate phages, resulting

in lysis of only a small portion of the target bacteria (6). Phage absorption to receptors on bacterial cell walls requires the chance collision of phages with bacterial cells. This interaction can be hampered by physical barriers in plants, animals, and foods such as the viscous environment of the intestine or rumen, or the particulate nature of food environments (180). Other ecological considerations include the pH of the environment, temperature, immune responsiveness and physiology of bacterial strains in vivo (143).

Using bacteriophages to reduce contamination of foods with various pathogens requires in-depth understanding of the ecology of the pathogen (332). Phages have been evaluated to control pathogens in a variety of foods of animal origin. Phages of *E. coli* O157:H7 have been characterized and their antibacterial activities have been determined in vitro (203, 270, 300). Several studies have been conducted to assess the efficiency of phages in eliminating *E. coli* O157:H7 from the digestive tracts of ruminants (296), mice (340), sheep (65), and beef meat surface (270). The use of bacteriophages to reduce the number of foodborne pathogen on various RTE foods has been examined with fresh-cut fruits and vegetables, such as fresh-cut melons and apples stored at various temperatures (218) and in combination with nisin (216), or on iceberg lettuce and cantaloupe (315).

1.3. Research Hypotheses, Goals, and Objectives

Goal: The ultimate goal of this project is to develop a collection of bacteriophages to reduce the viability of EHEC on typical food processing hard surfaces and fresh produce.

Null Hypothesis I:

Bacteriophages and bacteriophage cocktails cannot significantly reduce high titers of EHEC strains.

Null Hypothesis II:

Bacteriophage cocktails are not effective antimicrobials against liquid or dried strains of EHEC placed on typical food processing hard surfaces.

Null Hypothesis III:

Bacteriophage cocktails do not inactivate EHEC strains inoculated on fresh leafy green vegetables.

Objectives:

1. Develop a diverse collection of bacteriophages capable of infecting EHEC.
2. Determine the spectrum of activity and characterize individual bacteriophages specific against EHEC.
3. Identify a group of bacteriophage strains capable of killing high titers of EHEC.
4. Determine the effectiveness of a diverse bacteriophage mixture to inactivate multiple EHEC strains in liquid media and dried on hard surfaces typically found in food processing environments
5. Determine the effectiveness of a diverse bacteriophage mixture to inactivate multiple EHEC strains on lettuce and spinach surfaces.

CHAPTER 2

ISOLATION AND CHARACTERIZATION OF LYTIC BACTERIOPHAGES AGAINST ENTEROHEMORRHAGIC *ESCHERICHIA COLI*

Bacteriophages are natural antimicrobial agents that have the potential to reduce the levels of bacterial pathogens, such as enterohemorrhagic *Escherichia coli* (EHEC), in the food chain. The objective of this study was to isolate, identify and characterize a diverse collection of lytic bacteriophages capable of infecting EHEC serotypes O157, O26 and O111. Phages were isolated from dairy and cattle feedlot manure using EHEC O157, O26 and O111 strains as hosts. Manure was enriched using tryptic soy (TS) broth and these enriched extracts were centrifuged, filtered, combined with the host strain in tryptone top agar, and plated on TS agar. Plaques were purified and screened against additional strains (14, O157; 10, O26; 10, O111) using the efficiency of plating method (EOP). Phage CEV2, and five other phages previously isolated, were able to lyse all 14 O157 strains with EOP values consistently above 0.001. Two phages isolated from fecal slurry from dairy and feedlot cattle were found to be highly effective against strains of *E. coli* O157 through EOP tests and against O26 strains through spot tests, but not against the O111 serotype. Bacterial challenges of using a cocktail of 8 selected phages against a four *E. coli* O157 strain culture (>10^7 CFU/mL) resulted in >5 log CFU/mL reductions at 37°C. Multiplex-PCR revealed that none of the 8 phages carry any typical EHEC virulence

factor genes, while some were positive for *gene23*, the major capsid protein gene of T4-like phages. These results indicated that the isolated bacteriophages were highly effective against multiple strains of two EHEC serotypes. This collection of phages can be grouped and potentially used as an antimicrobial cocktail to inactivate O157 and O26 serotypes and reduce their incidence in the food chain.

2.1. Introduction

In recent years, Enterohemorrhagic *Escherichia coli* (EHEC) have caused multiple foodborne outbreaks worldwide related to the consumption of contaminated meat and fresh produce (29). One of these serotypes, *E. coli* O157:H7 is a leading cause of produce-related infections, accounting for 20% of the outbreaks in which the etiological agent was identified (275). Over the last 10 years, despite the best efforts of the food industry we have seen an increase in foodborne outbreaks through the consumption of fresh vegetables, such as the spinach outbreak of 2006, that have stressed the importance of developing new more potent antimicrobial strategies (75, 232).. The fresh characteristic of produce limits the number of antimicrobial treatments that can be used.

Bacteriophages are viruses of bacteria, that are highly specific in their host cell recognition often only infecting specific members of a single genus and as such offer an alternative natural method to reduce bacterial pathogens within the food supply chain (65). This high level of specificity leads to little disturbance of commensal bacteria through the oral consumption of phages targeted at pathogens and while bacteria develop

specialized phage defense mechanisms, phages also continuously adapt to these changed host systems (149). To address this issue, it is highly recommended to create cocktails of several phages to obtain sufficient breadth of host range and to reduce the probability of phage resistance (58). Due to their origin phages are considered "natural" and therefore highly applicable in the production of organic foods.

Advantages of using phages over traditional antimicrobial systems for foods have been reviewed on the pre-harvest (26, 180) and post-harvest level (216, 218). 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 (334).

In 2006, the U. S. Food and Drug Administration announced the approval of bacteriophages as antimicrobial food additives for control of *Listeria monocytogenes* in ready-to-eat meat and poultry products (119). Lately, using specific phages to eliminate or reduce the levels of contaminated bacteria on various foods such as fresh-cut fruits and vegetables is continually under investigation for *E. coli* O157:H7. As part of an ongoing study, Sharma et al. (315) 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.

A study by Niu et al (265) evaluated the host range and lytic capability of four phages against Shiga toxin-producing *E. coli* (STEC) O157 from cattle and humans. They found that the phages were effective against the majority of the bovine and human *E. coli* O157 isolates and suggested that lytic capability and host range should be considered when selecting a therapeutic phage for on-farm control of *E. coli* O157. Furthermore, they advocated for the use of phage cocktails as an effective mitigating approach for *E. coli* O157 due to the observation that some *E. coli* O157 isolates exhibited resistance to some but not all phages.

This study is part of a larger research effort focused on the development and application of a mixture of bacteriophages against foodborne pathogens. The specific objective of this study was to develop a collection of bacteriophages capable of infecting different serotypes of EHEC, determine their spectrum of activity, and characterize them. This could eventually lead to the creation of a mixture of bacteriophages that would potentially be used in food and food processing environments.

2.2. Materials and Methods

2.2.1. Phage Isolation, Purification, and Propagation

Phages CEV2 and CBA65 were previously isolated from sheep and cattle fecal samples against *E. coli* O157:H7 NCTC 12900 at The Evergreen State College, as per their standard protocol (277,296). Dr. Lawrence Goodridge (Colorado State University)

provided 8 pure phages and 31 lysate mixtures from his collection. Additional phages were isolated from dairy and feedlot manure using EHEC O157 (*E. coli* O157:H7 NCTC 12900, ATCC 43895), O26 (#41) and O111 (#10) and *E. coli* B strains as hosts as listed in Table 1. Stock cultures were stored in glycerol at -50°C and cultivated in TSB prior to use in experiments. Six batches of liquid sewage slurry, collected in August and September were used as the initial source for phage enrichment and isolation. Samples (10 mL) were mixed with 1 mL 10×tryptic soy broth (TSB) (Neogen, Corp., Lansing, MI), containing 1 mM $CaCl_2$ to assist in phage attachment (19) and 1 mL exponentially growing cultures of specific bacterial hosts, and incubated overnight at 37°C. This initial phage enrichment culture was then centrifuged for 5 min at 14,500 × *g* and filtered through a 0.45 μm filter before being, being plated with the specific host strain (0.2 mL) in tryptone top agar (TTA) on tryptic soy agar (TSA). Twenty four hours later individual phage plaques were plucked and resuspended, before being replated. Phages were considered purified after at least three passages of this process, and stored in phage storage buffer (PSB) comprised of pH 7.2 phosphate buffer saline (PBS) with added gelatin (0.4%). The phage suspension titers were determined through mixing serial dilutions with liquid cultures of the bacterial host strains in TTA. 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 x *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.

2.2.2. Initial screening/Efficiency of plating

All phages were screened against EHEC strains (15 strains O157; 1 strain O26) using the efficiency of plating method (EOP = phage titer on target bacterium / phage titer on host bacterium) to determine the effectiveness of each phage against a variety of target bacteria. Ten fold serial dilutions of phage suspensions (100 μL) were mixed with 200 μL of the target and host bacterium and incubated for 5 min at room temperature (24°C) before being mixed with 400 μL of molten TTA and plated as double-layers on TSA (361). The *E. coli* strains used for screening included: *E. coli* O157:H7 NCTC 12900, ATCC 43895, M4882, M4489, M4522, 4477, 2027, 2309, 2321, 2336, 6058, I 2005003658**,** EK1 TWO8609, EK27 TWO8635, E32511 TWO2883, *E. coli* O26 #41, and the non-pathogenic *E. coli* strains C, B, and CR63.

2.2.3. Host Range/Spot tests

Phages with the highest EOP's were spot tested against 123 *E. coli* O157: H7 strains, 10 O26 strains, 10 O111 strains, 4 O55 strains, 19 commensal *E. coli*, and 27 *Salmonella* strains (Table 1). Phage activity was examined by a spot test assay that entailed placing 20 μL of each phage (10^5 PFU/mL) on TSA seeded with each strain of *E. coli*. The plates were checked for clearance zones after incubating for 18-24 h at 37°C. The presence of a lytic zone was considered evidence of phage susceptibility; no lysis was considered evidence of phage resistance.

2.2.4. Bacterial challenge tests

The phages with the highest EOP's and a mixture of eight of them designated as "BEC8" were tested in liquid culture (approx. 10^6 CFU/mL) of 4 EHEC O157:H7 strains (EK1, EK27, I 2005003658-462, ATCC 43895). Tubes of TSB were inoculated with each O157 strain, incubated at 37°C overnight and a 100 μL aliquot was transferred in fresh TSB (9 mL) with 10 mM $CaCl_2$ for 3 hours to reach mid-exponential phase. Each tube was inoculated with different concentrations of a phage mixture or TSB to reach MOI of 1, 10, and 100. The tubes were placed at room temperature and 37°C for 5 h, followed by serial dilution in PBS and spread plating on TSA. Standard deviations were determined.

2.2.5. Molecular analysis

Purified phage suspensions (>10^6 PFU/mL) were heated at 95°C for 5 min, cooled to -20°C for 20 min, heated back up to 95°C for 5 min, and stored at 4°C until ready for use. Each phage was examined by multiplex-PCR for the presence of the Shiga toxin 1 (*stx1*), Shiga toxin 2 (*stx2*), intimin (*eae*), and/or the enterohemolysin (*hly*) gene(s) (283) (Table 2). Samples were subjected to 35 PCR cycles, each consisting of 95°C (1 min); 65°C (2 min) for the first 10 cycles, decrementing to 60°C by cycle 15; 72°C (1.5 min), incrementing to 2.5 min from cycles 25 to 35. *E. coli* ATCC 43895 and ATCC 43890 were used as positive controls, while *E. coli* ATCC 25922 and sterile water were used as negative and non-template controls. With this protocol approximately 180, 255, 384, and 534 bp amplicons were produced for *stx1, stx2, eaeA,* and *hlyA*, respectively. Phages also underwent PCR for the detection of gene *23*, the major capsid protein gene present in T4-like phages (347). Samples were subjected to 38 PCR cycles consisting of 96°C (30 s), 62°C (2 min), and 72°C (3 min). With this protocol, an approximately 850 bp amplicon

was produced. Phages T4 and T5 were used as positive and negative controls for *gene23*, respectively. PCR reaction mixtures were electrophoresed on 2% agarose gels stained with ethidium bromide.

Pulsed-field gel electrophoresis of phage DNA were run for 18 h at 6 V/cm and 14ºC with a pulse period of 45 to 90 s as shown previously (296) and were observed on an ethidium bromide-stained agarose gel (1%, wt/vol).

2.2.6. Statistical analysis

Statistical comparison of bacterial count as log CFU/mL of inactivation between the individual phages and its mixture were done by ANOVA (PROC MIXED) and the least-squares method was used to determine significant differences ($P < 0.05$) (SAS Statistical Analysis Software, version 8.0, SAS Institute, Cary, N.C.).

2.3. Results

Phage activity was detected in as many as six batches of fecal slurry samples (1 L), yet only isolates that were active against the 16 *E. coli* strains used in EOP testing were kept in the collection. Specifically, phages using *E. coli* ATCC 43895 and O26 #41 as a host were isolated from feedlot cattle fecal slurry samples. No isolates were recovered when using *E. coli* O111 as a host.

As a result of an initial screening of phage preparations provided by other researchers and recently isolated phages, as many as 88 lytic phages comprised the collection (Table 3). Isolated phages that resulted in cloudy plaques on the bacterial host

were not subjected to further study as incomplete clearing is indicative of temperate phages.

Efficiency of plating (EOP) values of 0.5 to 1 were ranked as "high" efficiency; 0.2 to 0.5 as "medium" efficiency; 0.001 to 0.2 as "low" efficiency; 0.0 was considered as not effective against the target strain. Phages that were unable to lyse (EOP of 0.0) any of the 16 *E. coli* O157:H7 strains used were not subject to further study. The 8 most efficient phages through EOP testing were phages 38, 39, 41, AR1, 42, CEV2, ECB7, and ECA1 (Table 4). Seven out of eight phages were found to be more than 50% "high" efficient against the 16 EHEC strains tested. CEV2 was 40% "high" efficient, and had "medium" efficiency against 53.3% of the strains. "Medium" efficiency among the phages ranged from 6.2 to 53.3 %, while "low" efficiency ranged from 0 to 43.8%. ECB7 was the only strain among the 8 that was not active against *E. coli* O26, but was highly efficient against the *E. coli* O157:H7 strains.

Phages 38, 39, 41, AR1, 42, CEV2, ECB7, and ECA1 lysed from 94 to 98% of the STEC O157:H7 (N=123) strains screened, but when tested against O26 strains (N=10) three of the phages inhibited only 70% of the strains. None of the phages were active against O111 (N=10) strains. The individual phages were poorly lytic against commensal *E. coli* strains (N=19) since their effectiveness ranged from 5 to 32% of the strains tested. Spot testing against 27 strains of different serovars of *Salmonella* was used to determine the cross species infectivity of each phage. Phage infectivity of *Salmonella* ranged from 0 to 47% of the strains tested. There were no O157 strains that were resistant to all 8 phages. Thus, the spot test allowed us to determine the range of each phage and

ultimately create a mixture of phages that would be effective against 100% of all O157 strains.

To investigate the ability of each phage and a mixture of all of them to lyse *E. coli* O157:H7 *in vitro*, challenge tests were performed that included the addition of phage (10^8 PFU/ml) at MOI of 100 to mid-exponential-phase cultures. The in vitro *E. coli* O157:H7 challenge tests were performed at room temperature and 37ºC. Significant decreases of viability of bacterial strains were observed at both temperatures (Figures 1 and 2) however, the lytic ability was significantly reduced at room temperature. Titers (approx. 10^6 CFU/mL) of EHEC O157 strains ATCC 43895, EK1, EK27, and 472 were all reduced by each individual phage as well "BEC8". BEC8 performed consistently as well as or better than the most effective single phage against all 4 EHEC O157. At 37°C, the mixture reduced 5.53 and 5.28 log CFU/mL of *E. coli* ATCC 43895 and 472, respectively, performing as well as or better than the individual phages. However, BEC8 reduced the population of strain EK1 by 5.06 log CFU/mL at 37°C, significantly lower than phage 39 that reduced the strain by 5.75 log CFU/mL. Similarly, BEC8 reduced strain EK27 by 1 log CFU/mL less, than phage CEV2 at 37°C. At room temperature, BEC8 was able to perform as well as or better than the individual phages, reducing strains EK1, EK27, and 472 by 3.3, 3.1, and 2.6 log CFU/mL.

All phages were subjected to PCR for typical EHEC virulence factors *stx1*, *stx2*, *eaeA*, and *hlyA,* as well as *gene23*, a capsid protein gene present in T4-like phages (347). None of the EHEC virulence factors were detected in any of the BEC8 phages. Phages 38, 41, ECA1, and ECB7 were found positive for *gene23*. PFGE analysis revealed the

genomic size of the phages was approx. 120 kb for CEV2, 38, ECA1, and ECB7, while the rest of the phages were all approx. 180 kb.

2.4. Discussion

Isolation of phages has been reported to be easily accomplished through environmental and fecal samples with medium enrichment and exponentially growing host bacteria (203, 251, 300). Since the natural niche of EHEC are ruminants, in one of the first steps of the study phages from fecal slurry samples of dairy and feedlot cattle were isolated (Table 3). The purpose of this study was not to determine the prevalence of EHEC-specific phages within a number of fecal samples. Instead, the study focused on isolating EHEC-specific phages to ultimately create an effective phage cocktail.

The reception and isolation of a large number of phages specific for pathogenic and non-pathogenic *E. coli* provided a starting point from which an antimicrobial cocktail could be developed. This study sought to investigate the lytic capability and host range of phages specific to *E. coli* O157 and non-O157 isolated from various sources. The ease of isolating phages from fecal slurry from cattle was in accordance with a recent study by Niu et al. (264), in which they found that the prevalence of phages was highest in slurry compared to pooled fecal pats, water with sediment from troughs, and rectal fecal samples. The same study reported that the prevalence of phages fluctuated in a fashion similar to that described for *E. coli* O157:H7. This pattern contributed to the success of isolating phages in our study since the fecal slurries samples were obtained during late summer when the prevalence of *E. coli* O157 is highest in cattle (24).

In studies addressing the distribution of phages attacking *E. coli* O157 in samples taken from different pens in a cattle feedlot, no phage was obtained without enrichment, but increasing extensive enrichment techniques resulted in isolating phages in 65 and 97% of samples specific against *E. coli* O157 and *E. coli* strain B, respectively (277). In a previous study, different phages were isolated from the same stool samples when using different indicator bacterial host cells (80). Similarly, in our study, enrichment of fecal slurries with 10×TSB resulted in many isolated phages from a small number (six) of batches from dairy and feedlot cattle.

Phages, such as T4, could potentially have an EOP of 100% in ideal conditions. Ideally, every viral particle attaching to a host cell can enter and result in a plaque on the appropriate bacterial strains under optimum conditions. However, there are a number of factors that affect plating efficiency, such as the specific host strain, thus highlighting the importance of investigating the relative EOP on a variety of susceptible hosts (206). The EOP method was used in our study as a tool to distinguish between efficient and inefficient phages against a random selection of *E. coli* O157:H7 strains. This lead to the screening of a large number of isolated phages and ultimately select those highly efficient phage isolates specific for *E. coli* O157:H7 and O26.

The spot testing conducted in this study was aimed to determine the host range of the 8 most effective phages in the collection (Table 5). All phages were highly effective against strains of EHEC O157 and O26 isolated from various sources which continuously pose a threat to the food supply (284). Previous studies have also shown that phages are capable of a wide host range in infecting EHEC (265, 360) and other pathogenic bacteria

(43, 335). When the lytic properties of the phages against non-pathogenic *E. coli* were investigated, the phages' lysis ability ranged from 5.3 to 31.6% of the collection. Viscardi et al (360) tested two phages against the ECOR collection of non-pathogenic *E. coli* and found that they lysed 13.9 and 20.8% of the strains. Even though the collection of non-pathogen *E. coli* is different, the low percentage of lysis by the phage was similar between the phages tested.

Of particular interest is the cross-genus activity of the phages with the *Salmonella* strains used in the spot tests. Phage ECB7 lysed 48.2% of the *Salmonella* strains, while the rest ranged from none to 22.2%. Villegas et al (359) found that the EHEC O157:H7-specific bacteriophage wV8 had genome characteristics very similar to the *Salmonella*-specific phage Felix O1, yet could not lyse any of the 12 *Salmonella* serovars through spot testing. In a previous report of an *E. coli* O157:H7-specific coliphage, Ronner and Cliver (300) described a phage that lysed Shiga toxin-producing bacteria as well as *Shigella dysenteriae*.

The EOP and host range experimental results emphasized the importance of considering these techniques when selecting phages for therapeutic or antimicrobial application purposes (360). Comparison of the bacterial cocktail against the performance of each individual phage in the mixture was also important. This assay determined whether there is a competition between the strains rather than a cooperative or synergistic effect. Cocktails of multiple phages may be necessary to obtain sufficient breadth of host range and to significantly reduce the probability of developing resistance (58). Host species specificity of phages may be an asset for phage applications, but can also be a

liability when a reasonable coverage of the targeted pathogen has to be achieved with a relatively small phage cocktail (93). O'Flynn et al used a three phage cocktail against 10^3 CFU/g *E. coli* O157:H7 on beef (270). In seven out of nine samples, no viable cells could be retrieved after storage at 37ºC, while in the remaining two samples counts were below 10 CFU/g. The *in vitro* challenge test in our study demonstrated that the phage mixture could be used to inactivate strains of pathogenic *E. coli* and have potential to be used in food applications.

The presence of *gene 23*, a major capsid gene typical of T4-like phages, varied among phages in the collection. The lack of EHEC virulence factors by the selected phages was not only a good safety indicator, but it indicates that the phages genome did not exchange potentially questionable genes even if the host strains were EHEC. Recombination between short regions of homology led to chimeric fibres and the acquisition of new host-range determinants (346). A study by O'Flynn reported that one of the phages isolated that was a member of the *Myoviridae* family had recently acquired the *eaeA* virulence factor from the bacterial host that was used (270).

Many important human bacterial pathogens attribute their virulence factors to prophages integrated into their genome (59, 364). Pathogenic *E. coli* O157 strains encode the major virulence factor, the Shiga-like toxin within prophages. However, T4 phages are considered as a paradigm of obligate lytic phages and do not seem to exhibit the same sort of reshuffling of groups of functionally related genes as described for lambdoid coliphages (80). Recent genome analysis and 40 years of genetic research with T4 have shown its chromosome contains no virulence genes (248). Actually, early in its infection

cycle, T4 rapidly degrades the host genome to the nucleotide level, thus preventing integration of phage DNA into its chromosome (248). Our study confirmed past findings of no toxin genes found in T4-like phages (58). T4 differs clearly from lambda-like phages, which are able to often carry virulence genes (48, 59).

In the region between aa 162 and aa 209 of the designed *gene23* sequence, the AR1 DNA sequence differs by 27% from the T4 sequence, while the rest of the gene diverges by less than 2% (347). This is a possible result of these phages acquiring the particular segment of *gene23* through genetic swap with another related phage. It has been shown that non-conserved sequences in *gene23* sequence provide binding sites for various head accessory proteins (378) that are able to interact with exposed patterns of *gp23* on the capsid surface. Evidently, any alteration of the accessory capsid proteins could change the physical and antigenic properties of the virion head.

2.5. Conclusion

These recently identified and characterized bacteriophages were highly effective against multiple strains of two EHEC serotypes. None of the phages contained EHEC virulence factors as shown through PCR. The combinations of all the phages in a single mixture lead to significant reductions of high titers of EHEC at 37°C and room temperature, thus suggesting that a phage mixture is feasible and effective. This collection of phages can be grouped and potentially used as an antimicrobial cocktail to inactivate O157 and O26 serotypes and reduce their incidence in the food chain**.** It has long been recognized that non-transducing, lytic phages pose relatively little risk to

humans (150), and at the same time can be used as a valuable tool for food safety and public health. A complete sequencing and bioinformatic analysis of the phage genomes will be a logical next step in the characterization before practical food applications (361).

Table 2.1. Bacterial strains used in this study

Bacteria	Strain Designation	Strain #	Source
E. coli O26	36, 37, 38, 39, 40, 41, 42, 43, 44, 45	10	FSML
E. coli O111	1, 2, 3, 4, 5, 6, 7, 8, 9, 10	10	FSML
	I 2005003658-472	1	MDH
E. coli	ATCC 43895, ATCC 43890, ATCC 35150	3	ATCC
O157:H7	A02, A03, A04, A05, A06, A07, A08, A09, A10, A11, A12, A13, A14, A15, A16, A17, A18, A19, A20, A21, A22, A24, A25, A26, A28, A29, A30, A31, A32, A33, A34, A35, A36, A37, A38, A39, A40, A41, A44, A47, A51, A52, A53, A54, A55, A57, A60, A61, A63, A64, A66, A68, A69, A70, A71, A72, A73, E10, E11, E12, E48, E56, EC261, EC263, EC265, (r)3W, (L)OD, (r)OD, (UC)7W, (M)3W, (c)OD, (M)OD	72	FSML
	M4882, M4489, M4522, 4477, 2027, 2336, 6058, 1144, 1145, 1146, 1147, 1148, 1149, 1150, 1151, 1152, 1153, 1154, 1155, 1156, 1157, 1158, 1159, 1160, 1161, 1162, 2026, 2027, 2028, 2029, 2030, 2031, 2079, 2206, 2255, 2257, 2266, 2309, 2317, 2321, 2324, 3081, MAC, SEA1 31388, 2727(8727), 4489, 4719	48	ARS
	EK1 TWO8609, EK27 TWO8635, E32511 TWO2883, EHEC1, EHEC2, EHEC3, EHEC4, EHEC5, EHEC6, EHEC7, EHEC8	11	MSU
E. coli (non-pathogenic)	KS(gr)2, KS(S)1, KS(S)2, F(S)1, PG(GR)3, PG(gr)2, PG(gr)1, CSH50, SD(L)2, DJ(L)1, KS(S)2, GR(L)1, GR(c)1, F(S)3, KS(L)2, PG(gr)4, K12, W3110, DH5α,	17	FSML
	C, B, CR63	3	CSU
Salmonella Typhimurium	NE200, I649, I503, E200, I535, I536, UK1, I527, I740, I600, 3010907, I4028, I598, I534, I526, 758	17	FSML
	ATCC700408	1	ATCC
S. Newport	B4442, AM07073, AMO7076, AMO5104, 2006036	5	CDC
S. Enteritidis	I823, 95657613	2	
S. Montevideo	95573473	1	MDH
S. Saintpaul	E200	1	MDH
S. Agona		1	FSML

Abbreviations: FSML, Food Safety Microbiology Laboratory, University of Minnesota; MSU, Microbial Evolution Laboratory, Michigan State University; MDH, Minnesota Department of Health; CSU, Colorado State University; ARS, Agricultural Research Service's Southern Plains Agricultural Research Center; CDC, Centers for Disease Control and Prevention.

Table 2.2. Primers used in PCR analysis of virulence genes and gene 23 in isolated phages.

Primer Name		Sequence	Amplicon Size (bp)
Stx 1 F		5’ ATA AAT CGC CAT TCG TTG ACT AC 3’	180
Stx 1 R		5’ AGA ACG CCC ACT GAG ATC ATC 3’	
Stx 2 F		5’ GGC ACT GTC TGA AAC TGC TCC 3’	255
Stx 2 R		5’ TCG CCA GTT ATC TGA CAT TCT G 3’	
eaeA F		5’ GAC CCG GCA CAA GCA TAA GC 3’	384
eaeA R		5’ CCA CCT GCA GCA ACA AGA GG 3’	
hlyA F		5’ GCA TCA TCA AGC GTA CGT TCC 3’	534
hlyA R		5’ AAT GAG CCA AGC TGG TTA AGC T 3’	
Gene 23	Mzia1 F	5' TGT TAT IGG TAT GGT ICG ICG TGC TAT 3'	850
	CAP8 R	5' TGA AGT TAC CTT CAC CAC GAC CGG 3'	

Table 2.3. List of phages used in this study, their source, and host bacterium

Source	Phage Designation	Number of Phages	*E. coli* Host
Provided by Dr. Goodridge	AR1, 38, 39, 41,42, RB33, RB34, 56, 1, 2, 3, 4, 5, 6, 7, 8, 8a, 9, 10, 10a, 11, 11a, 12, 13, 15, 16, 17, 20, 21, 22, 23, 24, 25, 27, 50, 52, 53, 54, 57	39	B, C, or CR63
Provided by Dr. Brabban	CEV2, CBA65	2	O157:H7 NCTC12900
Dairy cattle	ECA1, ECA2, ECC4	3	O157:H7 ATCC 43895
	ECC5, ECC6, ECC7, ECC8a, ECC8b, ECC9, ECC10, ECC11, ECC12, ECC13	10	O26 #41
Feedlot cattle	ECB3, ECB4, ECB5, ECB6, ECB7, ECB8, ECB9	7	O157:H7 NCTC 12900
	ECC1, ECC2, ECC3a, ECC3b, ECC14, ECC15	6	O26 #41
	ECD1, ECD2, ECD3	3	B, C, or CR63

Table 2.4. Bacteriophage host range ranking as number of strains falling within each efficiency of plating (EOP) range of the total number of *E. coli* strains used (N=16) determined using the EOP method.

EOP range	**Phages**							
	38	**39**	**41**	**AR1**	**42**	**CEV2**	**ECB7**	**ECA1**
0.5-1*	6	8	11	13	11	6	10	9
0.2-0.5	5	1	4	3	3	8	1	7
0.001-0.2	5	7	1	0	2	2	4	0
0.0	0	0	0	0	0	0	1	0

*Efficiency of plating range.

Table 2.5. Determination of host range by using spot testing of bacteriophages against target bacteria.

Target bacterium	% Positive[a]							
	38	**39**	**41**	**AR1**	**42**	**CEV2**	**ECB7**	**ECA1**
O157:H7 (N=130)	96.2	93.1	97.7	93.1	99.2	96.0	93.1	93.1
O26 (N=10)	70.0	70.0	90.0	70.0	80.0	80.0	90.0	100.0
O111 (N=10)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Commensal *E. coli* (N=19)	26.1	31.6	15.8	15.8	31.6	21.1	21.1	5.3
E. coli O55 (N=4)	25.0	0.0	0.0	75.0	25.0	50.0	50.0	25.0
Salmonella (N=27)	22.2	11.1	7.4	7.4	18.5	0.0	48.2	7.4

[a] Able to create a clearance zone on the lawn of the specific bacterial strain.

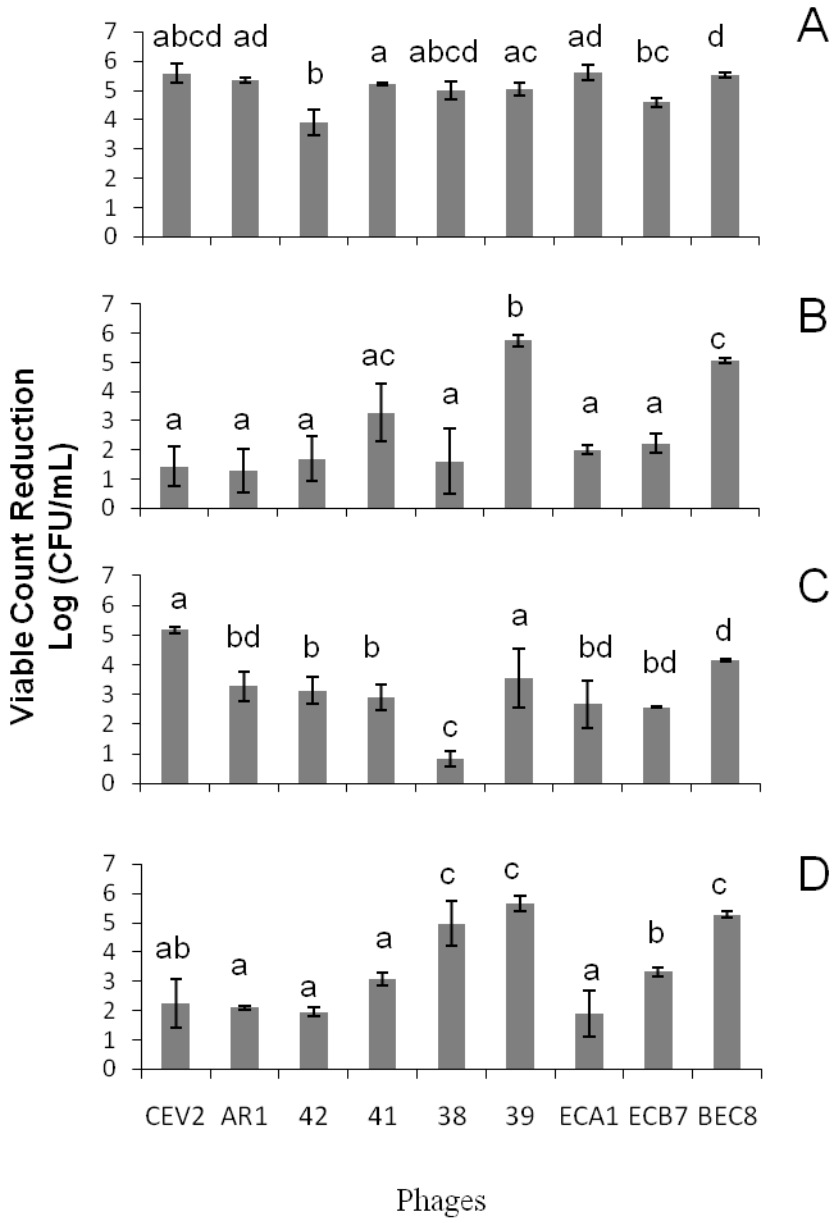

Figure 2.1. High titer inactivation of *E. coli* O157:H7 strains A) ATCC 43895, B) EK1, C) EK27, and D) I2005003658 (472) by phages CEV2, AR1, 42, 41, 38, 39, ECA1, ECB7, and the phage mixture BEC8 after 5 hours of incubation at 37°C.

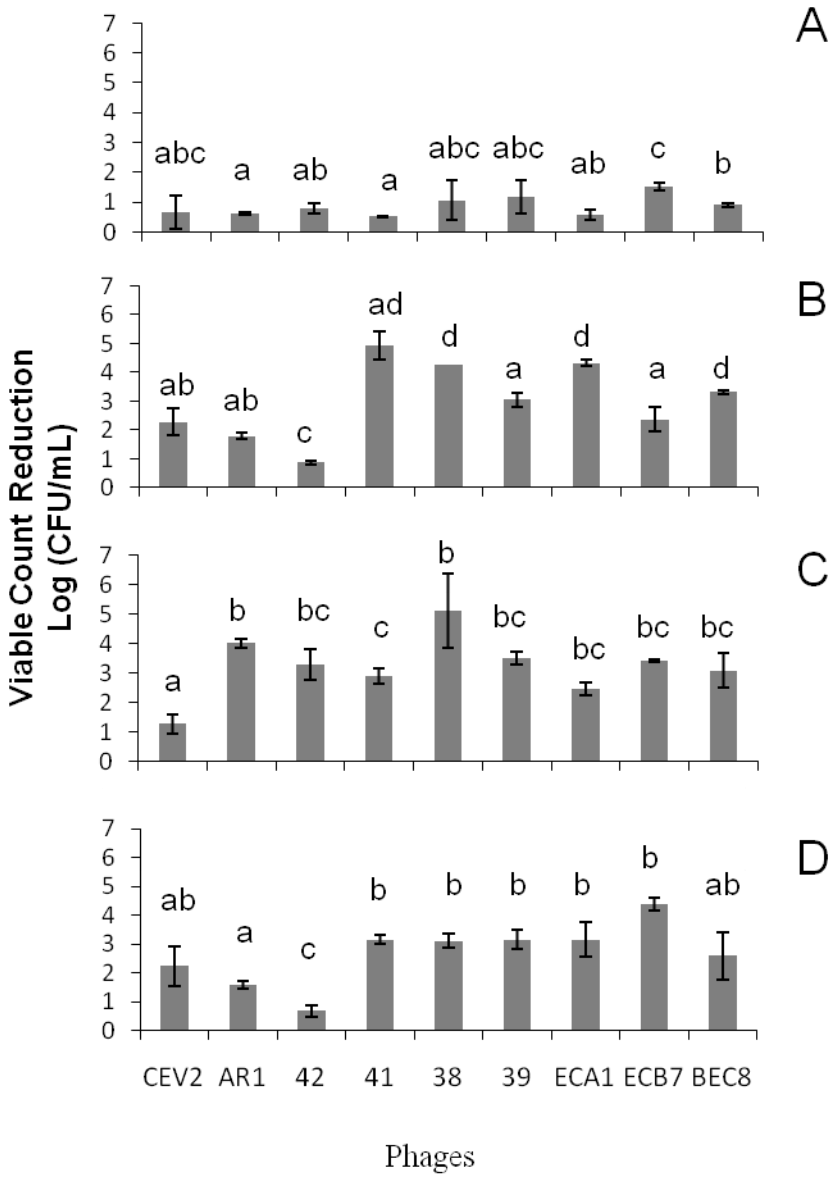

Figure 2.2. High titer inactivation of *E. coli* O157:H7 strains A) ATCC 43895, B) EK1, C) EK27, and D) I2005003658 (472) by phages CEV2, AR1, 42, 41, 38, 39, ECA1, ECB7, and the phage mixture BEC8 after 5 hours of incubation at room temperature.

CHAPTER 3

REDUCTION OF *ESCHERICHIA COLI* O157:H7 VIABILITY ON HARD SURFACES BY TREATMENT WITH A BACTERIOPHAGE MIXTURE

This study determined the effect of the phage cocktail BEC8 on enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 strains applied on food processing surfaces. Sterile stainless steel chips (SSC), ceramic tile chips (CTC), and high density polyethylene chips (HDPEC) were used. Cultures of EHEC O157:H7 strains EK27, ATCC 43895, and 472 were combined, spot inoculated on surfaces, and dried. Chips were inoculated with 10^6, 10^5, and 10^4 CFU/chip, to obtain 1, 10 and 100 multiplicity of infection (MOI) values, respectively. Controls and BEC8 (approx. 10^6 PFU/chip) were applied on inoculated surfaces and incubated at 4, 12, 23, and 37^{o}C. EHEC survival was determined using standard plate count on tryptic soy agar. At 37°C and 12^{o}C on SSC, no survivors were detected (detection limit 10 CFU/chip) after BEC8 treatment at MOI of 100 after 10 min and at 23°C after 1 h on SSC. Similar result was obtained on CTC at 37°C after 10 min, and after 1 h at 23°C. The rate of phage inactivation at a 100 MOI followed first order kinetics. D-values on any surface material ranged from 5 min at 37°C to 55 min at 12^{o}C. All three Z values calculated for each material resulted in approximately 22^{o}C. These results indicated that the phage cocktail was effective within an hour against low levels of the EHEC mixture at above room temperature on all 3 hard surfaces.

3.1. Introduction

Shiga toxin-producing *Escherichia coli* (STEC) and specifically *E. coli* O157:H7 are considered as emerging foodborne pathogens that occur worldwide but are most common in the US, UK and Canada (11, 257, 344). In the US, *E. coli* O157:H7 is estimated to cause approximately 73,000 illnesses, roughly 2,000 hospitalizations, and 60 deaths (242). The associated economic costs related to this pathogen have been estimated to be $405 million USD (129). In recent years, EHEC has been responsible for multiple food outbreaks that have involved ground beef and fresh produce (29, 91). *E. coli* O157:H7 is now one of the leading causes of produce related outbreaks, accounting for 20% of the cases in which the etiological agent was identified (275).

Contamination of food processing equipment, surfaces, and facilities by pathogenic bacteria can be a serious problem (2). Some bacteria attach to surfaces as their predominant form of survival in nature (222) and can survive on hands, sponges/cloths, utensils and currency for hours or days after initial contact (311). Food particles are usually cleaned from the surface when good hygienic practices are applied, but bacteria attached to these surfaces are not visible to the eye and may therefore not be removed (205). Decontamination presents a serious challenge due to the increased resistance of many potentially pathogenic bacteria to traditional sanitizers, such as hypochlorous acid and benzalkonium chloride (89). In addition, numerous chemical sanitizers can be corrosive and toxic and thus, unacceptable for foods or surfaces that come in direct contact with food. To this end, new sanitizers are needed to aid in decontaminating food processing surfaces and equipment.

The interest in bacteriophages is driven by the demand for natural non-antibiotic methods that could meet organic food standards to reduce pathogens from the food supply. Bacteriophages are obligate parasites of bacteria capable of killing specific species and offer a natural method to control contamination of foods (65). *E. coli* phages can be isolated from sewage, soil, waste water, polluted rivers and fecal samples of humans or animals (58). The molecular mechanism of phage invasion into bacterial cells has been extensively elucidated (248). It has been long recognized that bacteria can also develop resistance to phages and this consideration should be considered in phage applications.

The use of cocktails of several phages can circumvent the potential for resistance development by offering sufficient breadth of host range (58). Because phages are known to use different protein receptors at the bacterial membrane, the use of several phage strains at once reduces the probability of phage resistance. Phages are highly specific and their use in food products is not likely to select for phage resistance in untargeted bacterial species. Advantages of using phages over traditional antimicrobial systems for foods have been reviewed at the pre-harvest (26, 180) and post-harvest level (216, 218).

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 (334). A recent study conducted

by Abuladze et al (2) reported that high levels (10^{10} PFU/mL) of a three lytic bacteriophage mixture inactivated as much as 99.99% of a mixture of three *E. coli* O157:H7 strains on glass cover slips. That finding suggested that bacteriophages could be useful to decontaminate hard food processing surfaces.

The objective of this study was to determine the effect of a previously characterized collection of bacteriophages, BEC8 on the viability of a mixture of EHEC O157:H7 strains applied on surfaces of materials commonly found in food processing plants.

3.2. Materials and Methods

3.2.1. Phage 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. All eight phages were members of the family Caudovirales, and were 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. The phage cocktail was suspended in a solution of TSB.

3.2.2. Bacterial strains

The bacterial strains used to inoculate hard surfaces were *Escherichia 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). Strains were stored in vials containing trypic soy broth (TSB; Neogen, Inc. Lansing MI) and 10% glycerol (Sigma Chemical Co., St. Louis, MO) at -55°C. For inoculum preparation, frozen suspensions were streaked on tryptic soy agar (TSA; Neogen, Inc. Lansing MI) and the TSA plates were incubated at 37°C for 24 h.

3.2.3. Hard surfaces

Stainless steel chips (SSC) (1 cm diameter), ceramic tile chips (CTC) (2.0 cm ± 0.2 cm × 2.3 cm ± 0.4 cm), and high density polyethylene chips (HDPEC) (1.5 cm ± 0.4 cm × 1.8 cm ± 0.5 cm) were used to represent three different food processing materials. Matrices were cleaned with 70% ethanol, autoclaved, placed in Petri plates, and used immediately or stored at room temperature until ready to use.

3.2.4. Inoculation

The EHEC strains were streaked on tryptic soy agar (TSA; Neogen, Inc. Lansing MI), incubated overnight at 37°C, individual colonies were transferred to 9 mL of TSB, and incubated overnight at 37°C. Aliquots (100 µL) were transferred to fresh tubes of 9 mL TSB and incubated at 37°C for 3 h resulting in mid-exponential phase cultures. Aliquots of 0.5 mL of each strain were combined in 1 mL microcentrifuge tubes, vortexed, and centrifuged at 14,500 × g for 5 min. Supernatants were decanted and fresh 1.5 mL TSB were added and mixed. Three different levels of bacteria were added to each hard surface. Mixtures of the three EHEC strains were diluted in TSB to provide 4, 5, and 6 log CFU/chip of inoculum designated as low (L), medium (M), and high (H) levels,

respectively. The mixture was spot inoculated (20 µL) onto the hard surfaces and allowed to dry for 1 h in a biosafety cabinet.

3.2.5. Phage application

Aliquots (100 µL) of BEC8 (10^7 PFU/mL) or TSB (treatments and negative control, respectively) were applied on top of areas of the hard surfaces inoculated with mixtures of the three *E. coli* O157:H7 strains. This set three levels of multiplicities of infection (MOI) of 100, 10, and 1, corresponding to the L, M, and H inoculation levels, respectively. Positive controls included 20 µL of bacterial mixtures spotted on the hard surfaces and mixed with 100 µL of BEC8 without drying. Chips were then incubated inside of Petri plates at 4, 12, 23 and 37°C for 10 min, 1 h, and 24 h. For the determination of D and Z values, measurements were done every 5 to 10 min depending on the temperature at a MOI of 100. Three pieces of filter paper and 5 mL of sterile water were added to the Petri plates to avoid complete evaporation of BEC8 or TSB that were incubated for 24 h at 37°C.

3.2.6. Processing

Treated chips were placed in 50 mL centrifuge tubes that contained 1 mL of phosphate buffer saline (PBS) (pH 7.2) and 10-15 glass beads (3 mm) using sterile forceps. The tubes were then vortexed for approximately 1 min, followed by serial dilution in PBS and plating on TSA. The TSA plates were then incubated at 37°C overnight and enumerated. Bacterial counts as CFU were calculated per chip processed and the data transformed to logarithm base 10.

3.2.7. Data Analysis

Decimal reduction time (D) values and the respective 95% CL (time to inactivate 90% of the population) were determined from the linear portion of the survival curves by plotting the survival counts against incubation times at each temperature using a semi-log plot. Calculations were made using the procedures developed by Labuza and Kamman (1983) using the spreadsheet available at:

http://www.ardilla.umn.edu/00fscn8334-1f/FScN8334_Graphs%20and%20Spreadsheets.html.

Inactivation trials were performed in at least duplicate with duplicate samples at each time interval. Note that the Excel Trendline function allows for the calculation of slope of a linear function but does not have a built in 95% CL function for the line slope k. Based on the exponential regression equation for survival plots i.e. N= No exp(-kt) where No is the initial inoculum, N is the CFU count at time and k is the slope of the line ie the death rate constant with units of reciprocal time, The D values were calculated from D =2.303/k and corresponds to the time for 1 log reduction. The Excel spreadsheet allows for creating a plot with both the upper and lower 95% CL as well as for projection to the future value corresponding to the limit of detection. The increase of temperature (°C) which would cause a 10-fold reduction of the cell count were determined from the slopes of the linear regression of the decimal reduction time curves (log D-values vs. temperature) where $Z = -\text{slope}^{-1}$.

3.3. Results

The detection limit for the recovery of cells of *E. coli* O157:H7 from the hard surfaces was 1 log CFU/chip. All the results are presented under the assumption that the

survival of bacterial mixture at levels below the detection limit cannot be quantified. Therefore, when no surviving cells were found, they were assumed to be at a 1 log CFU/chip level.

Treatment of the experimentally contaminated SSC, CTC, and HDPEC with BEC8 significantly reduced ($p<0.05$) the number of viable dried *E. coli* O157:H7 cells that could be recovered (Figures 1-3). Treating the contaminated SSC, CTC, and HDPEC at three different MOI levels (1, 10, and 100) with BEC8 produced a reduction of at least one log CFU in the number of the *E. coli* O157:H7 cells after 24 h at all temperatures tested above 12°C. The highest levels of inactivation were observed with increasing values of time, temperature, and MOI. The inactivation trends observed were similar for all three surfaces at all MOIs and time-temperature combinations. No survivors were detected after BEC8 treatment at low inoculum level (approx. 10^4 CFU/chip) at the following incubation conditions: 37^oC for 10 min and RT after 1 h on SSC and CTC; 12^oC after 10 min on SSC, 1 h for CTC, and 24 h for HDPEC. At low levels there was at least a 1 log CFU reduction of the pathogen at 4°C. At temperatures above 12°C there was more than a 3 log CFU reduction within 10 min of applying BEC8 on all 3 surfaces. Inactivation of the pathogen below detection limits was observed for all surfaces at low inoculum levels after 24 h at 23 and 37°C. At medium inoculum levels, the pathogen was inactivated below detection levels after 24 h at 37°C, while at temperatures less than 23^oC reductions of no more than 2 log CFU were observed with all materials. Medium inoculum levels of dry cells were only markedly reduced after 24 by approximately 5, 2.5, and 3 log CFU at 37^oC SSC, CTC, and HDPEC, respectively. The loss of viability of

high inoculum levels of EHEC was less than 1 log CFU at all conditions with the exception of 3 log CFU after 24 h at 37°C for all three of the surfaces (Figures 3.1c, 3.2c, and 3.3c).

Treatment of the experimentally contaminated liquid cultures of *E. coli* O157:H7 on SSC, CTC, and HDPEC with BEC8 served as positive controls for the same phage preparations used on dried cells. Under these conditions, BEC8 significantly reduced ($p<0.05$) the number of viable cells in liquid TSB that could be recovered (Tables 3.1-3.3). Treating the SSC, CTC, and HDPEC containing liquid cultures at three different MOI levels (1, 10, and 100) with BEC8 produced a reduction of at least one log CFU in the number of the *E. coli* O157:H7 cells after 24 h at all temperatures tested above 12°C. At low bacterial levels (high MOI) BEC8 inactivated *E. coli* O157:H7 cells in liquid TSB at levels similar to those acting against dry cells resulting in inactivation of 1.14 to 1.95 log CFU after 24 h. At temperatures above 12°C, BEC8 resulted in inactivation of at least 3 log CFU for all surfaces. Similar to the dry cell inactivation, BEC8 inactivated liquid cells at low inoculum levels with increasing efficiency from 4 to 37°C.

When the kinetics of inactivation with BEC8 at MOI of 100 were calculated, D-values were consistently lower for liquid cells than for dry cells, but these differences were not significant at a level of $p = 0.05$. D-values of inactivation ranged from approximately 5 min at 37^{o}C to 55 min at 12^{o}C, and they were significantly different when comparing the different incubation temperatures used (Table 3.4). However, D-values were not statistically different among the different hard surfaces. Z-values for SSC, CTC, and HPEC for liquid positive controls were 22.86, 21.43, and 22.74°C (R^2

values >0.91), respectively and for dry cells treated with BEC8 Z values were 26.58, 22.50, and 27.38°C (R^2 values >0.93), respectively.

3.4. Discussion

The bacteriophage cocktail used in this study was previously characterized and included the phages 38, 39, 41, AR1, 42, CEV2, ECB7, and ECA1 (submitted for publication). Extensive spot testing against STEC O157:H7 showed that the BEC8 phages were capable of causing lysis of 94 to 98% of the strains (N=123) screened. When BEC8 phages were subjected to PCR for typical EHEC virulence factors *stx1*, *stx2*, *eaeA*, and *hlyA*, none of the virulence factors were detected.

The purpose of this study was to test the efficacy of the bacteriophage cocktail BEC8 against a mixture of *E. coli* O157:H7 strains at different MOIs on hard surfaces commonly used in the food industry. The bacterial mixture was markedly inactivated, with rates of viable cell count losses increasing from low to high MOIs, low to high temperatures, and short to longer exposure times. Other researchers have reported that bacteriophages were able to effectively inactivate bacterial pathogens when attached to hard surfaces found in food processing environments, but only at high MOI values of 1000 (2, 159) or greater (100,000) (314). Phages have also been applied to control biofilms significant to the medical community, since they often form on the surfaces of embedded medical devices such as catheters but they usually use high MOIs (142). A relevant study by Fu et al (134) demonstrated that phages applied on catheters made of

silicone at a MOI of 1,000 can inactivate up to 4.37 log CFU/cm^2 after 24 h. In this work, relatively rapid rates of inactivation were observed on all surfaces at an MOI ratio of 100.

Phages are considered to have potential as a viable alternative or complement to chemical sanitizers on food processing surfaces. A study by Sharma et al (314) sought to determine the effectiveness of the lytic bacteriophage KH1 against *E. coli* O157:H7 ATCC 43895 and the *rpoS*-deficient strain FRIK 816-3 attached to stainless steel coupons. Initial populations of 2.6 log CFU/coupon of attached cells of both strains were reduced by 1.2 log CFU/coupon when treated with bacteriophage KH1 at 7.7 log PFU/mL for up to 4 days at 4^{o}C at an MOI of approx. 100,000. Reductions in the number of cells of *E. coli* O157:H7 attached to stainless steel coupons or in liquid form were similar, suggesting that attachment under the conditions tested did not provide additional protection against bacteriophage attack (314). Similarly, in our study, the efficiency of the phage cocktail on the bacterial pathogen stored at low temperatures of 4^{o}C resulted in an inactivation of approx. one log CFU and there was no significant difference between dry and liquid cells. However, the highest MOI used in our study was 100 which are several orders of magnitude lower than the MOI used in the Sharma et al study. Those early results of bacteriophages against *E. coli* O157:H7 supported the idea that they could be a potential treatment of surfaces.

The use of phages has been investigated to control a wide variety of pathogenic microorganisms. In a study by Goode et al (141), high phage titers were needed to inactivate *Salmonella* and *Campylobacter* from chicken skin. This phenomenon is typical of the diffusion limitation of phage–food pathogen on a surface contaminated by a low

inoculum level of the pathogen. Various studies have shown that there are threshold concentrations below which phage infection can be interrupted (79) and that in general a higher MOI results in higher levels of bacterial inactivation (146, 216). O'Flynn et al (270) used a mixture of three phages at a MOI of one million to reduce *E. coli* O157:H7 on nine steak meat samples. Seven of the nine samples were completely free of *E. coli* O157:H7 as determined by viable plate count after enrichment. Likewise, in this study, higher levels of reduction were obtained with increasing MOI.

In a study by Roy et al (304), phages were used to disinfect stainless steel and polypropylene surfaces contaminated with *Listeria monocytogenes* strains10401 and 8427 at room temperature. They found that the phage mixture, at a MOI of approx. 10,000, was able to reduce bacterial levels by 3.7 and 3.5 log units after an hour on stainless steel and polypropylene, respectively, showing a significant difference between the phage efficacy on each surface. In our study, at a much lower MOI of 100 and at the same temperature conditions there was a similar inactivation of approx. 4 log CFU of *E. coli* O157:H7 after an hour of treatment. Usually, contamination of foods by *E. coli* O157:H7 is due to low concentrations of cells and outbreaks are sometimes caused by less than 20 *E. coli* O157:H7 CFU/g of food and, often, with less than 1 CFU/g of food (242). For comparison purposes, the levels of *E. coli* O157:H7 used in our study were 100 to 10,000 times higher than those found in naturally contaminated foods or surfaces.

An average phage lytic cycle can take from 20 to 40 min (237). This suggests, in agreement with the findings of Abuladze et al (2), that the significant reductions in *E. coli* O157:H7 counts observed in our experiments, specifically at higher temperatures and

lower bacterial inocula, were not endpoints of the lytic process on the hard surfaces examined. Instead, they were the result of initial adsorption of the phages to the bacterial membrane and ensuing lysis of the bacteria. When O'Flynn et al (270) investigated the activity of phages against *E. coli* O157:H7 in broths, they found that phages could eliminate the bacteria at temperatures of 30 or 37°C where the organism was growing under optimal conditions. However, the phages could not lyse the cells in the absence of growth at 12°C. In other studies though, phages have been able to significantly reduce the numbers of *Salmonella* in cheese (249) chicken (141) and melon slices (218) in the absence of or very limited bacterial growth at low temperatures. In our study, low temperatures of 4°C resulted in decreased inactivation of the bacterial mix at every MOI and storage time. However, there was still a 1.5 to 2 log CFU reduction in the bacterial mixture at high MOI. The importance of the phage-to-bacterium contact time depends on the application the antimicrobial is intended for. In this case, this project was developed on the hypothetical scenario that phage cocktail was intended for decontamination of surfaces typically present in food processing plants when there is ample time due to the presence of positive pathogen detection results. The phage cocktail could then be left on surfaces for a prolonged period of time allowing for maximal efficacy through a complete phage lysis cycle.

A major concern for bacteriophage applications is the emergence of bacterial resistance. There are a number of studies that have suggested that bacterial resistance to phages is not a very frequent event and ultimately does not irreversibly inhibit the effectiveness of the treatment. In addition, O'Flynn et al (270) observed in that the rate of

appearance of phage-resistant mutants was very low and did not reduce the treatment's efficacy in beef samples experimentally contaminated by *E. coli* O157:H7 and treated with a phage preparation. By using a three phage cocktail the risk of selecting for phage-resistant mutants was decreased, as proposed by several authors (218, 334). Similarly, we did not find any phage-resistant mutants of *E. coli* O157:H7 when randomly selected surviving colonies were tested for their susceptibility to BEC8.

The *E. coli* O157:H7 cells used in this study were grown to mid-exponential phase and then spot inoculated on the hard surfaces as seen in other studies (146, 217). This was done to simulate the conditions found in a typical food processing setting in which the pathogen could have been growing rapidly in a nutrient dense environment before contaminating and ultimately drying on the hard surface. It is also known that bacterial cells are most susceptible to bacteriophage attack during exponential-phase growth (1), and the reduction of *E. coli* O157:H7 would be optimal under these conditions. It is imperative that further work is conducted to determine whether stationary-phase cells of *E. coli* O157:H7 would be less susceptible to BEC8 on hard surfaces.

3.5. Conclusions

E. coli O157:H7 is a prevalent pathogen with a low infectious dose. Using bacteriophages to reduce contamination of food processing equipment and surfaces by bacterial pathogens has the potential to be one of the most environmentally friendly and natural approaches for reducing the incidence of food-borne disease. The data presented

in this study suggests that *E. coli* O157:H7-specific phages can be a potential intervention against food-borne pathogens that may contribute to prevent future cases of bloody diarrhea.

Table 3.1. Effect of the bacteriophage cocktail BEC8 on cells of *E. coli* O157:H7 in liquid TSB placed on stainless steel chips at a multiplicity of infection (MOI) of 1, 10, and 100.

MOI	**Temperature (°C)**	**Reduction (log CFU/chip)**		
		10 min	**1 h**	**24 h**
1	4	0.52	0.62	0.52
	12	0.70	1.17	1.43
	23	1.26	1.86	3.09
	37	1.90	3.57	5.25
10	4	1.33	2.00	2.05
	12	1.86	1.60	2.12
	23	2.27	2.62	2.83
	37	2.34	3.21	4.30
100	4	1.40	1.25	1.95
	12	1.71	2.91	3.30
	23	2.37	3.34	3.22
	37	3.26	3.35	3.28

Table 3.2. Effect of the bacteriophage cocktail BEC8 on cells of *E. coli* O157:H7 in liquid TSB on ceramic tile chips at a multiplicity of infection (MOI) of 1, 10, and 100.

MOI	Temperature (°C)	Reduction (log CFU/chip)		
		10 min	1 h	24 h
1	4	0.29	0.49	0.45
	12	0.56	0.55	0.66
	23	0.30	0.58	1.93
	37	0.37	2.7	3.36
10	4	0.92	1.31	1.16
	12	0.77	1.66	1.48
	23	1.92	2.94	3.69
	37	2.36	2.81	4.32
100	4	1.02	0.88	1.14
	12	1.37	1.78	2.92
	23	1.71	2.54	2.69
	37	1.37	1.78	2.92

Table 3.3. Effect of the bacteriophage cocktail BEC8 on cells of *E. coli* O157:H7 in liquid TSB on high density polyethylene chips at a multiplicity of infection (MOI) of 1, 10, and 100.

MOI	Temperature (°C)	Reduction (log CFU/chip)		
		10 min	1 h	24 h
1	4	0.75	0.73	0.65
	12	0.57	1.05	0.90
	23	0.93	1.53	1.23
	37	0.97	1.95	3.75
10	4	0.67	1.26	1.58
	12	1.56	2.25	1.40
	23	0.95	2.15	3.21
	37	3.16	4.11	3.84
100	4	0.29	0.67	1.79
	12	0.72	1.56	2.72
	23	1.73	2.89	2.75
	37	3.12	3.45	3.08

Table 3.4. D-values of a mixture of dry and liquid (positive control) *E. coli* O157:H7 strains inactivated by bacteriophage cocktail BEC8 on three surface chips (SSC, CTC, HDPEC). Bacterial mixture included strains I 2005003658-472, TWO8635- EK27, and ATCC 43895

Surface materials	**Temp. (°C)**	**Liquid cells**				**Dry cells**			
		D-Value	**Lower (min)**	**Upper**	**R^2**	**D-Value**	**Lower (min)**	**Upper**	**R^2**
SSC	12	42.9 aA*	33.5	59.4	0.87	46.0 aA	35.6	65.1	0.85
	23	23.0 aA	17.4	33.8	0.83	30.0 aA	25.3	37.1	0.93
	30	4.6 bB	3.9	6.0	0.91	8.8 bA	6.4	13.9	0.83
	37	4.8 bA	4.7	7.8	0.94	5.9 bA	4.7	7.8	0.91
CTC	12	32.6 aA	23.9	51.1	0.79	46.7 aA	32.1	85.5	0.71
	23	21.3 aA	15.6	33.6	0.79	29.3 aA	22.3	42.8	0.83
	30	3.8 bA	2.7	6.1	0.82	6.6 bA	4.6	12.0	0.79
	37	3.9 bA	2.8	6.1	0.84	5.1 bA	3.6	8.6	0.80
HDPEC	12	39.1 aA	26.8	72.1	0.70	46.5 aA	33.5	76.1	0.77
	23	24.9 aA	18.6	37.5	0.81	32.1 aA	26.7	40.2	0.92
	30	5.2 bA	3.7	8.5	0.81	7.0 bA	4.7	14.3	0.72
	37	4.8 bA	3.2	9.9	0.71	7.1 bA	4.9	12.9	0.77

*D-values with the same lower case and upper case letter are not significantly different within the same column and row, respectively.

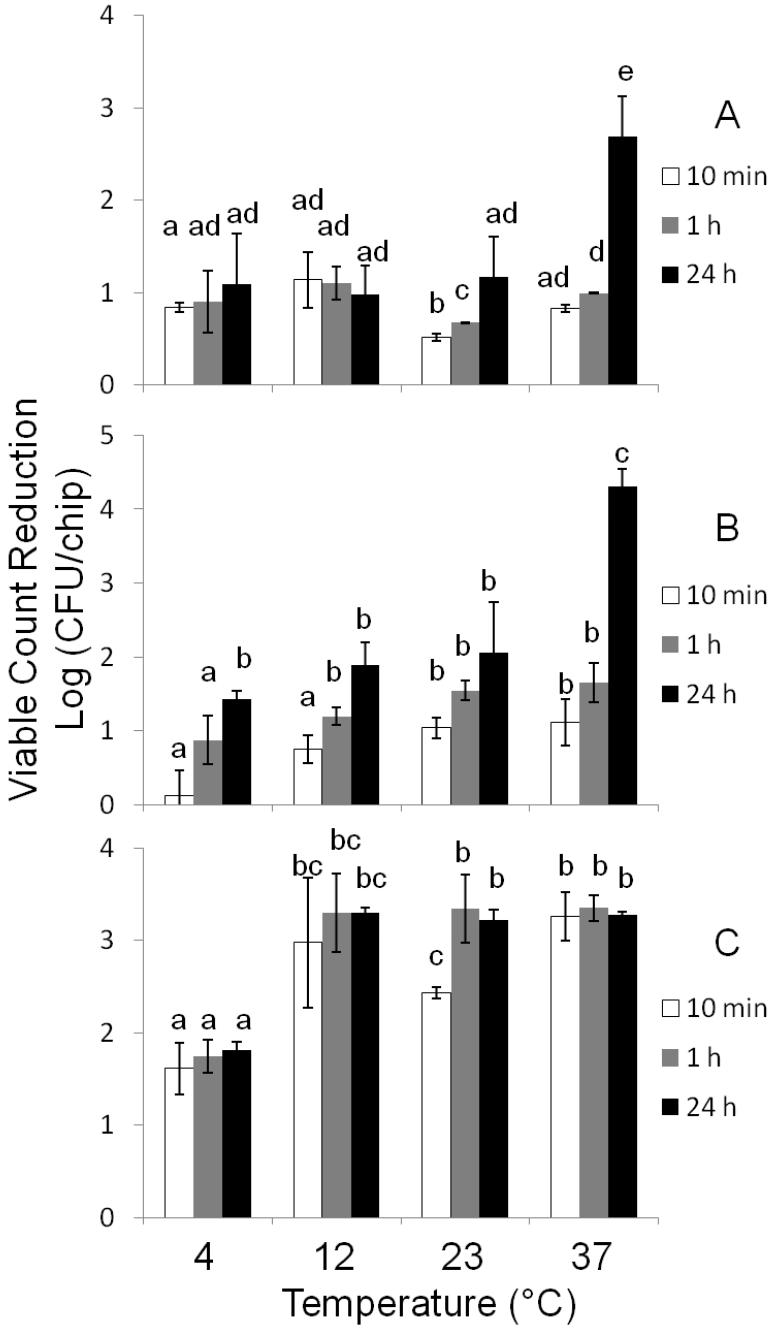

Figure 3.1. Effect of BEC8 bacteriophage mixture on *E. coli* O157:H7 cells dried on stainless steel chips at a MOI of A) 1, B) 10, and C) 100.

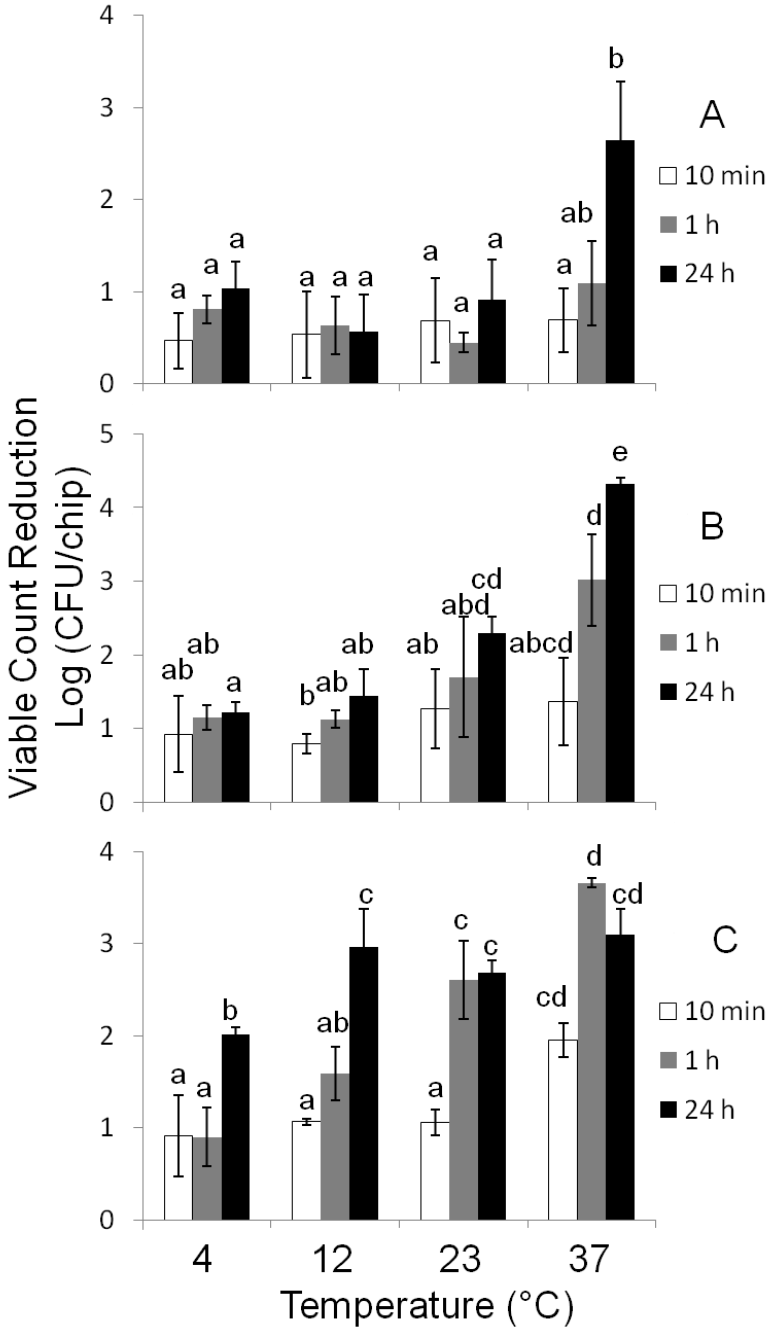

Figure 3.2. Effect of BEC8 bacteriophage mixture on *E. coli* O157:H7 cells dried on ceramic tile chips at a MOI of A) 1, B) 10, and C) 100.

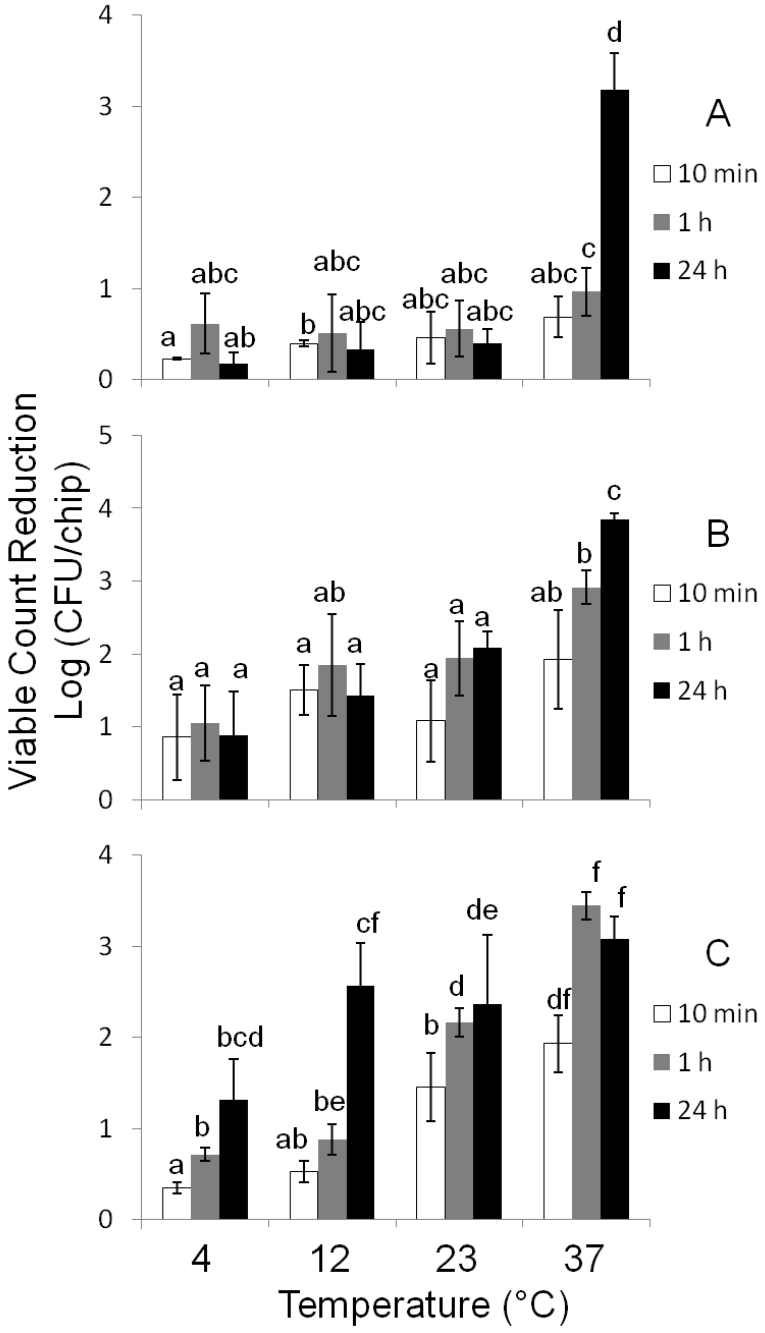

Figure 3.3. Effect of BEC8 bacteriophage mixture on *E. coli* O157:H7 cells dried on high density polyethylene chips at a MOI of A) 1, B) 10, and C) 100.

CHAPTER 4

REDUCTION OF *ESCHERICHIA COLI* O157:H7 VIABILITY ON LEAFY GREEN VEGETABLES BY TREATMENT WITH A BACTERIOPHAGE MIXTURE AND *TRANS*-CINNAMALDEHYDE

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/mL), and TC (0.5% v/v) were applied on treated leaves. The leaves were incubated at 4, 8, room temperature (RT), and 37^{o}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.

4.1. Introduction

Fresh vegetables are increasingly being identified as a source of foodborne outbreaks around the world (227). There have been many outbreaks associated with the consumption of fresh vegetables linked to Shiga toxin-producing *Escherichia coli* (STEC). Some examples include the radish sprout outbreak in Japan (246), fresh lettuce in Sweden (327), and bagged spinach and lettuce in the US (75). In December of 2006, a 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 (118). Also in 2006, Utah and New Mexico health departments investigated a multistate cluster of STEC O157 associated with consuming bagged spinach (345).

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 (117). 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 (51). In

Australia, fresh produce has been responsible for 4% of all foodborne outbreaks reported between 2001 and 2005 (196).The incidence of foodborne illness associated with the consumption of minimally processed ready-to-eat salad vegetables has been consistently increasing (35, 188, 344). In the US, the percentage of outbreaks associated with fresh produce increased from less than 1% in the 1970s to 6% in the 1990s (320). 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% to 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 (7, 71, 160, 185, 232, 345, 353, 370).

Bacteriophages are obligate parasites of bacteria capable of killing specific species and offer a natural method to control contamination of foods (65). *E. coli* phages can be isolated from sewage, waste water, polluted rivers and fecal samples of humans or animals (58). 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. (315) 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 (26, 180) and post-harvest level (216, 218).

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 (334). In addition, there has been recent exploration in different phage delivery systems. Puapermpoonsiri et al. (293) 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 (153). A study by Helander et al (153) 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 (76). Baskaran et al (27) investigated the antimicrobial effect of low concentrations of *trans*-cinnamaldehyde on *Escherichia 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 (19, 25, 33, 35). Furthermore, using combined treatments is consistent with the hurdle concept (211) 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 (187, 368, 379) 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 (382) and sprouting mung beans and alfalfa seeds (381). 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 *Enterobacter asburiae* could be enhance 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.

4.2. Materials and Methods

4.2.1. 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. 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. 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.

4.2.2. Bacterial strains

The bacterial strains used to inoculate leafy green vegetables were nalidixic acid resistant mutants of *Escherichia 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 50 μg/mL. Strains were stored in vials containing trypic soy broth (TSB; Neogen, Inc. Lansing MI) and 10% glycerol (Sigma Chemical Co., St. Louis, MO) at -55°C. For inoculum preparation, frozen suspensions were streaked on tryptic soy agar (TSA; Neogen, Inc., Lansing, MI) and the TSA plates were incubated at 37°C for 24 h.

4.2.3. Leafy green vegetables

Organic baby spinach (5.0 cm ± 0.5 cm × 3.5 cm ± 0.5 cm) and baby romaine lettuce (4.0 cm ± 0.5 cm × 2.5 cm ± 0.5 cm) were purchased at a local supermarket. The leafy greens were rinsed with tap water immediately after purchasing, followed by rinsing with 70% ethanol, placed in Petri plates and used right away. The purpose of the pretreatment was to reduce the number of naturally occurring microflora.

4.2.4. Inoculation

The Nal^R EHEC strains were streaked on tryptic soy agar (TSA; Neogen, Inc. Lansing MI), incubated overnight at 37°C, individual colonies were transferred to tubes of 9 mL of TSB, and incubated overnight at 37°C. Aliquots of 100 µL were transferred to fresh tubes of 9 mL TSB and incubated at 37°C for 3 h resulting in mid-exponential phase cultures. Aliquots of 0.5 mL of each strain were combined in 1 mL microcentrifuge tubes, vortexed, and centrifuged at 14,500 × g for 5 min. Supernatants were decanted and fresh 1.5 mL TSB were added and mixed. Three different levels of bacteria were added to each hard surface. Mixtures of the three Nal^R EHEC strains were diluted in TSB to provide 4, 5, and 6 log CFU/leaf of inoculum designated as low (L), medium (M), and high (H) levels, respectively. The mixture was spot inoculated (20 µL) onto the leaves and allowed to dry for 1 h in a biosafety cabinet.

4.2.5. BEC8 and TC application

Aliquots (100 µL) of BEC8 (10^7 PFU/mL), TC, or TSB (phage and essential oil treatments and negative control, respectively) were applied on top areas of the leafy green vegetables inoculated with mixtures of the three Nal^R *E. coli* O157:H7 strains. This set three levels of multiplicities of infection (MOI) of 100, 10, and 1 for the phage treatment, corresponding to the L, M, and H inoculation levels, respectively. Positive controls included 20 µL of bacterial mixtures spotted on the leaves and mixed with 100 µL of BEC8 or TC without drying. Leaves were then incubated inside of Petri plates at 4, 8, 23 and 37°C for 10 min, 1 h, and 24 h. Five pieces of filter paper and 10 mL of sterile water were added to the Petri plates to avoid complete evaporation of BEC8, TC, or TSB, and wilting of the lettuce or spinach leaves.

4.2.6. Processing

Treated leafy greens were placed in 50 mL centrifuge tubes that contained 1 mL of phosphate buffer saline (PBS) (pH 7.2) and 15-20 glass beads (3 mm) using sterile forceps. The tubes were then vortexed for approximately 1 min, followed by serial dilution in PBS and plating on NA-SMAC. The NA-SMAC plates were then incubated at 37°C overnight and enumerated. Bacterial counts as CFU were calculated per leaf processed and the data transformed to logarithm base 10.

4.2.7. BEC8 viability in TC solution

To determine the viability of the BEC8 bacteriophage cocktail in the 0.5% v/v solution of *trans*-cinnamaldehyde the two components were combined at the same levels as described above and incubated at 23 and 37°C for 24 h. Bacteriophage survival was determined using the plaque assay by serially diluting the solution in PBS (pH 7.2) and combining 100 μL of the BEC8/TC aliquot with 200 μL of exponentially growing cells of *E. coli* B and 0.4 μL of tryptone top agar (TTA) followed by plating on TSA.

4.2.8. Data Analysis

Inactivation log CFU was calculated by subtracting the surviving log CFU/leaf of each treatment from the surviving log CFU/leaf of the negative control. Statistical comparison of bacterial survival as log CFU/leaf between each treatment and incubation time done by ANOVA (PROC MIXED) and the least-squares method was used to determine significant differences ($p < 0.05$) (SAS Statistical Analysis Software, version 8.0, SAS Institute, Cary, N.C.).

4.3. Results

The detection limit for the recovery of cells of *E. coli* O157:H7 from the leafy green vegetables was 1 log CFU/leaf. All the results are presented under the assumption that the survival of bacterial mixture at levels below the detection limit cannot be quantified. Therefore, when no surviving cells were found, they were assumed to be at a 1 log CFU/leaf level.

4.3.1. Effect of BEC8 on *E. coli* O157:H7 on leafy greens

Treatment of the experimentally contaminated baby spinach and baby romaine lettuce with BEC8 significantly reduced ($p<0.05$) the number of viable dried *E. coli* O157:H7 cells that could be recovered (Figures 4.1 and 4.2). Treating the contaminated leafy greens at three different MOI levels (1, 10, and 100) with BEC8 produced a reduction of at least one log CFU in the number of the *E. coli* O157:H7 cells after 24 h at all temperatures tested above 8°C. The highest levels of inactivation were observed with increasing values of time, temperature, and MOI. The inactivation trends observed were similar for both types of leafy greens at all MOIs and time-temperature combinations. No survivors were detected after BEC8 treatment at high MOI/low inoculum level (approx. 10^4 CFU/leaf) at the following incubation conditions: 37^oC for 1 h and RT after 24 h on both leafy green vegetables. At high MOI/low inoculum levels there was at least a 1 log CFU reduction of the pathogen at 4°C within 10 min. At temperatures above 8°C there were more than 2 log CFU reduction within 10 min and 3 log CFU reduction within 24 h of applying BEC8 on both leafy greens. At medium inoculum levels, the pathogen was reduced by 2 log CFU within 24 h at temperatures above 8°C, while at 4^oC

reductions of less than 2 log CFU were observed on both leafy greens. The loss of viability of high inoculum levels of EHEC was less than 1 log CFU at all conditions with the exception of 3 log CFU after 24 h at RT and 37°C for both leafy greens (Figures 4.1a and 4.2a).

Treatment of the experimentally contaminated liquid cultures of *E. coli* O157:H7 on the leafy green vegetables with BEC8 served as positive controls for the same phage preparations used on dried cells. Under these conditions, BEC8 significantly reduced ($p<0.05$) the number of viable cells in liquid TSB that could be recovered (Tables 4.1-4.3). Treating the leafy green vegetables containing liquid cultures at three different MOI levels (1, 10, and 100) with BEC8 produced a reduction of at least 1 log CFU in the number of the *E. coli* O157:H7 cells after 24 h at all temperatures. At low bacterial levels (high MOI) BEC8 inactivated *E. coli* O157:H7 cells in liquid TSB at levels similar to those acting against dry cells after 24 h. At temperatures above 8°C, BEC8 resulted in inactivation of at least 2.5 and 3 log CFU for both leaves at medium and low MOI, respectively. Similar to the dry cell inactivation, BEC8 inactivated liquid cells at low inoculum levels with increasing efficiency from 4 to 37°C.

4.3.2. Effect of TC on *E. coli* O157:H7 on leafy greens

Treatment of the experimentally contaminated baby spinach and baby romaine lettuce with 0.5% v/v TC significantly reduced ($p<0.05$) the number of viable dried *E. coli* O157:H7 cells that could be recovered (Figures 4.3 and 4.4). Treating the contaminated leafy greens at three different MOI levels (1, 10, and 100) with TC produced a reduction of at least 3 log CFU in the number of the *E. coli* O157:H7 cells

after 24 h at all temperatures tested. The highest levels of inactivation were observed with increasing values of time and temperature. The inactivation trends observed were similar for both types of leafy greens at all time-temperature combinations. No survivors were detected after TC treatment at high MOI/low inoculum level (approx. 10^4 CFU/leaf) at the following incubation conditions: 37°C for 10 min and 4, 8°C, and RT after 24 h on both leafy green vegetables. At low inoculum levels there was at least a 1 log CFU reduction of the pathogen at 4°C within 10 min. At temperatures above 8°C there was more than a 2 log CFU reduction within 10 min and 3 log CFU reduction within 24 h of applying TC on both leafy greens. At medium inoculum levels, the pathogen was reduced by 3 log CFU within 24 h at temperatures above 8°C, while at 4°C reductions of less than 3 log CFU were observed on both leafy greens. The loss of viability of high inoculum levels of EHEC was less than 4 log CFU at all conditions with the exception of 5 log CFU after 24 h at RT and 37°C for both leafy greens (Figures 4.3a and 4.4a).

TC significantly reduced ($p<0.05$) the number of viable cells in liquid TSB that could be recovered (Tables 4.4-4.6). Treating the leafy green vegetables containing liquid cultures at three different inoculums levels with TC produced a reduction of at least 2 log CFU in the number of the *E. coli* O157:H7 cells after 24 h at all temperatures. At low bacterial levels TC inactivated *E. coli* O157:H7 cells in liquid TSB at levels similar to those acting against dry cells. At temperatures above 8°C, TC resulted in inactivation of at least 3 log CFU for both leafy greens. Similar to the dry cell inactivation, TC inactivated liquid cells at low inoculum levels with increasing efficiency from 4 to 37°C.

4.3.3. Effect of BEC8 and TC combined on *E. coli* O157:H7 on leafy greens

Treatment of the experimentally contaminated baby spinach and baby romaine lettuce with the BEC8/TC combination significantly inactivated ($p<0.05$) the number of viable dried *E. coli* O157:H7 cells that could be recovered (Figures 4.5 and 4.6). Treating the contaminated leafy greens at three different inoculum levels with the BEC8/TC produced a complete inactivation of the number of the *E. coli* O157:H7 cells after 10 min at all temperatures tested. The inactivation trends observed were similar for both types of leafy greens at all inoculums levels and time-temperature combinations except for the medium and high inoculums levels after 10 min where they differed by one log CFU. The BEC8/TC combination resulted in complete inactivation of *E. coli* O157:H7 within 10 min on baby spinach leaves, while on baby romaine lettuce there was complete inactivation after 1 h of treatment.

BEC8/TC significantly inactivated ($p<0.05$) the number of viable cells in liquid TSB that could be recovered (Tables 4.7-4.9). Treating the leafy green vegetables containing liquid cultures at three different inoculum levels with BEC8/TC also resulted in complete inactivation in the number of the *E. coli* O157:H7 cells within 10 min at all temperatures. Similarly to dry cells, liquid cells of *E. coli* O157:H7 were also completely inactivated within 10 min on baby spinach leaves, while on baby romaine lettuce they were completely inactivated within 1 h.

4.3.4. Effect of TC on BEC8 viability

Treatment of the BEC8 bacteriophage cocktail with TC at 23 and 37°C for 24 h resulted in 81.25% ± 8.8% and 79.2% ± 5.9% survival of the phages, respectively.

4.4. Discussion

The bacteriophage cocktail used in this study was previously characterized and included the phages 38, 39, 41, AR1, 42, CEV2, ECB7, and ECA1 (submitted for publication). Extensive spot testing against STEC O157:H7 showed that the BEC8 phages were capable of causing lysis of 94 to 98% of the strains (N=123) screened. When BEC8 phages were subjected to PCR for typical EHEC virulence factors *stx1*, *stx2*, *eaeA*, and *hlyA*, none of the virulence factors were detected.

The purpose of this study was to determine the effect of BEC8 and TC, individually and combined, on cells of *E. coli* O157:H7 dried on the surfaces of baby spinach and baby romaine lettuce. The bacterial mixture was markedly inactivated by the bacteriophage cocktail, with rates of viable cell count losses increasing from low to high MOIs, low to high temperatures, and short to longer exposure times. Other researchers have reported that bacteriophages were able to effectively inactivate bacterial pathogens when attached to fresh produce and fruit such as lettuce (146, 315), honeydew melon (217) and apples (216), tomatoes (2, 382), spinach, and broccoli (2), sprout seeds (280), sprouting mung bean and alfalfa seeds (381), and others. However, these studies used high MOIs ranging from 1,000 to 100,000 and above. In this study, no survivors were detected from an initial inoculum of aprox. 10^4 CFU/leaf after 24 h at room temperature and after 1 h at 37°C with a MOI of 100.

Phages are increasingly being investigated as an alternative antimicrobial treatment for fresh-cut produce as well as food processing surfaces. A study by Sharma et al (314) investigated the effect of phage KH1 on *E. coli* O157:H7 attached to stainless

steel surfaces. They observed that the reductions in the number of cells of *E. coli* O157:H7 attached to stainless steel coupons or in liquid form were similar; suggesting that attachment under the conditions tested did not provide additional protection against bacteriophage attack. Similarly, in our study, the efficiency of the phage cocktail on the bacterial pathogen was independent of whether the cells were dry or in liquid form on the leafy green surfaces. This suggests that drying of the bacterial cells on the surfaces of leafy greens does no provide extra protection against the BEC8 cocktail.

Cinnamon oil is commonly used in the food industry because of its special aroma and its antimicrobial activity has attracted great attention from many researchers. However, not many studies have been conducted testing the effect of TC on *E. coli* O157:H7 on leafy green vegetables. A closely related compound, cinnamic acid, is a naturally occurring organic acid found in many fruits and spices that has antimicrobial activity against pathogenic microorganisms, but has low aqueous solubility. A study used a solubility-enhancing alpha-cyclodextrin-cinnamic acid inclusion complex against *E. coli* O157:H7 suspended in apple cider (350). When the cider contained 400 mg/L of the cinnamic acid complex, the pathogen was reduced by 4.38 log at 4^{o}C and a 6.38 log at 26^{o}C.

In a related study, the activity of cinnamaldehyde in vapor phase was tested against *E. coli* O157:H7 on intact and damaged lettuce and spinach tissue (267). The vapor of the lowest concentration (40 μL per L of air) employed inactivated more than 4 log CFU of *E. coli* O157:H7 at 0 and 4°C in 4 days and at 10°C in 2 days. At the tissue damaged by cutting, the highest antimicrobial concentration (80 μL per L of air) reduced

the pathogen by 4 log CFU at 0°C and 2 to 4 log CFU at 4°C in 4 days. The pathogen concentration on spinach surface was reduced by 1 log less compared to lettuce surface. Overall, greater inactivation occurred on lettuce than spinach leaves. In this study, TC was able to inactivate low levels of the *E. coli* O157:H7 mixture dried on spinach and lettuce surfaces by more than 3 log CFU/leaf after 1 h of application, medium levels by more than 3 log CFU/leaf after 24 h, and high levels by more than 3 log CFU/leaf after 1 h at all temperatures and more than 5 log after 24 h at temperatures higher than 23°C. On the other hand, there was no significant difference between the inactivation trends of *E. coli* O157:H7 by TC between spinach and lettuce.

The combination of the bacteriophage cocktail with an essential oil applied on dry and liquid cells of *E. coli* O157:H7 attached to spinach and lettuce leaves is a novel idea. In the past, there have been studies involving the combination of phages with other antimicrobials such as nisin (216), antibiotics (166, 225), chlorine compounds (304), and antagonistic bacteria (381, 382). In the most relevant study, Leverentz et al (216) applied phages in combination with nisin against *Listeria monocytogenes* on fresh-cut honeydew melons and fresh-cut apples. They found that the phages alone inactivated *L. monocytogenes* by 2.0 to 4.6 log CFU more than the control, while nisin on its own resulted in a 3.2 log CFU reduction on melons. When the two treatments were used in combination, there was a 5.7 log CFU inactivation. On the other hand, the synergy between phages and nisin exhibited on melons was not demonstrated on fresh-cut apples. Similarly, in this study, application of TC and BEC8 on their own resulted in an inactivation of high levels of *E. coli* O157:H7 by 3 and 1 log CFU/leaf, respectively at

temperatures below 8°C after 24 h. But when the two treatments were combined, there was complete inactivation (5 log CFU/leaf reduction) within 10 min and 1 h at all temperatures for spinach and lettuce, respectively.

In an effort to understand the increased activity of the BEC8/TC combination, the effects of TC on the bacterial membrane must be taken under consideration. A study by Helander et al (153) used different essential oils to inhibit *E. coli* O157:H7 and *Salmonella* and they found that TC 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. When assessing the antimicrobial action of essential oil components, the lipophilic character of their hydrocarbon skeleton and the hydrophilic character of their functional groups are of the main importance. The activity rank of essential oil components is as follows: phenols > aldehydes > ketones > alcohols > ethers > hydrocarbons. Di Pasqua et al (96) used fatty acid extraction and gas chromatographic analysis to assess changes in membrane fatty acid composition of *E. coli* treated with TC. Substantial changes were observed on the long chain unsaturated fatty acids when the *E. coli* strains grew in the presence of TC. This could allow bacteriophages to attach on to the bacterial membrane more readily, inject their genetic material, and ultimately dooming the host much faster than it would without the altered bacterial membrane.

The *E. coli* O157:H7 cells used in this study were grown to mid-exponential phase and then spot inoculated on the hard surfaces as conducted in other studies (146, 217). The purpose of using this method to simulate the conditions found in a typical fresh produce processing facility in which the pathogen could have been growing rapidly in a

nutrient dense environment before contaminating and ultimately drying on the spinach or lettuce's surface. Close association with the plant increases the likelihood of survival for *E. coli* through access to nutrients in exudates released from the roots (301). In general, enteric pathogens can adapt to plant surfaces (52) and it has been shown that some epiphytes such as *Wausteria paucula* can assist *E. coli* O157:H7 in surviving (85) by modifying the plant tissue and supplying a suitable growth niche by releasing nutrients from the plant (55, 309). It is also known that bacterial cells are most susceptible to bacteriophage attack during exponential-phase growth (1) and the reduction of *E. coli* O157:H7 would be optimal under these conditions. It is imperative that further work is conducted to determine whether stationary-phase cells of *E. coli* O157:H7 would be less susceptible to BEC8 and TC individually and in combination on surfaces of leafy green vegetables.

4.5. Conclusion

E. coli O157:H7 is a prevalent pathogen with a low infectious dose. Using bacteriophages in combination with another natural antimicrobial such as TC to reduce contamination of leafy green vegetables by bacterial pathogens has the potential to be one of the most environmentally friendly and natural approaches for reducing the incidence of food-borne disease. The data presented in this study suggests that *E. coli* O157:H7-specific phages combined with TC can be a potential intervention against food-borne pathogens that may contribute to prevent future cases of bloody diarrhea.

Table 4.1. Effect of the bacteriophage cocktail BEC8 on cells of *E. coli* O157:H7 in tryptic soy broth placed on leaves of baby spinach at a multiplicity of infection (MOI) of 1, 10, and 100.

MOI	Temperature (°C)	Viable Count Reduction (log CFU/leaf)		
		10 min	1 h	24 h
1	4	0.26	0.16	0.40
	8	0.39	0.68	1.21
	23	1.00	1.28	1.32
	37	0.81	1.94	2.86
10	4	0.56	0.98	1.66
	8	0.83	1.30	2.45
	23	0.75	2.3	3.00
	37	1.64	2.47	2.53
100	4	0.41	1.79	1.50
	8	1.37	2.18	2.81
	23	2.01	2.43	3.53
	37	1.73	3.12	3.08

Table 4.2. Effect of the bacteriophage cocktail BEC8 on cells of *E. coli* O157:H7 in tryptic soy broth placed on leaves of baby romaine lettuce at a multiplicity of infection (MOI) of 1, 10, and 100.

MOI	Temperature (°C)	Viable Count Reduction (log CFU/leaf)		
		10 min	1 h	24 h
1	4	0.22	0.51	0.67
	8	0.37	0.81	0.87
	23	0.97	0.59	1.68
	37	0.22	0.94	3.68
10	4	0.99	1.32	1.83
	8	0.77	1.16	1.51
	23	1.40	1.42	2.05
	37	1.66	2.10	3.21
100	4	1.51	1.77	2.02
	8	1.81	1.92	1.94
	23	1.99	2.17	3.78
	37	2.77	3.49	3.57

Table 4.3. Effect of *trans*-cinnamaldehyde on cells of *E. coli* O157:H7 in tryptic soy broth placed on leaves of baby spinach at an inoculum level of high (H), medium (M), and low (L).

Inoculum level	Temperature (°C)	Viable Count Reduction (log CFU/leaf)		
		10 min	1 h	24 h
H	4	1.63	3.28	3.96
	8	3.19	3.99	4.02
	23	3.26	3.61	5.08
	37	3.90	4.24	5.38
M	4	2.59	2.26	2.79
	8	1.96	2.51	2.69
	23	1.65	1.52	3.72
	37	1.64	4.05	4.62
L	4	2.49	2.58	2.45
	8	2.60	1.76	2.99
	23	1.51	2.22	3.74
	37	3.75	3.83	3.43

Table 4.4. Effect of *trans*-cinnamaldehyde on cells of *E. coli* O157:H7 in tryptic soy broth placed on leaves of baby romaine lettuce at an inoculum level of high (H), medium (M), and low (L).

Inoculum level	Temperature (°C)	Viable Count Reduction (log CFU/leaf)		
		10 min	1 h	24 h
H	4	2.38	2.28	3.67
	8	2.61	2.60	3.33
	23	2.16	3.22	3.50
	37	1.87	3.15	4.85
M	4	1.72	2.23	2.71
	8	1.59	2.46	3.79
	23	1.85	3.38	3.60
	37	2.35	4.15	3.85
L	4	1.09	2.77	3.31
	8	1.89	2.82	3.35
	23	1.93	2.81	3.08
	37	3.26	3.35	3.26

Table 4.5. Effect of BEC8 and *trans*-cinnamaldehyde on cells of *E. coli* O157:H7 in tryptic soy broth placed on leaves of baby spinach at an inoculum level of high (H), medium (M), and low (L).

Inoculum level	**Temperature (°C)**	**Viable Count Reduction (log CFU/leaf)**		
		10 min	**1 h**	**24 h**
H	4	5.61	5.31	5.29
	8	5.14	5.21	5.20
	23	5.29	5.26	5.33
	37	5.25	5.34	5.23
M	4	4.33	4.29	4.35
	8	4.25	4.30	4.32
	23	4.31	4.25	4.14
	37	4.39	4.21	4.22
L	4	3.31	3.12	3.19
	8	3.35	3.08	3.24
	23	3.31	3.17	3.39
	37	3.42	3.20	3.36

Table 4.6. Effect of BEC8 and *trans*-cinnamaldehyde on cells of *E. coli* O157:H7 in tryptic soy broth placed on leaves of baby romaine lettuce at an inoculum level of high (H), medium (M), and low (L).

Inoculum level	**Temperature (°C)**	**Viable Count Reduction (log CFU/leaf)**		
		10 min	**1 h**	**24 h**
H	4	3.90	5.16	5.36
	8	4.71	5.24	5.26
	23	3.78	5.03	5.21
	37	5.38	5.46	5.47
M	4	2.53	7.19	4.22
	8	3.99	4.38	4.19
	23	3.34	4.31	4.32
	37	4.14	4.03	4.25
L	4	3.11	3.39	3.33
	8	3.31	3.34	3.23
	23	3.19	3.37	3.19
	37	3.35	3.42	3.30

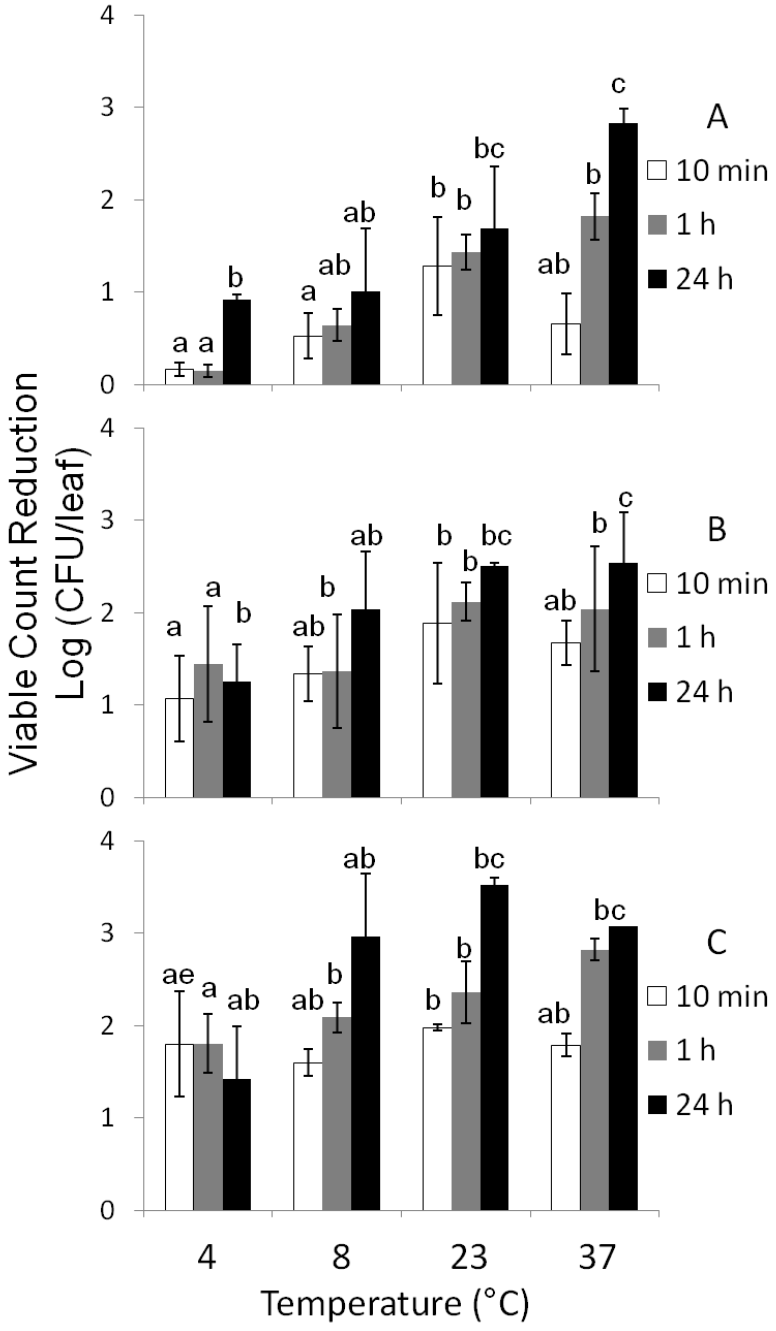

Figure 4.1. Effect of BEC8 bacteriophage mixture on *E. coli* O157:H7 cells dried on baby spinach leaves at a MOI of A) 1, B) 10, and C) 100.

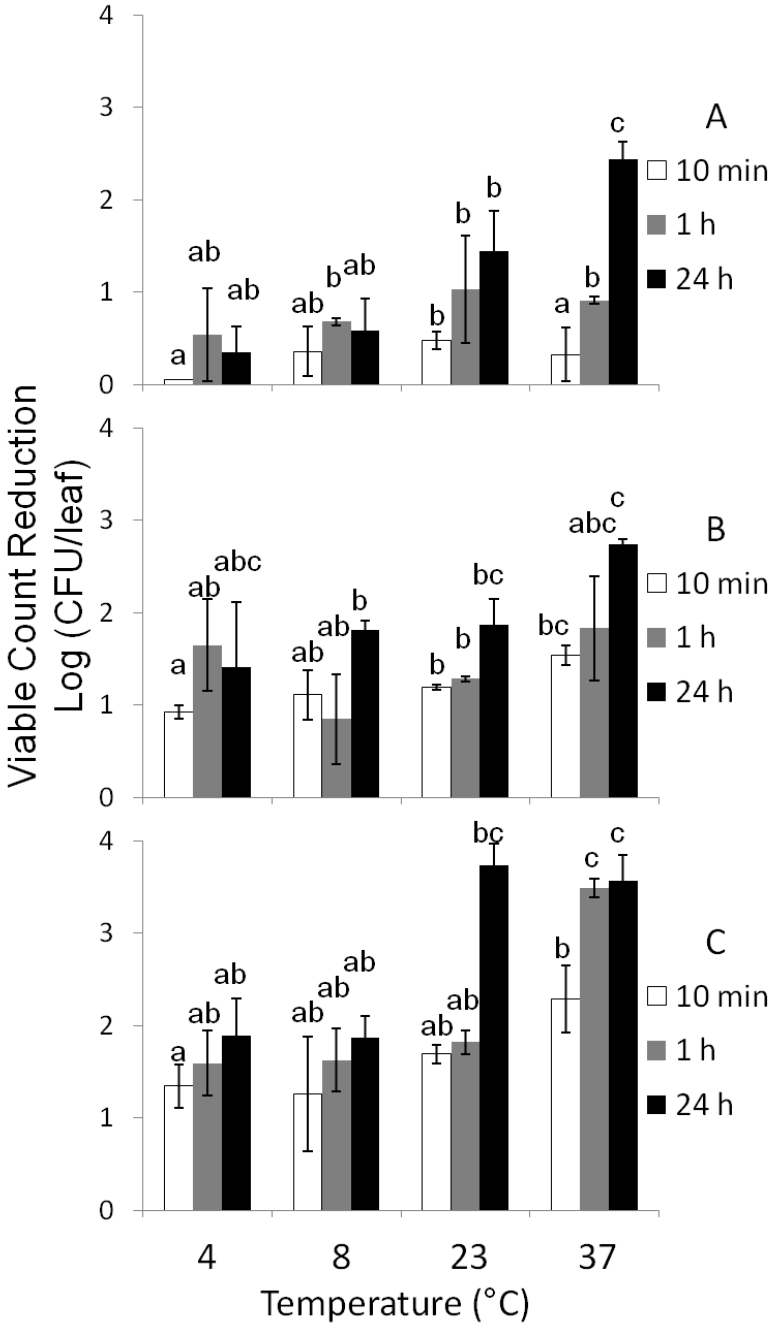

Figure 4.2. Effect of BEC8 bacteriophage mixture on *E. coli* O157:H7 cells dried on baby romaine lettuce leaves at a MOI of A) 1, B) 10, and C) 100.

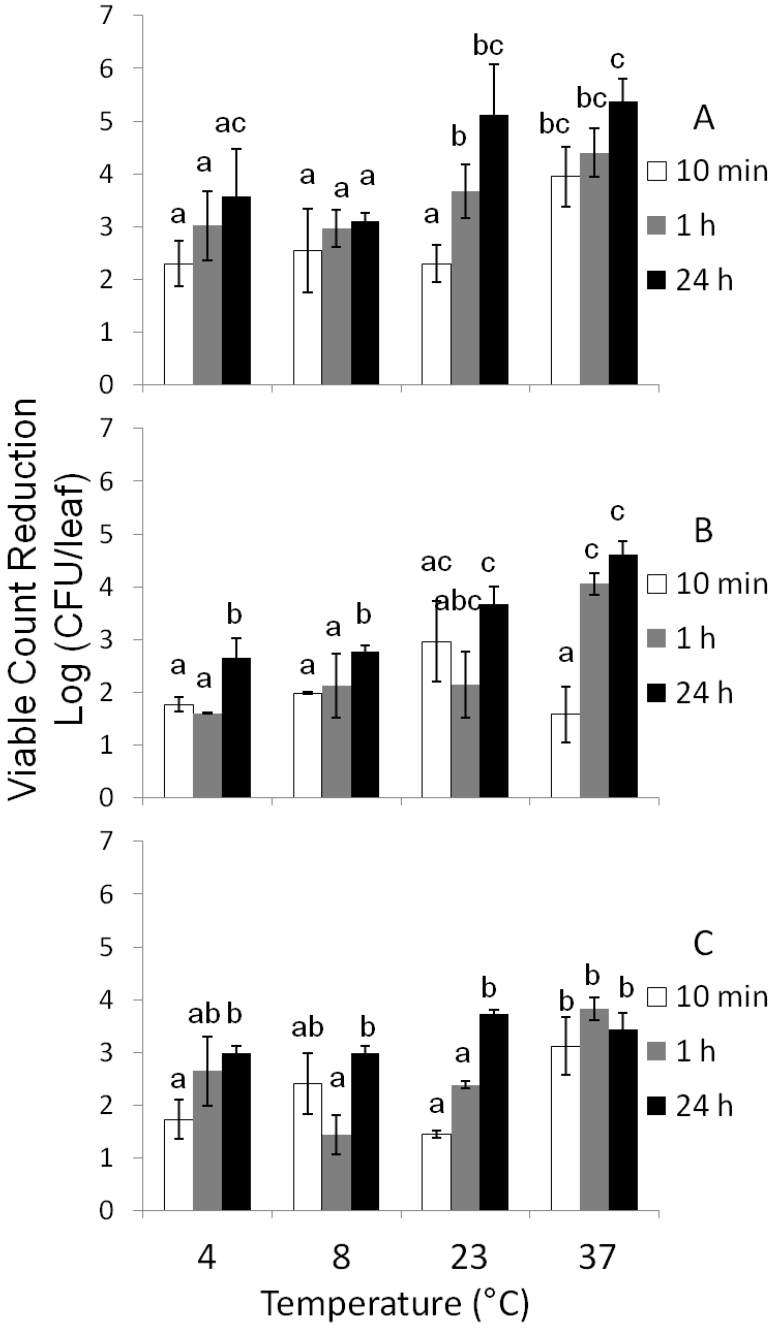

Figure 4.3. Effect of 0.5% *trans*-cinnamaldehyde on *E. coli* O157:H7 cells dried on baby spinach leaves at a MOI of A) 1, B) 10, and C) 100.

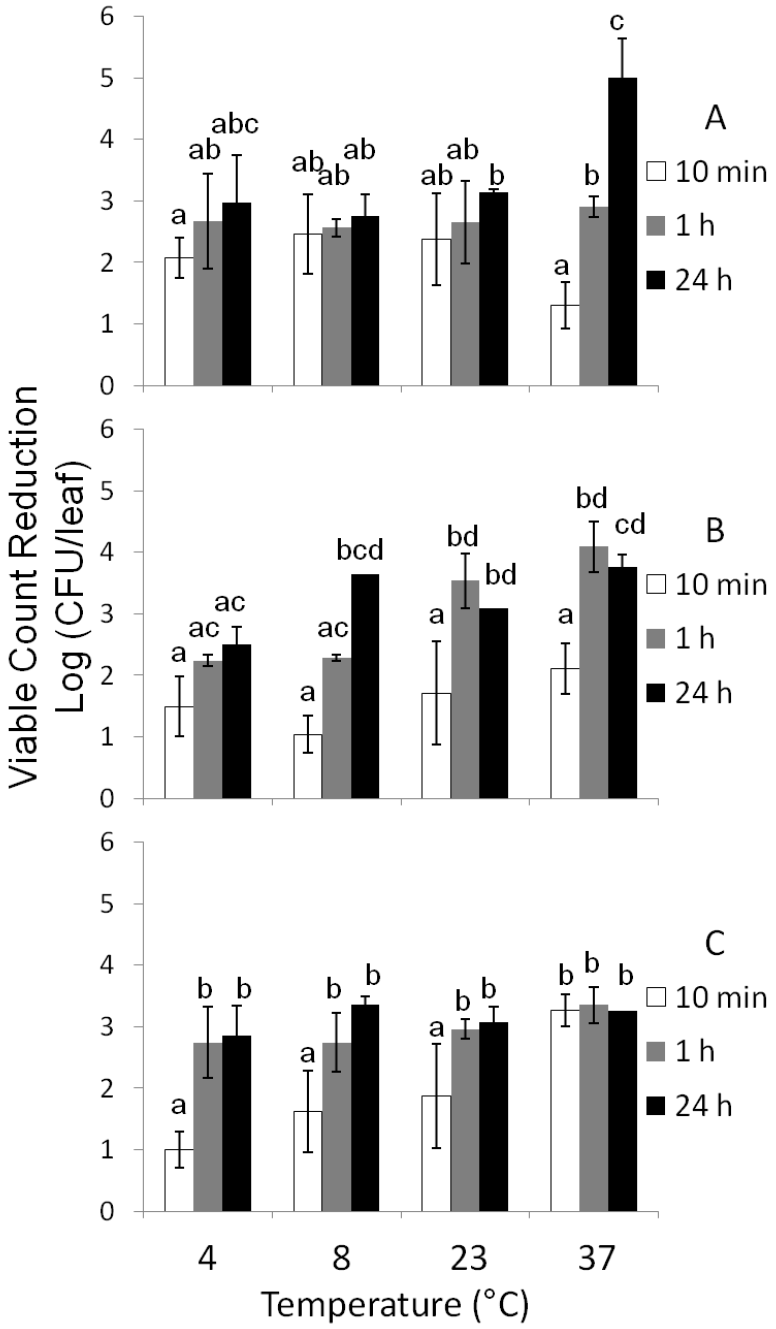

Figure 4.4. Effect of 0.5% *trans*-cinnamaldehyde on *E. coli* O157:H7 cells dried on baby romaine lettuce leaves at a MOI of A) 1, B) 10, and C) 100.

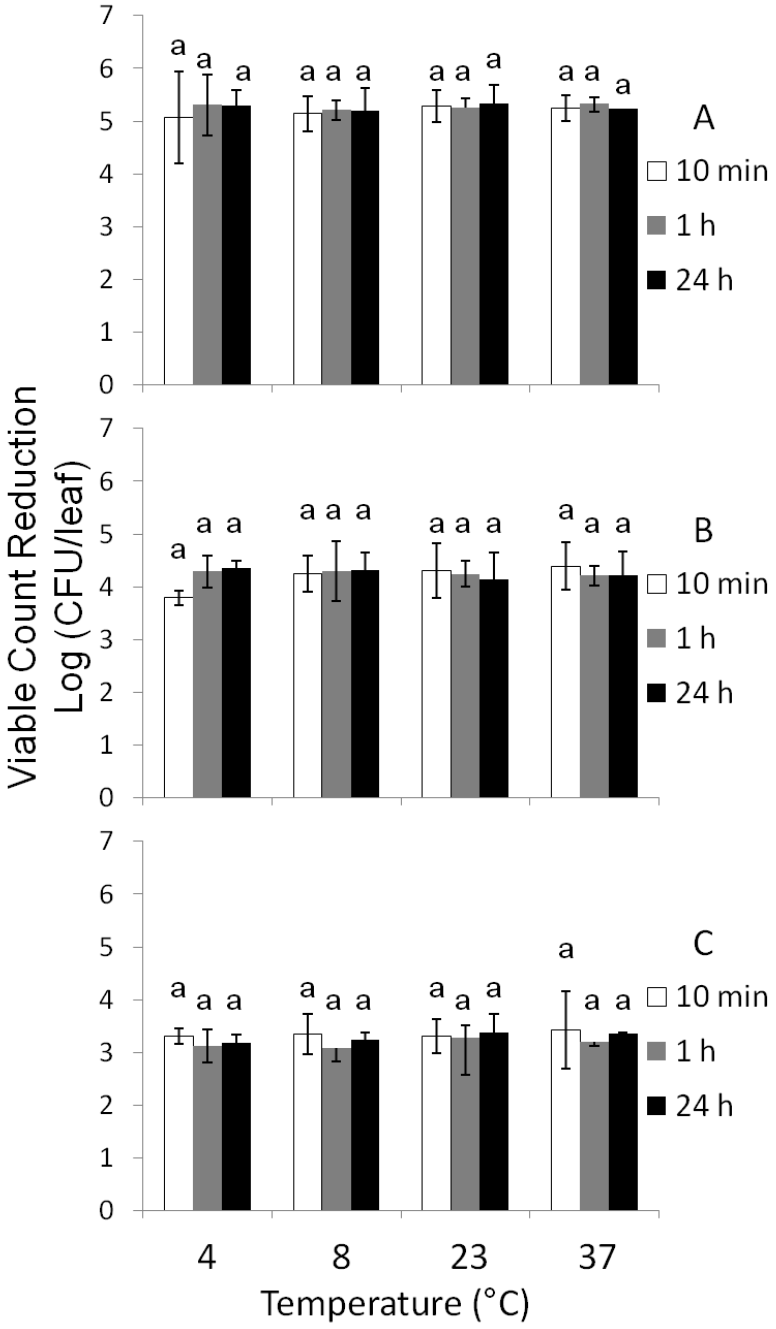

Figure 4.5. Effect of BEC8 bacteriophage mixture and 0.5% *trans*-cinnamaldehyde on *E. coli* O157:H7 cells dried on baby spinach leaves at a MOI of A) 1, B) 10, and C) 100.

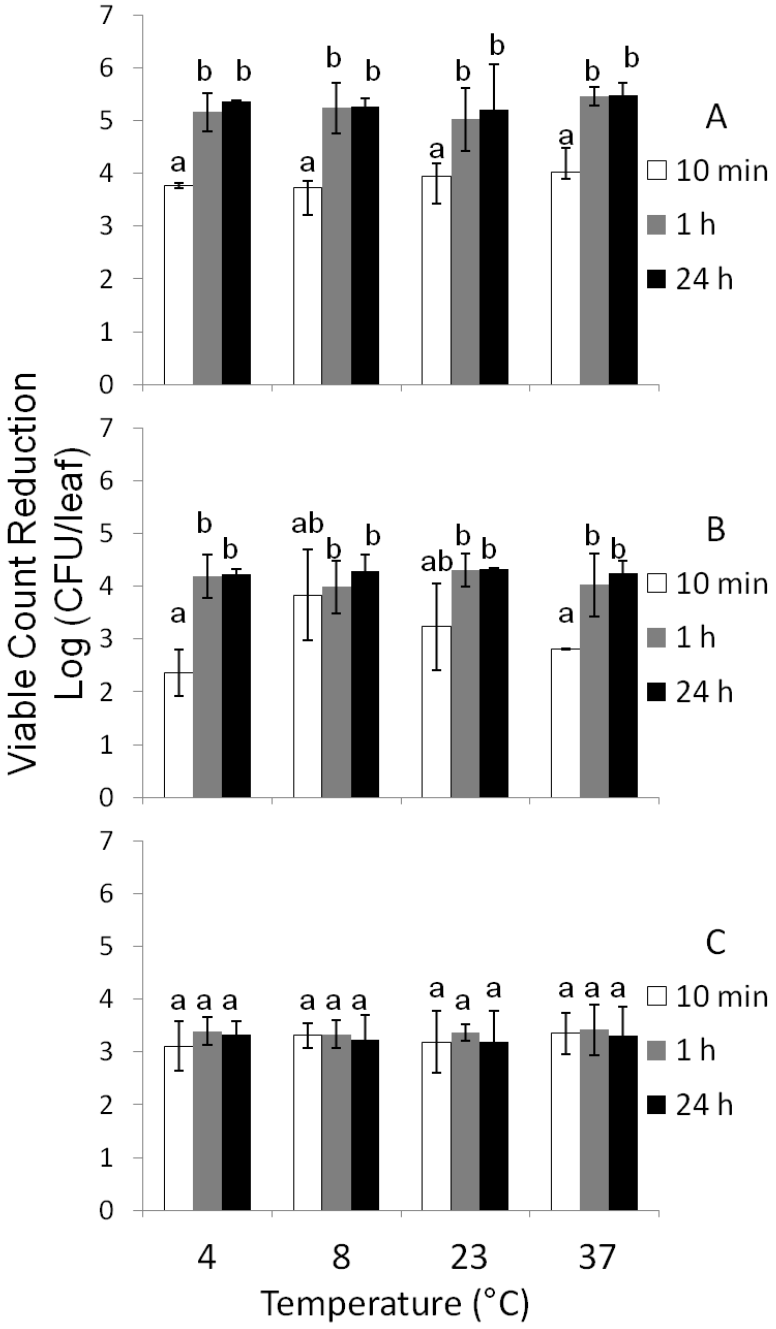

Figure 4.6. Effect of BEC8 bacteriophage mixture and 0.5% *trans*-cinnamaldehyde on *E. coli* O157:H7 cells dried on baby romaine lettuce leaves at a MOI of A) 1, B) 10, and C) 100.

CHAPTER 5

SUMMARY AND CONCLUSIONS

EHEC O157:H7 has been recognized as a major foodborne pathogen responsible for frequent gastroenteritis outbreaks. EHEC cause human infections that involve HC, other milder forms of diarrheal illness, and they are the major etiologic agent responsible for the HUS (144, 282). The incidence of foodborne illness associated with the consumption of minimally processed ready-to-eat salad vegetables has been consistently increasing (35, 188, 344). The increase of foodborne outbreaks due to the consumption of fresh vegetables stresses the importance of developing antimicrobial strategies to reduce their microbial load (7, 71, 160, 185, 232, 345, 353, 370). The most recent outbreaks of foodborne disease caused by bagged lettuce and spinach have exposed the limited effect of current conventional washes. The fresh produce industry as a whole would benefit from research on alternative effective antimicrobial treatments (71, 232). Unfortunately, due to the fresh characteristic of produce, the number of antimicrobial treatments is rather limited.

Chlorine is used under widely varying postharvest procedures (38). In general, the chlorine dosages (50–200 ppm) and contact times (1–2 min) used by produce processors generally result in 1–2 log CFU (90–99%) bacterial inactivation (74). Past research has indicated that organic fresh produce did not pose greater risk of contamination with foodborne pathogens and indicator organisms than conventional produce (254, 255). However, the greatest number of *E. coli*-positive samples in both organic and conventional produce has been found in leafy vegetables such as lettuce, spinach, kale

and collard. These results stress the importance of targeting high risk vegetables for developing interventions. Mixtures of bacteriophages with a wide spectrum of activity could potentially serve as one of such antimicrobial treatments when applied to fresh produce.

Organic agriculture is a production system that in principle bans the use of synthetic materials in food manufacture (202). Current regulations acknowledge the lack of available organic materials for many stages of production and have allowed the use of a limited number of non-organically produced substances (116, 289). Most synthetic sanitizers cannot be currently used in washing of organic produce because they are in direct contact with the product. Organic producers of fresh fruits and vegetables have almost no available effective sanitizers to wash fresh produce. The limited choice of approved sanitizers for organic food processing has forced most certification agencies to allow the use of unlisted synthetic sanitizers as long as they are rinsed with water before the equipment or surface can be in contact with food. The practice of rinsing sanitizers, not only can pose serious safety risks since it leads to product contamination, but it seriously compromises the principles of organic production systems. It is critical to identify and develop technologies that will not only be allowed by organic regulatory agencies, but that will be produced in accordance with organic regulations. One such potential solution is the use of bacteriophages.

Phages can be used as a natural antimicrobial method to reduce bacterial pathogens from the food supply. On August 18, 2006, the FDA announced the approval of the use of bacteriophage as an antimicrobial food additive targeting *Listeria*

monocytogenes on ready-to-eat (RTE) meat and poultry products (119). The use of bacteriophages to reduce the number of foodborne pathogen on various RTE foods has been examined with fresh-cut fruits and vegetables, such as fresh-cut melons and apples stored at various temperatures (218) and in combination with nisin (216), or on iceberg lettuce and cantaloupe (315).

The focus of this thesis was to develop a collection of lytic bacteriophages, specific to EHEC, and evaluate their ability to inactivate EHEC. Specifically, the collection of bacteriophages was screened to find the phages most effective against EHEC by using EOP and spot tests. Once the most effective phages were identified they were grouped together to form BEC8. BEC8 was applied on EHEC strains in pure culture and compared with the lytic ability of its individual phage-components and was found to be as or more effective. Next, the bacteriophage cocktail was applied on EHEC strains that were in dry and liquid form on hard surfaces commonly found in food processing environments. BEC8 was able to completely inactivate the EHEC mixture when used at high MOI. BEC8 was also applied on baby spinach and baby romaine lettuce against EHEC strain alone and in combination with TC. Both treatments were successful, particularly for low levels of the pathogen, but were highly effective when used in combination exhibiting a synergy that resulted in complete inactivation of the EHEC strains at high levels. The BEC8 cocktail was not affected by TC after 24 h of suspension.

The thesis was divided into five chapters. Chapter 1 provided introductory material, described the problem and stated the goals, hypotheses, and objectives of the study. Chapters 2, 3, and 4 were research studies with different objectives. These research

chapters include methods, results, and discussion on the results pertinent to each chapter. In this chapter, specific outcomes from each research chapter are highlighted, followed by overall conclusions and future perspectives.

Chapter 2: The objective of the second chapter was to isolate, identify and characterize a diverse collection of lytic bacteriophages capable of infecting EHEC serotypes O26, O111 and O157. Phage CEV2 and five other phages previously isolated were able to lyse all 14 O157 strains with EOP values consistently above 0.001. Two phages isolated from fecal slurry from dairy and feedlot cattle were highly effective against strains of *E. coli* O157 through EOP tests and against O26 through spot tests, but not O111. Bacterial challenges against high titers of four *E. coli* O157 strains suggested that a mixture of the 8 most effective phages was just as effective as or more than each individual phage. This collection of phages was grouped and labeled "BEC8". It was subsequently used as an antimicrobial cocktail to inactivate EHEC O157 in chapters 3 and 4.

Chapter 3: The objective of the third chapter was to determine the effect of the bacteriophage cocktail, BEC8, on the viability of a mixture of EHEC O157:H7 strains applied on surfaces of materials representative of food processing plants. No survivors were detected after BEC8 treatment at low inoculum level at the following incubation conditions: 37^{o}C for 10 min and RT after 1 h on SSC and CTC; 12^{o}C after 10 min on SSC, 1 h for CTC, and 24 h for HDPEC. These results indicated that the phage cocktail

was effective within an hour against low levels of the EHEC mixture at RT on all 3 hard surfaces.

Chapter 4: The objective of the fourth chapter was to determine the effect of the bacteriophage cocktail, BEC8, on its own and in combination with TC on the viability of a mixture of EHEC O157:H7 strains applied on whole baby romaine lettuce and baby spinach leaves. No survivors were detected when treated with BEC8 or TC separately at low inoculum level after 24 h at RT on lettuce and spinach. However, when the EHEC inoculum size increased or the incubation temperature decreased, the efficacy of BEC8 and TC decreased. When the two treatments were combined, no survivors were detected after 10 min at all temperatures on lettuce and spinach. These results indicated that the phage cocktail and TC combination was highly effective against EHEC on both leafy greens.

The findings of this study could assist in elucidating the use and application of bacteriophages against low levels of EHEC. Future studies could include using stationary-phase cells of EHEC O157 instead of exponential phase cells. Furthermore, a mixture of EHEC O26 could be inoculated on hard surfaces or leafy green vegetables and determine the effect of BEC8 on the survival of the pathogen. In addition, different susceptible serovars of *Salmonella* could be used in the same type of experiment. Other vegetables such as tomatoes or sprouts or fruit such as fresh cut apples or cantaloupe can be used as shown in similar studies. This is the first study to combine the use of TC and a bacteriophage cocktail against EHEC on leafy green vegetables. It would be of great

interest to determine the mode of action of the combined use of phages and the essential oil TC. Also, other natural antimicrobials or other essential oils such as carvacol or thymol could be used in combination with phages.

An additional surface typically found in food processing facilities is gypsum board that is used for ceilings and sometimes walls. This could be used to test a typical porous material and determine whether BEC8 and the BEC8/TC suspension would be effective. The BEC8 bacteriophage cocktail could also be used in combination with other bacteriophage cocktails specific for *Salmonella* or *Listeria* strains to investigate whether the resulting cocktail could be effective against a mixture of different bacterial pathogens. If the BEC8 bacteriophage cocktail is to be used in any real-world food application, then the genome of the individual phages would have to be sequenced. This would provide information on whether the phages are truly lytic or lysogenic and whether there are any other virulence factors besides the ones already tested for through the mPCR in chapter 2.

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APPENDIX A

INABILITY OF EXPONENTIALLY GROWING CELLS OF *ESCHERICHIA COLI* O157:H7 TO INDUCE GLUTAMATE-DEPENDANT ACID RESISTANCE AND SURVIVE PH 2 ACID SHOCK

A.1. Abstract

Stationary-phase *Escherichia coli* cells have a relatively high acid resistance (AR), but little is known on the ability of exponentially growing cultures to induce the glutamate decarboxylase (GAD)-dependent AR system. The purpose of this study was to determine the ability of log-phase *E. coli* O157:H7 to become AR and survive acid shock (AS) in glutamic acid media. We hypothesized that exposure to conditions typically observed with stationary cultures would induce AR in growing cells. Overnight cultures of *E. coli* O157:H7 ATCC 43895 and K-12 in tryptic soy broth (TSB) were diluted 100-fold into fresh TSB tubes and grown for 3 h at 37^{o}C. Aliquots of log-phase cells were transferred to media containing different compositions, pH and ox/redox conditions. After incubations from 0.5 to 3 h, cell suspensions were subjected to AS in pH 2 glutamic acid solutions for 1 h. Survival rates (SR) greater than 20% were observed for both strains of *E. coli* in the same TSB cultures grown overnight. Low SR (<0.1%) or no growth were observed for the conditions used. The inability of exponentially-growing cells to become AR may have potential implications for food safety.

A.2. Introduction

Acid resistance (AR) in *Escherichia coli* is defined as the ability to survive an acid shock (AS) of pH 3.0 or less for a few hours. Acid resistance has been typically observed in stationary-phase cells. *E. coli* possess three overlapping acid survival systems that may protect the stationary-phase cells under acidic environments (73). Three AR systems have been identified, the AR1 system, which relies on the general stress sigma factor RpoS, the AR3 arginine-dependent acid resistance system and the AR2 glutamate-dependent acid resistance system (72). The third and most effective AR system requires glutamate for protection. At pH 2.5, one of two genes encoding glutamate decarboxylase (*gadA* or *gadB*), and the gene encoding the putative glutamate:γ-aminobutyric acid antiporter (*gadC*) protect the cell (157, 351). At pH 2.0, both of the gad isozymes are necessary for survival (366). Stationary phase cells are more acid resistant compared to exponentially growing cells, but it is commonly accepted that log-phase cells can become acid induced by exposure to mild pH condition and eventually survive acid shock.

Studies conducted by Foster (120), show that once an AR system has been induced, *E. coli* O157:H7 can remain acid-resistant for at least a month during refrigeration. The ability of *E. coli* to survive acid exposure depends on the growth stage that they reach before acid challenge (208). In addition, after exposure to acidic condition, the survival rate is significantly lower for log-phase cells compared to stationary-phase cells under the same conditions (120, 208). Some genes expressed in stationary phase can cause the same gene expression as when *E. coli* is pre-exposed to

physical stress and can ultimately lead to protection from other stresses also known as cross protection (78, 181).

Aerobic log-phase cultures grown at pH 5.0 have been shown to be acid resistant, while their survival decreases 10- to 100-fold as the pH of growth is increased to pH 8.0. Extended growth in log phase also decreases acid resistance substantially (322). Furthermore, *gadA*, *gadBC*, and *gadE* genes were induced by low pH in growing cells incubated in minimal glucose media (229). Acidic anaerobic media induced acid resistance in *E. coli* cells, while a number of genetic systems are known to be co-induced by acid and anaerobiosis (276, 322). Adding $CdCl_2$ and ethanol to an exponentially growing culture of *E. coli* W3110 inhibited growth by 50% and shifted the growth curve to stationary phase, during which cells had increased AR (357). In most of these reports that observed increased acid resistance of log-phase cells, however, acid resistance was determined at pH values greater than 2.5.

The induction of *gadA* through cell exposure to *N*-acyl-L-homoserine lactone, was reported to result in an increased acid tolerance of *E. coli* in exponential phase, but the ability to survive acid conditions was assayed at pH 4.0 (355). Rowbury and Goodson reported that neutralized medium filtrates from *E. coli* grown to stationary phase at pH 5 induced acid tolerance in log phase cells growing at pH 7 (303). This latter study, however, subjected cultures to a pH of 3.0. Mates and coworkers recently suggested that compounds expelled by *E. coli* when reaching stationary phase could confer protection against an acid challenge to log-phase cells, however the data was not provided (236).

These studies supported the idea that log-phase cells could become acid resistant, but none of them demonstrated its occurrence at an extreme pH of 2.0.

We hypothesized that exposure to conditions typically observed with stationary cultures would induce the expression of acid resistance in exponentially growing cells of *E. coli* O157:H7 ATCC 43895. The objective of this study was to induce acid resistance in exponentially growing cells of *E. coli* O157:H7 ATCC 43895.

A.3. Methods

E. coli strain ATCC 43895 cells were exposed to various stresses and incubation conditions previously reported to induce some form of AR in stationary and log-phase cells. In addition, treatments were aimed at activating various cross protection mechanisms, SOS regulons, and RpoS-regulated genes, as well as simply shifting exponentially growing cultures to starvation conditions (Table 1). Cultures were incubated in tryptic soy broth (TSB) at 37^{o}C for 24 h, diluted 100-fold into fresh TSB tubes and grown for 3 h at 37^{o}C. Aliquots (0.1 mL) of log-phase cells were transferred to tubes containing 10 mL of different acid induction (AI) media, each with a different composition and pH. After incubations from 0.5 to 2 h, cell suspensions were subjected to acid shocks (AS) in pH 2.0 glutamic acid (6 mM) solutions for 1 h. Survivors were determined by dilution, plating, and comparing cell counts before and after AS. The AR-inducing media included: to 6.0, supernatant from stationary phase cultures in TSB and EC, anaerobic LB broth with glucose (LBG) at pH 4, M9 minimal media at pH 4, 5.5, and 6, and media containing different concentrations of ferric ammonium citrate, $MgCl_2$,

cumene hydroperoxide, ethanol, $CdCl_2$, and/or N-hexanoyl-L-homoserine lactone (HHL) (Table 1). *E. coli* K-12 strain W3110 cells were exposed only to the following conditions: peptone water (PW) with and without sodium acetate at pH 4.0, LBG with and without sodium acetate at pH 4, and M9 minimal media at pH 4, 5.5, and 6.

A.4. Results and Discussion

Overall, pre-incubation in AI media produced very low survival rates (SR) of exponentially growing cells after 1 h of AS (Tables 2-4). When exponential phase TSB-growing cells of *E. coli* O157:H7 were transferred into PW with sodium acetate at pH 4 and incubated at 37^{o}C for as long as 2 h, the survival rates after AS for 1 h were less than 0.1%. SR greater than 20% were observed in the same TSB cultures grown overnight that reached stationary phase. Relatively low SR (<0.6%) were also observed when pH 4 LBG was used. Addition of sodium acetate also resulted in SR of less than 0.6%. When using spent supernatant of stationary phase cells of *E. coli* ATCC 43895 as AI medium in TSB and EC broth at a range of pH (4-6), SR of less than 0.2% were observed. Using anaerobic media, minimal media, sub-inhibitory toxic compounds, Fe-Mg solutions, and HHL as AI media resulted in complete inactivation of the acid shocked cells in exponential phase regardless of concentration and pH used. Only exception to this observation was the use of 6 μM $CdCl_2$, which resulted in SR of 0.415 and 0.197% after incubating log phase cells for 0.5 and 1 h, respectively.

When exponential phase TSB-growing cells of *E. coli* K-12 were transferred into PW with and without sodium acetate and incubated at 37^{o}C for as long as 2 h, the

survival rates after AS for 1 h were at approximately 0.1%. SR greater than 30% were observed in the same TSB cultures grown overnight that reached stationary phase. When LBG was used the SR reached 1.69%, while when sodium acetate was added or M9 minimal media were used the SR were less than 1 and 0.2% respectively.

This study was part of a larger project aimed at developing a new detection method for non-O157 EHEC by taking advantage of the pathogen's acid resistance at pH 2 in the presence of glutamate compared to non-*E. coli* organisms. In an effort to shorten the detection method's duration, mid-exponential phase cells were subjected to acid inducing conditions. The acid induction media and approach employed in this study were not adequate in inducing AR in exponentially growing cells of *E. coli* O157:H7 ATCC 43895. The results indicate that acid sensitive *E. coli* O157:H7 could not readily become resistant to low pH and suggest that there may be additional factors regulating their acid resistance. Sudden transfer of log-phase cultures of *E. coli* O157:H7 to conditions that typically induce AR in stationary phase failed to enhance recovery rates after pH 2.0 acid shock in the presence of glutamate. These findings have potential implications on food safety. They suggest *E. coli* O157:H7 that survive the acidic environment of humans' stomachs are most likely in a stationary rather than exponential growth phase. Furthermore, previously described conditions that promote induction of acid tolerance in log-phase cells do not lead induction of acid resistance at pH 2 in the presence of glutamate.

It is commonly accepted that *E. coli* possess log-phase and stationary phase acid survival mechanisms (122, 302). Furthermore, this study provides new information on the

ability of exponential phase cells to undergo acid induction. Previous studies have shown that as long as exponential phase cells of K-12 and diarrheagenic strains of *E. coli*, including *E. coli* O157:H7, are grown in minimal media they can survive acid shock for several hours (39). However, our study found that 3 h of incubation of a 1:100 diluted stationary phase culture does not allow the organism to induce acid resistance mechanisms capable of survival at pH 2 in glutamic acid.

E. coli O157:H7 strain ATCC 43895, originally isolated from raw hamburger meat implicated in a hemorrhagic colitis outbreak (strain 933 (371)), was used throughout the study as used in numerous acid resistance studies (78, 97, 220, 291). This EHEC isolate was used as a model organism and has been previously demonstrated to undergo log phase acid resistance (17). Many of the past studies that discuss acid induction of log-phase cells have used *E. coli* strain K-12, however, the acid resistance of enterohemorrhagic and commensal strains of *E. coli* is similar when challenged in LBG broth at pH 2.0, despite some strain differences observed for individual systems (220). Similarly, in our study, the AS SR of the two *E. coli* strains used was similar under the AI conditions used. Interestingly, the SR was significantly higher when K-12 cells were treated with LBG and LBG with acetate compared to the other treatment conditions. However, they did not display SR typical of acid resistant strains as they were significantly lower than the SR of the overnight acid resistant cells of *E. coli* O157:H7 and K-12.

Both strains of *E. coli* were allowed to grow for 3 h to reach mid exponential phase and therefore their growth phase was not only dependant on diluting the stationary

phase culture. It is apparent from the results that RpoS and other stationary phase factors for acid resistance were either not present, or in minute concentrations since acid resistance was not achieved with any of the treatments. Throughout the studies used as a guide to test the acid inducing media in mid-exponential-phase cells the pH of the AS media was always higher than 2. In most studies the pH was 2.5, while in some it was either 3 or 4. This difference in the pH of the AS media could potentially explain the inability of the cells to survive such low pH levels.

Table A.1. Composition and pH of acid inducing (AI) treatments tested

Type of treatment	Composition/Concentration	pH Tested
Peptone water (PW)	With and without 10 mM sodium acetate	4, 5
Luria Bertani broth with 4 g l^{-1} Glucose (LBG):	With and without 10 mM sodium acetate	4
Anaerobic LBG (containing 0.6 g l^{-1} cysteine HCl, 10-5 g l^{-1} resazurin under O_2-free nitrogen)	With and without 10 mM sodium acetate	4
Spent supernatant from stationary phase cultures of same strain	Previously grown in tryptic soy broth and in EC broth	4, 4.5, 6
M9 minimal media	Standard formulation	4, 5.5, 6
Pyruvic acid solution	10 g l^{-1} sodium pyruvate	4, 5, 6.5
Sub-inhibitory toxic compounds	0.001, 0.01, 0.1% cumene hydroperoxide	6.5
	0.04, 0.4, 4% ethanol	6.5
	0.6 μM to 60 mM cadmium chloride	6.5
Fe-Mg salts	10 mM ferric ammonium citrate 10 mM magnesium chloride	4, 5, 5.7
Signal molecules	0.5 mM N-hexanoyl-L-homoserine lactone	4, 4.5, 5

Table A.2. Survival rate of exponentially growing *Escherichia coli* ATCC 43895 after a 1 h acid shock following incubation in different growth media previously reported to induce acid-resistance after reaching stationary phase (AI). Cells were grown in tryptic soy broth. Detection limit was 10 CFU/mL. Initial counts are shown as log CFU/mL.

Media	pH	Incubation time (h)					
		0.5		1		2	
		Initial	% Survival	Initial	% Survival	Initial	% Survival
PW	4	6.43	0.02±0.01	6.41	0.01±0.005	6.64	0.01±0.005
PW + acetate	4	6.21	0.05±0.0002	6.14	0.05±0.004	5.58	0.09±0.0006
LBG	4	6.61	0.49±0.33	6.43	0.29±0.27	6.42	0.56±0.49
LBG+ acetate	4	6.74	0.60±0.02	6.50	0.06±0.03	6.45	0.02±0.01
S.S. EC	4	N.T.	N.T.	6.9	N.S.	7.03	0.01
S.S. EC	4.5	N.T.	N.T.	6.71	N.S.	6.71	N.S.
S.S. EC	6	N.T.	N.T.	7.52	0.04	7.46	0.05
S.S. TSB	4	N.T.	N.T.	7.64	0.03	7.45	0.03
S.S. TSB	4.5	N.T.	N.T.	7.44	0.18	6.85	0.19
S.S. TSB	6	N.T.	N.T.	7.64	0.13	7.57	0.15
Anaerobic LBG	4	5.40	N.S.	5.39	N.S.	5.30	N.S.
Anaerobic LBG +acetate	4	5.35	N.S.	5.41	N.S.	5.40	N.S.
M9	4	N.T.	N.T.	5.17	N.S.	5.49	N.S.
M9	5.5	3.11	N.S.	3.50	N.S.	3.78	N.S.
M9	6	3.11	N.S.	3.46	N.S.	3.50	N.S.

N.S. = no survival above detection limit, N.T. = not tested
Media abbreviations: PW = peptone water, LBG= Luria-Bertani-glucose broth, S. S. = spent supernatant, EC= EC media, TSB= tryptic soy broth

Table A.3. Survival rate of exponentially growing *Escherichia coli* ATCC 43895 after a 1 h acid shock following incubation in solutions of individual compounds previously reported to induce acid-resistance (AI). Cells were grown in tryptic soy broth. Detection limit was 10 CFU/mL. Initial counts are shown as log CFU/ml.

Solutions	**pH**	**Incubation time (h)**					
		0.5		**1**		**2**	
		Initial	**% Survival**	**Initial**	**% Survival**	**Initial**	**% Survival**
PA 10 g/L	4	4.71	N.S.	4.73	N.S.	4.82	N.S.
PA 10 g/L	5	4.95	N.S.	5.01	N.S.	5.09	N.S.
PA 10 g/L	6.5	4.82	N.S.	4.99	N.S.	5.09	N.S.
CH 0.001%	6.5	4.61	N.S.	4.81	N.S.	4.62	N.S.
CH 0.01%	6.5	3.69	N.S.	2.73	N.S.	2.43	N.S.
CH 0.1%	6.5	N.D.	N.S.	N.D.	N.S.	N.D.	N.S.
Fe-Mg salts	4	4.59	N.S.	4.50	N.S.	4.54	N.S.
Fe-Mg salts	5	4.86	N.S.	4.99	N.S.	5.00	N.S.
Fe-Mg salts	5.7	4.77	N.S.	4.89	N.S.	4.84	N.S.
Ethanol 0.04%	6.5	4.85	N.S.	5.13	N.S.	5.23	N.S.
Ethanol 0.4%	6.5	4.90	N.S.	5.12	N.S.	5.17	N.S.
Ethanol 4%	6.5	4.69	N.S.	4.92	N.S.	4.95	N.S.
$CdCl_2$ 0.6 μM	6.5	4.79	N.S.	5.01	N.S.	5.19	N.S.
$CdCl_2$ 6 μM	6.5	4.79	0.415±0.05	4.80	0.197±0.05	4.78	N.S.
$CdCl_2$0.6 mM	6.5	2.74	N.S.	1.18	N.S.	N.S.	N.S.
$CdCl_2$ 60 mM	6.5	2.74	N.S.	2.22	N.S.	N.S.	N.S.
HHL	4	4.69	N.S.	4.97	N.S.	5.02	N.S.
HHL	4.5	4.80	N.S.	5	N.S.	5.21	N.S.
HHL	5	4.94	N.S.	5.03	N.S.	5.30	N.S.

N.S. = no survival above detection limit, N.T. = not tested
Chemical compound abbreviations: PA= pyruvic acid, CH=cumene hydroperoxide, HHL=N-hexanoyl-L-homoserine lactone

Table A.4. Survival rate of exponentially growing *Escherichia coli* K-12 after a 1 h acid shock following incubation in different growth media previously reported to induce acid-resistance (AI). Cells were grown in tryptic soy broth. Detection limit was 10 CFU/mL. Initial counts are shown as log CFU/ml.

Media	**pH**	**Incubation time (h)**					
		0.5		**1**		**2**	
		Initial	**% Survival**	**Initial**	**% Survival**	**Initial**	**% Survival**
PW	4	5.76	0.11±0.08	5.91	0.01±0.005	6.04	0.10±0.05
PW + acetate	4	5.82	0.12±0.04	5.82	0.07±0.02	5.98	0.09±0.006
LBG	4	6.43	1.62±0.6	6.03	3.45±0.44	6.12	1.69±0.50
LBG+ acetate	4	6.21	2.07±0.32	6.10	1.66±0.53	6.15	0.79±0.05
M9	4	5.17	0.24±0.05	6.40	N.S.	5.90	0.15±0.004
M9	5.5	5.49	1.41±0.42	6.22	0.03±0.0002	5.88	0.007±0.001
M9	6	5.58	0.01±0.002	6.14	0.03±0.0002	5.92	0.003±0.001

APPENDIX B

DETECTION OF ENTEROHEMORRHAGIC *ESCHERICHIA COLI* IN CLINICAL SAMPLES USING A CONVENTIONAL AND NOVEL CULTURE-BASED METHOD

B.1. Abstract

The performance of the Selective Acid Shock (SAS) protocol was compared to a conventional method for detection of EHEC for a selected mixture of clinical samples. The two detection methods were not significantly different in EHEC recovery. An EHEC isolate was always obtained from the SAS protocol, unlike the conventional protocol.

B.2. Introduction

Most pathogenic strains of *Escherichia coli* behave like any other non-pathogenic *E. coli* from a biochemical and ecological standpoint. This makes their detection among commensal *E. coli* an important problem, especially for Shiga toxin-producing *E. coli* (STEC) and enterohemorrhagic *E. coli* (EHEC) (33). After the first outbreak in 1982, *E. coli* O157:H7, the most widely known EHEC strain, was found unable to ferment sorbitol (107, 144, 298, 342), providing a phenotypic characteristic that led to the effective detection of the pathogen. Non-O157 serotypes lack any obvious phenotypic characteristic that would allow for differentiation from commensal *E. coli* and therefore, no standard reliable method exists to isolate, characterize, and identify these pathogens. Unfortunately, they are only found and reported by the most enthusiastic laboratories since very few clinical laboratories actively screen for them (33). Development of rapid,

cost-effective diagnostic methods are of critical importance due to the pathophysiological changes of HUS that occur by the time that the patient suffers from diarrhea (184). Currently, no selective culture medium exists for isolation of non-O157 STEC. To this end, the assessment of the effectiveness of novel detection methods is of great importance as it has the potential of improving the isolation of EHEC.

The selective acid shock (SAS) protocol was recently developed by combining the use of glutamic acid shock (GAS) at pH 2 and plating on sorbitol-MacConkey (SMAC) and rhamnose-MacConkey (RMAC) agar plates containing potassium tellurite and ceftazidime (204). The sensitivity and specificity of this method in detecting *E. coli* O157 in ground beef was comparable to a USDA-approved method. The Minnesota Department of Health (MDH) clinical microbiology laboratory routinely tests all stool cultures received for EHEC, using sorbitol-MacConkey (SMAC) agar, followed by a multiplex-PCR performed on a mixture of bacteria from the plate, used to identify virulence factors typical of EHEC strains as described by Paton and Paton (283). Once the mixture is identified as positive, then the mixture of bacteria is re-streaked onto SMAC and isolated colonies are tested again through the multiplex-PCR.

We hypothesized that the incorporation of acid shock and novel selective agents employed in the SAS protocol can enhance the recovery rate of non-O157 EHEC. The objective of this study was to determine if the rate of isolation of EHEC from mixed clinical specimens obtained from the MDH can be enhanced using the SAS protocol.

B.3. Materials and Methods

Samples were obtained from the MDH and included all Minnesota residents that have had EHEC symptoms and subsequently had their stool culture forwarded to the MDH. This included portions of all SMAC plates received from January of 2007 until June of 2008. They belonged to the following categories: a) presumptive positive and positive isolation, b) presumptive positive, but negative isolation, and c) presumptive negative and negative isolation, but from patients that have had suspected EHEC symptoms.

In collaboration with the Health Partners Center Care laboratory, the MDH routinely receives fecal specimens that have been tested positive for shiga toxin by EIA, commonly used by clinical laboratories. Furthermore, the MDH also collaborates with CentraCare, which provided the majority of the samples in this study. Patients with EHEC symptoms placed their stool samples in ParaPak^{TM} transport containers with Cary-Blair and after streaking on SMAC plates, they were sent to the MDH. The microbial growth on the SMAC plates was mixed to provide a homogeneous mixture defined as a "sweep". A sweep of the microbial growth was then suspended in 200 µl of molecular grade water, followed by boiling for 15 minutes. A portion of the sweep was also stored in trypticase soy broth and 50% glycerol at -80^{o}C for archiving. The samples were then subjected to multiplex-PCR as conducted previously (283) (Table 1). Each extract was amplified in 25-µl reaction mixtures with 0.650 units of AmpliTaq Gold (Table 2). Samples were subjected to 35 PCR cycles, each consisting of 1 min of denaturation at 95^{o}C; 2 min of annealing at 65^{o}C for the first 10 cycles, decrementing to 60^{o}C by cycle 15; and 1.5 min of elongation at 72^{o}C, incrementing to 2.5 min from cycles 25 to 35. The

PCR reaction mixtures were electrophoresed on 2% agarose gels and stained with ethidium bromide (282). If a sweep was found to be positive for any shiga toxin genes, then the mixture was re-streaked on SMAC and isolated colonies were tested again with multiplex-PCR.

All samples were blinded from the Food Safety Laboratory investigators. An equivalent sample size from the same sweep was evaluated by the MDH to allow for comparison to the novel detection method. The specimens were received in Styrofoam boxes with dry ice, and subsequently stored frozen (-20^{o}C). A loop-full of the mixed sweep samples were transferred to EC broth and incubated overnight (18-24h) at 37^{o}C. Aliquots (0.1ml) of the culture were transferred to 10 ml of Glutamic Acid pH 2 solution (GAS) for 2h at 37^{o}C, followed by plating on Selective Rhamnose MaConkey agar (S-RMAC) and Selective Sorbitol MaConkey agar (S-SMAC), which were incubated at 37^{o}C overnight. Up to 5 colonies from each plate were transferred onto TSA plates and subjected to multiplex PCR as described above. After processing, the information was sent to the MDH and all isolates that were found shiga toxin positive from the SAS protocol were streaked on TSA plates and submitted to the MDH for verification through multiplex-PCR as described above. This served as a quality control check and verification process.

Statistical comparison of recovery rates between the two methods was done using the chi-square test to determine significant differences ($p<0.05$). Sensitivity and specificity were calculated for each method by assigning the conventional method used

by the MDH as the reference standard method and using the SAS protocol as the reference method for the MDH method.

B.4. Results and Discussion

The SAS protocol has been shown to perform as well as the current USDA-approved method on ground beef samples (204). In this study we sought out to evaluate its performance on clinical samples and compare the findings to the conventional method used by the MDH. The overall results of the study are summarized in Table 3. Overall, the SAS protocol detected 15 less positive samples than the MDH-approved method. When using the MDH methods as a reference test, the sensitivity and specificity of the SAS protocol were found to be 85 and 94%, respectively, while the positive and negative predictive values were found to be 96 and 80%, respectively (Table 4). When using the SAS protocol as a reference method, the MDH methods' sensitivity and specificity were found to be 96 and 80%, respectively, while the positive and negative predictive values were found to be 96 and 94%, respectively (Table 5). Statistical analysis (chi-square test) showed that the recovery rates of the two methods were not significantly different ($p<0.05$).

Of the 208 samples processed, 19 samples were found positive by the MDH and were not recovered by the SAS protocol. Of these 19 positive samples, 5 (26.3% of missed positives) did not grow in EC broth after 24 hrs using the SAS protocol. The SAS protocol found 5 samples positive that were not recovered by the MDH. The MDH method was unable to recover an isolate for 18 (18.6% of positive samples) of the 97

samples that produced a positive PCR. The SAS protocol was able to provide an isolate from the samples it detected as positive at a rate of 100%.

Obtaining colony suspensions from the MDH that had previously been stored frozen; transporting them to the facilities at the UMN, storing them frozen there, and then processing them for detection may have had detrimental effects to the survival of EHEC. The specimen quality can be affected by the duration of storage (63), while freeze thaw cycles can lower the titer of the stock solution. This may partially explain the lower -yet not significantly different- recovery rate or the SAS protocol compared to the MDH methods used. On average, the SAS protocol takes about 42 hours to confirm the presence of EHEC virulence factors through mPCR on a recovered isolate. Conventional methods, such as the one used by the MDH takes up to 30 hours to confirm the presence of EHEC virulence factors on a mixture of bacteria able to grow on SMAC media, followed by additional 30 hours to recover an isolate and confirm its virulence through mPCR. The SAS protocol is shorter by 18 hours and provides an isolate 100% of the time. Furthermore, it does not require an extra multiplex-PCR step providing a cheaper, faster alternative to the conventional methods used for the isolation of EHEC. These findings suggest that further research is needed to confirm the ability of the SAS method to improve shiga-toxin producing *E. coli* recovery in clinical samples.

B.5. Conclusions

This novel detection method was found to be as effective as the conventional methods used by the MDH at isolating EHEC. Furthermore, an isolate was recovered

100% of the time using the novel detection method. However, a study in which identical initial samples are tested using both methods would provide important information on whether the novel detection method can recover isolates not recovered by the conventional method.

Table B.1. Primers used in multiplex-PCR

Primer Name	Sequence	Amplicon size (bp)
Stx 1 F	5’ ATA AAT CGC CAT TCG TTG ACT AC 3’	180
Stx 1 R	5’ AGA ACG CCC ACT GAG ATC ATC 3’	
Stx 2 F	5’ GGC ACT GTC TGA AAC TGC TCC 3’	255
Stx 2 R	5’ TCG CCA GTT ATC TGA CAT TCT G 3’	
eaeA F	5’ GAC CCG GCA CAA GCA TAA GC 3’	384
eaeA R	5’ CCA CCT GCA GCA ACA AGA GG 3’	
hlyA F	5’ GCA TCA TCA AGC GTA CGT TCC 3’	534
hlyA R	5’ AAT GAG CCA AGC TGG TTA AGC T 3’	

Table B.2. Concentration of PCR reagents

Component	µl/ reaction	Final Concentration
Sterile water	14.12	
10x PCR Buffer w/o $MgCl_2$	2.5	1x
$MgCl_2$, 25mM	3.5	3.5mM
dNTPs 12.5mM w/ dUTP	2.0	0.2 mM each dATP, dCTP, dGTP; 0.4 mM dUTP
Stx 1 Primer set, 20 µM	0.125	100 nM
Stx 2 Primer set, 20 µM	0.125	100 nM
eaeA Primer set, 20 µM	0.250	200 nM
hlyA Primer set, 20 µM	0.250	200 nM
AmpliTaq Gold, 5U/µl	0.13	0.650 units
Volume	23.00	

Table B.3. Summary of results

Method	Total	Positive	Negative	Negative from MDH found Positive by SAS	Positive from MDH not recovered by SAS
SAS	208	97	111	5	19
MDH	208	112	96		

Table B.4. Performance of SAS protocol compared to conventional method used by MDH[a]

Test under evaluation: SAS	Reference Test (MDH) Positive	Negative	Total	Sensitivity[a] (%)	Specificity (%)
Positive	107	5	112	85	94
Negative	19	77	96		
Total	126	82	208		

[a]Sensitivity: 107/(107+19) = 84.92%, specificity: 77/(77+5) = 93.90%, positive predictive value = 107/(107+5) = 95.54%, negative predictive value = 77/(77+19) = 80.21%

Table B.5. Performance of MDH conventional method compared to SAS protocol

Test under evaluation: MDH	Reference Test (SAS) Positive	Negative	Total	Sensitivity[a] (%)	Specificity (%)
Positive	107	19	126	91	80
Negative	5	77	82		
Total	112	96	208		

[a]Sensitivity: 107/(107+5) = 95.53%, specificity: 77/(77+19) = 80.20, positive predictive value: 112/(112+5) = 95.72%, negative predictive value: 77/(77+5) = 93.90%

Table B.6. Results of samples analyzed by SAS protocol and conventional MDH methods

Sample	SAS +/-	SAS serotype	MDH +/-	MDH serotype
I 2006 5329	+	N/A	+ N.I.	N/A
I 2006 5393	+	N/A	+	O1:H20
5454	+	N/A	+	OKO103
5538	+	O26	+	O26:H11
5517	+	N/A	+	UND:H2
I 2007 121	-	N.N.	+ N.I.	N/A
440	+	N/A	+ N.I.	N/A
462	+	N/A	+	O157:H7
517	+	O26	+ N.I.	N/A
649	+	O111	+	O111:NM
905	+	N/A	+	O124:NM
968	-(DNG)*		+ N.I.	N/A
1169	-		-	-
1268	-		-	-
1270	+	N/A	+ N.I.	N/A
1273	+	N/A	+	O157
1321	-		-	-
1369	-		-	-
1375	+	N/A	-	-
1420	-		-	-
1441	+	O157	+	O26:H11

1473	-		-	-
1525	+	N/A	+	O121:H19
1529	-	N.N.	+ N.I.	N/A
1537	-		-	-
1547	+	N/A	+	OUND:HUND
1560	-		-	-
1566	-	N.N.	+	OKO103:NM
1569	-	-	-	-
1573	+	N/A	+	O157:H7
1672	-		-	-
1675	-		-	-
1682	+	N/A	+	OKO103:H2
1683	+	N/A	+	O157:H7
1692	-		-	-
1694	-		-	-
1696	+	N/A	+	O143:NM
1723	-		-	-
1727	-		-	-
1767	-		-	-
1768	-		-	-
1790	+	N/A	+	O157:H7
1855	+	N/A	-	N.N.
1858	+	O157:NM	+	O157:H7

1859	-	N/A	+	OKO111:NM
1914	-	N.N.	-	N.N.
1919	+	N/A	+	O157:H7
1920	+	N/A	+	O157:H7
1935	-		+ N.I.	N/A
2013	-	-	-	-
2023	-		+	N.N.
2026	-	N/A	+	N/A
2027	+	N/A	-	N.N.
2091	-	-	+ N.I.	N/A
2102	+	N/A	+	O157
2103	+	O157	+	O157
2104	+	O157	+	O157
2105	+	O157	+	O157
2106	+	O157	+	O157
2107	+	N/A	+	O157
2178	-		+	OUND:HUND
2192	+	N/A	+	OKO103:H2
2236	+	N/A	-	-
2238	-		-	-
2246	+	N/A	+	O111:NM
2247	-		-	-
2248	-		-	-

2249	-		-	-
2253	-		-	-
2326	+	N/A	+ N.I.	N/A
2342	-		-	-
2348	-		-	-
2365	-		-	-
2366	-		-	-
2379	+	N/A	+	O111:NM
2398	-	N/A	+ N.I.	N/A
2401	+	N/A	+ N.I.	N/A
2412	-		-	-
2419	-		-	-
2420	-		-	-
2462	+	N/A	+	OKO111:NM
2470	-	N.N.	-	N.N.
2475	-		-	-
2494	+	O157	+	O157:H7
2495	-	N.N.	-	N.N.
2500	-		-	-
2503	-		-	-
2511	-		-	-
2521	+	N/A	+	OKO121:H19
2522	-		-	-

2524	-		-	-
2525	+	N/A	+	OKO121:H19
2527	-	N.N.	-	-
2534	-		-	-
2539	-		-	-
2541	-(DNG)		+ N.I.	N/A
2548	-		-	-
2553	+	O157	+	O157:H7
2567	+	N/A	+	OKO103:HUND
2569	+	N/A	+	O157:H7
2577	+	N/A	+	O157:H7
2584	+	N/A	+	OKO26:NM
2585	-	N.N.	-	N.N.
2617	-		-	-
2623	+	N/A	+	OKO103:HUND
2631	+	O157	+	O157:H7
2640	+	N/A	+	O26:NM
2641	+	N/A	+	N/A
2644	+	O157	+	O157:H7
2648	+	N/A	+	N/A
2676	-		-	-
2680	-		-	-
2683	-		-	-

2691	-		-	-
2697	-		-	-
2707	+	N/A	+	O157:H7
2708	+	O157	+	O157:H7
2704	-	N/A	-	-
2720	-		-	-
2732	+	N/A	+	N/A
2742	-		-	-
2758	+	N/A	+	N/A
2845	+	N/A	+	N/A
2877	+	N/A	+	O157:H7
2781	-	-	+ N.I.	N/A
2782	-		-	-
2805	-		-	-
2810	-		-	-
2845	-	-	+ N.I.	N/A
2877	-		+	O157:H7
2944	+	N/A	+	N/A
2979	+	N/A	+	O157:H7
3012	+	N/A	+	N/A
3018	+	N/A	+	N/A
3039	+	N/A	+	N/A
3040	+	N/A	+	N/A

3044	+	N/A	+	N/A
3059	+	N/A	+	N/A
3087	+	N/A	+	N/A
3129	+	N/A	+	N/A
3159	-		-	-
3185	-		-	-
3217	-		-	-
3218	+	N/A	+	N/A
3224	-	N/A	-	N/A
3241	+	N/A	+	N/A
3283	-		-	-
3347	-		-	-
3351	-		-	-
3396	+	N/A	+	N/A
2986	-		-	-
3132	+	O157	+	O157:H7
3157	+	O157	+	O157:H7
3218	+	O157	+	O157
3220	+	O157	+	O157:H7
3264	+	O157	+	O157:H7
3419	+	O157	+	O157:H7
3430	+	N/A	+	O26:H11
3436	-	-	-	-

3448	-	N.N.	-	-
3464	-	N.N.	-	N.N.
3492	-(DNG)		-	-
3502	-(DNG)		-	-
3672	-	-	-	-
3677	+	O157	+	OKO103:H2
3703	+	N/A	+	O26:H11
3714	-		-	-.
3724	-	N.N.	-	N.N.
3725	-		+	Orough:H19
3763	+	O111	+ N.I.	N/A
3771	+	O111	+	O111:NM
3788	+	O26	+	O26:H11
3789	+	O157	+	O157:H7
3803	-(DNG)		-	-
3810	+	O157	+	O157:H7
3811	+	O157	+	O157:H7
3839	-(DNG)		-	-
3941	-		-	-
3949	+	O26	+	OKO26
3957	+	O111	+	OKO111:NM
3962	+	N/A	+	OUND
4000	-	N.N.	-	N.N.

4033	-	-	-	-
4050	-	-	-	-
4055	-(DNG)		+ N.I.	N.N.
4063	-		-	-
4110	-		-	-
5206	-(DNG)	(orig 2236 resent)	-	-
5301	-(DNG)	(orig 1375 resent)	-	-
5507	-	-	-	-
2007 4133	-	N.N.	+ N.I.	N/A17
2008 0003	+	O157	+	O157:H7
38	-(DNG)		+	O157:H7
143	-(DNG)		-	N.N.
184	+	O157	+	O111
190	-(DNG)		+	Orough
246	-(DNG)		-	N.N.
281	+	N/A	+	O103:H2
332	-	-	-	-
517	-		-	-
547	-(DNG)		-	N.N.
569	+	N/A	+	O26:NM
594	-(DNG)		-	-
606	+	N/A	-	N.N.
642	-(DNG)	-	-	-

650	-(DNG)	-	-	-
687	+	N/A	+	O26
696	+	N/A	+	O103
Totals (208)	(+): 97	(-): 111	(+): 112	(-): 96

*DNG: Did not grow, N/A: Not available, N.N.: Not needed (isolate did not have stx marker), N.I.: no isolate

APPENDIX C
Using bacteriophages to control EHEC data

C.1. Bacteriophage host range determined using the efficiency of plating method

Strains/ phages	EOP ratio							
	38	**39**	**41**	**AR1**	**42**	**CEV2**	**ECB7**	**ECA1**
ATCC 43895	0.34	0.5	0.3	0.7	0.49	0.6	0.009	Host
NCTC 12900	0.5	0.8	0.6	0.9	1.2	Host	Host	3.7
O26	0.7	0.05	0.7	0.4	0.03	0.9	0.0	0.44
6058	0.7	0.5	0.7	1.6	0.6	0.46	1.9	1.1
4477	0.38	3.8	0.7	1.2	0.18	0.49	0.8	6.2
2336	1.2	0.03	0.003	1.4	0.3	0.09	0.007	0.3
2309	0.46	0.18	0.67	1.8	7.1	0.28	1.9	0.4
2027	0.01	0.01	0.62	0.8	0.5	0.45	1.3	0.8
2321	0.006	0.06	3.6	1.3	7.1	0.8	1.0	0.4
M4882	0.03	0.49	0.49	0.9	0.9	0.47	1.9	0.3
M4489	0.02	0.04	3.4	0.7	4.6	0.28	2.1	0.8
M4522	9.7	4.1	1.6	1.4	1.0	0.7	0.48	0.3
EK1	0.8	0.9	0.9	0.38	0.4	0.48	0.006	0.7
EK27	0.36	0.16	1.3	0.9	4.5	1.2	4.3	1.1

C.2. Effect of individual phages and of BEC8 on exponential-phase cells *E. coli* O157:H7 ATCC 43895 in TSB at a MOI of 100 after 5 h of incubation at 23 and 37°C.

Phage	**Inactivation at 37°C (log CFU/mL)**	**Inactivation at 23°C (log CFU/mL)**
CEV2	5.58±0.33	0.67±0.55
AR1	5.35±0.07	0.63±0.06
42	3.91±0.44	0.79±0.17
41	5.23±0.04	0.52±0.04
38	4.99±0.30	1.06±0.67
39	5.05±0.21	1.19±0.56
ECA1	5.60±0.27	0.59±0.19
ECB7	4.59±0.14	1.51±0.13
BEC8	5.53±0.07	0.91±0.07

C.3. Effect of individual phages and of BEC8 on exponential-phase cells *E. coli* O157:H7 EK1 in TSB at a MOI of 100 after 5 h of incubation at 23 and 37°C.

Phage	**Inactivation at 37°C (log CFU/mL)**	**Inactivation at 23°C (log CFU/mL)**
CEV2	1.42±0.68	2.27±0.46
AR1	1.28±0.76	1.79±0.09
42	1.69±0.77	0.86±0.08
41	3.28±0.98	4.91±0.49
38	1.61±1.11	4.26±0.01
39	5.75±0.21	3.04±0.26
ECA1	2.01±0.15	4.32±0.10
ECB7	2.22±0.33	2.37±0.41
BEC8	5.06±0.09	3.31±0.06

C. 4. Effect of individual phages and of BEC8 on exponential-phase cells *E. coli* O157:H7 EK27 in TSB at a MOI of 100 after 5 h of incubation at 23 and 37°C.

Phage	**Inactivation at 37°C (log CFU/mL)**	**Inactivation at 23°C (log CFU/mL)**
CEV2	5.17±0.12	1.28±0.33
AR1	3.28±0.49	4.02±0.14
42	3.13±0.46	3.31±0.52
41	2.90±0.42	2.90±0.27
38	0.83±0.27	5.12±1.28
39	3.53±0.99	3.52±0.22
ECA1	2.67±0.79	2.46±0.22
ECB7	2.58±0.01	3.41±0.05
BEC8	4.15±0.04	3.09±0.58

C.5. Effect of individual phages and of BEC8 on exponential-phase cells *E. coli* O157:H7 472 in TSB at a MOI of 100 after 5 h of incubation at 23 and 37°C.

Phage	**Inactivation at 37°C (log CFU/mL)**	**Inactivation at 23°C (log CFU/mL)**
CEV2	2.25±0.83	2.24±0.68
AR1	2.10±0.06	1.58±0.12
42	1.95±0.16	0.69±0.21
41	3.07±0.23	3.16±0.16
38	4.98±0.75	3.11±0.23
39	5.65±0.26	3.16±0.34
ECA1	1.91±0.80	3.16±0.60
ECB7	3.33±0.17	4.38±0.21
BEC8	5.28±0.12	2.59±0.83

C. 6. Mean time and 95% CL for a 5-log reduction (limit of detection) of dry and liquid (positive control) *E. coli* O157:H7 strains inactivated by bacteriophage cocktail BEC8 on three surface chips. Bacterial mixture included strains I 2005003658-472, TWO8635-EK27, and ATCC 43895

Surface materials	**Temp. (°C)**	**Liquid cells**			**Dry cells**		
		Time to 5-log reduction (min)	**Lower**	**Upper**	**Time to 5-log reduction (min)**	**Lower**	**Upper**
SSC	12	189 aA*	152	254	205 aA	164	281
	23	99 bA	79	135	135 bA	117	161
	30	20 cA	16	25	37 cB	28	56
	37	23 cA	20	28	26 cA	22	33
CTC	12	130 abA	101	192	190 abA	138	331
	23	85 abA	67	123	110 abA	89	153
	30	23 cA	13	16	25 cB	19	42
	37	15 cA	12	22	20 cA	16	31
HDPEC	12	168 abA	122	290	196 abA	147	306
	23	101 bA	80	141	129 abA	110	156
	30	22 cA	17	34	28 cA	20	52
	37	21 cA	15	37	27 cA	20	45

*D-values with the same lower case and upper case letter are not significantly different within the same column and row, respectively.

C.7. D-values of low levels of dry and liquid cells of *E. coli* O157:H7 strains inoculated on stainless steel chips at 12, 23, 30, and 37°C inactivated by BEC8 at a MOI of 100.

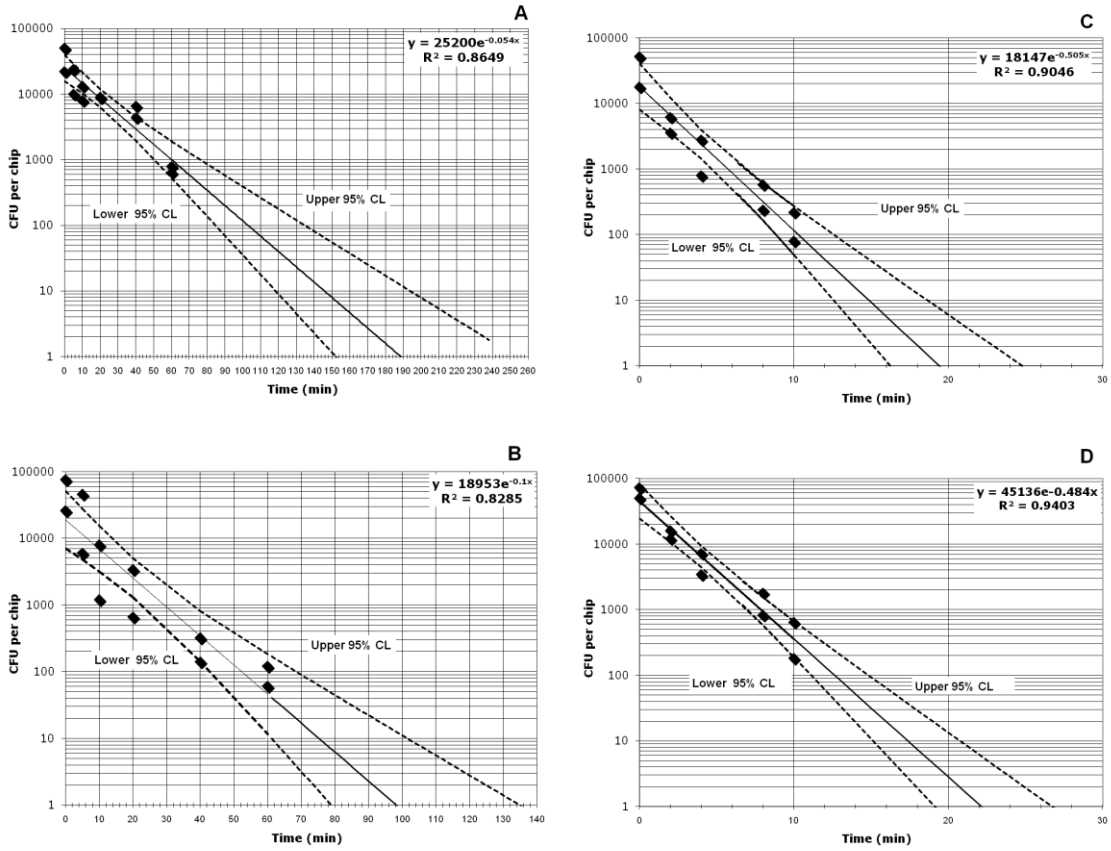
Temp. (°C)	Time (min)	CN+ Survival (CFU) Rep. 1	CN+ Survival (CFU) Rep. 2	D-value (min)	BEC8 Survival (CFU) Rep. 1	BEC8 Survival (CFU) Rep. 2	D-value (min)
12	0	50000	22000	33.5	34000	32000	46.0
	5	24000	10000		28000	8000	
	10	8000	13200		14000	13800	
	20	9000	8800		12800	10400	
	40	4400	6600		6600	7200	
	60	800	640		860	980	
23	0	76000	26000	17.4	50000	22000	30.0
	5	46000	6000		42000	14000	
	10	8000	1200		14000	7800	
	20	3400	660		6800	5600	
	40	320	140		3000	800	
	60	120	60		280	320	
30	0	52000	18000	3.9	24000	28000	8.8
	2	6200	3600		8000	4000	
	4	2800	800		5600	4800	
	8	580	240		3400	3200	
	10	220	80		800	1200	
37	0	50000	72000	4.7	36000	42000	5.9
	2	16000	12000		8000	6000	
	4	7200	3400		8600	3200	
	8	1800	820		1400	800	
	10	640	180		880	380	

C.8. D-values of low levels of dry and liquid cells of *E. coli* O157:H7 strains inoculated on ceramic tile chips at 12, 23, 30, and 37°C inactivated by BEC8 at a MOI of 100.

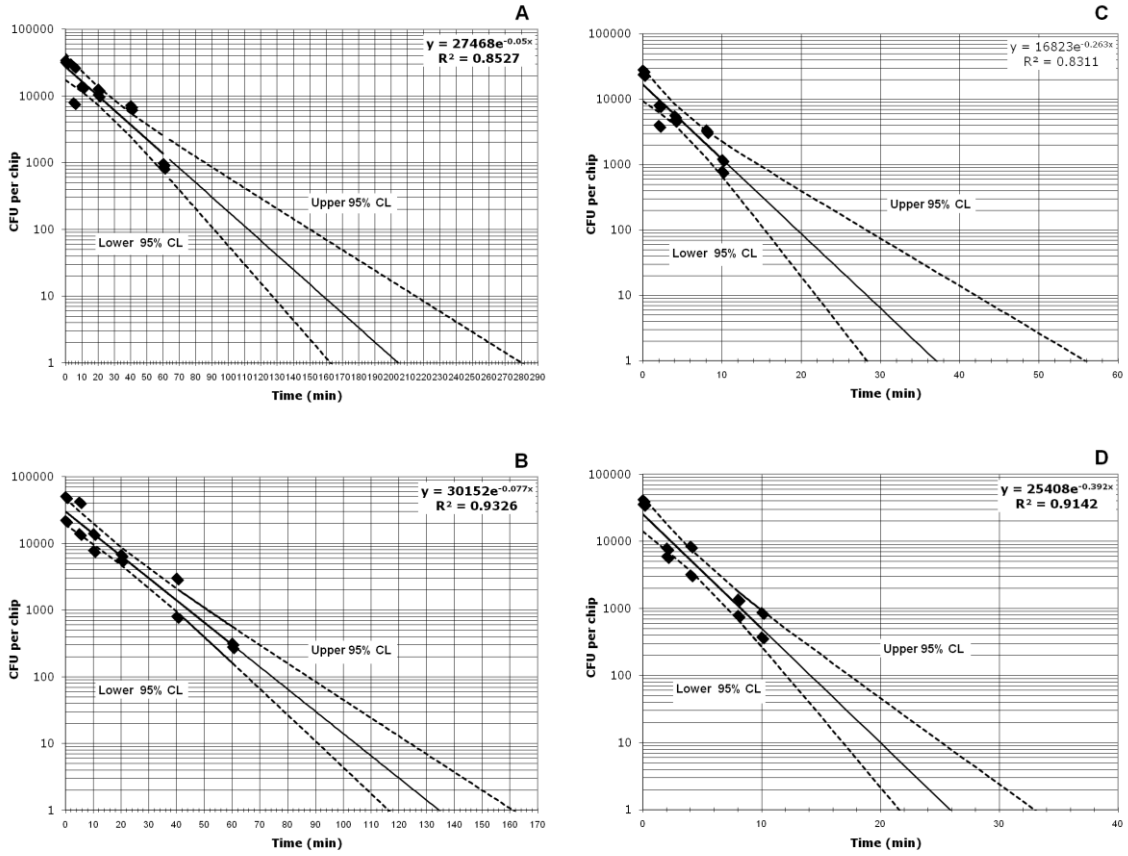
Temp. (°C)	Time (min)	CN+ Survival (log CFU) Rep. 1	CN+ Survival (log CFU) Rep. 2	D-value (min)	BEC8 Survival (log CFU) Rep. 1	BEC8 Survival (log CFU) Rep. 2	D-value (min)
12	0	40000	32000	32.6	12000	40000	46.7
	5	5800	5200		5000	10000	
	10	3000	1400		2800	8800	
	20	1600	800		1800	6600	
	40	520	420		1200	5200	
	60	240	200		400	780	
23	0	45000	38000	21.3	8000	24000	29.3
	5	3000	8000		5000	6800	
	10	2000	6400		1800	920	
	20	80	280		600	660	
	40	60	140		140	340	
	60	20	60		40	180	
30	0	16000	98000	3.8	14000	8000	4.6
	2	800	7400		2800	2400	
	4	400	2000		1600	400	
	8	520	240		800	380	
	10	40	20		540	80	
37	0	68000	12000	3.9	14000	12000	5.1
	2	1800	1400		4000	1400	
	4	280	180		2200	380	
	8	180	120		800	80	
	10	20	40		220	40	

C.9. D-values of low levels of dry and liquid cells of *E. coli* O157:H7 strains inoculated on high density of polyethylene chips at 12, 23, 30, and 37°C inactivated by BEC8 at a MOI of 100.

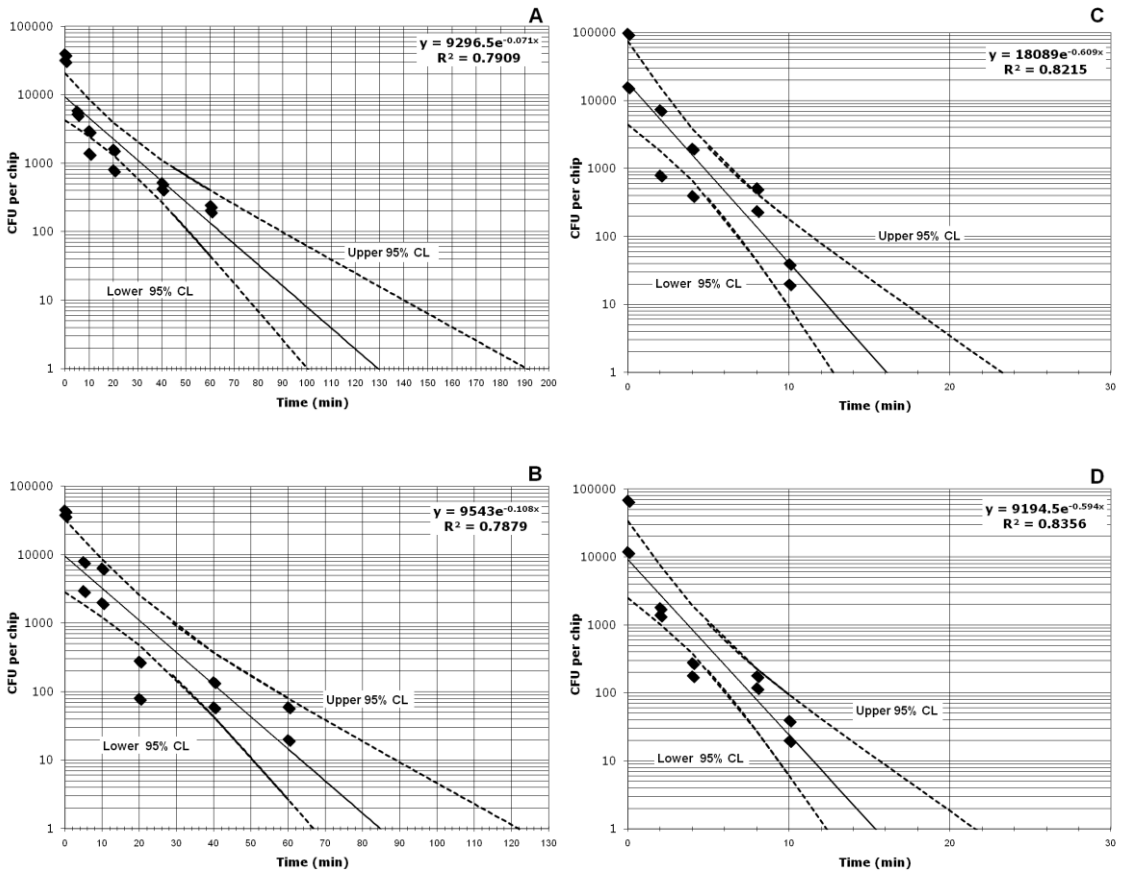
Temp. (°C)	Time (min)	CN+ Survival (log CFU) Rep. 1	CN+ Survival (log CFU) Rep. 2	D-value (min)	BEC8 Survival (log CFU) Rep. 1	BEC8 Survival (log CFU) Rep. 2	D-value (min)
12	0	26000	68000	39.1	12000	36000	46.5
	5	6000	38000		8200	22000	
	10	3200	18000		5200	8000	
	20	1800	4000		3000	8800	
	40	860	1800		1200	6600	
	60	720	1200		980	600	
23	0	58000	46000	24.9	18000	16000	32.1
	5	4200	6800		5400	9800	
	10	1000	1800		2600	4800	
	20	1260	800		1200	1400	
	40	120	380		420	880	
	60	40	120		180	140	
30	0	36000	38000	5.2	8000	38000	7.0
	2	9200	2800		2800	3000	
	4	7400	600		1800	600	
	8	1200	400		960	400	
	10	280	240		800	240	
37	0	38000	48000	4.8	4000	12000	7.1
	2	6400	800		3400	2800	
	4	2200	200		3000	400	
	8	620	280		900	320	
	10	240	80		420	120	



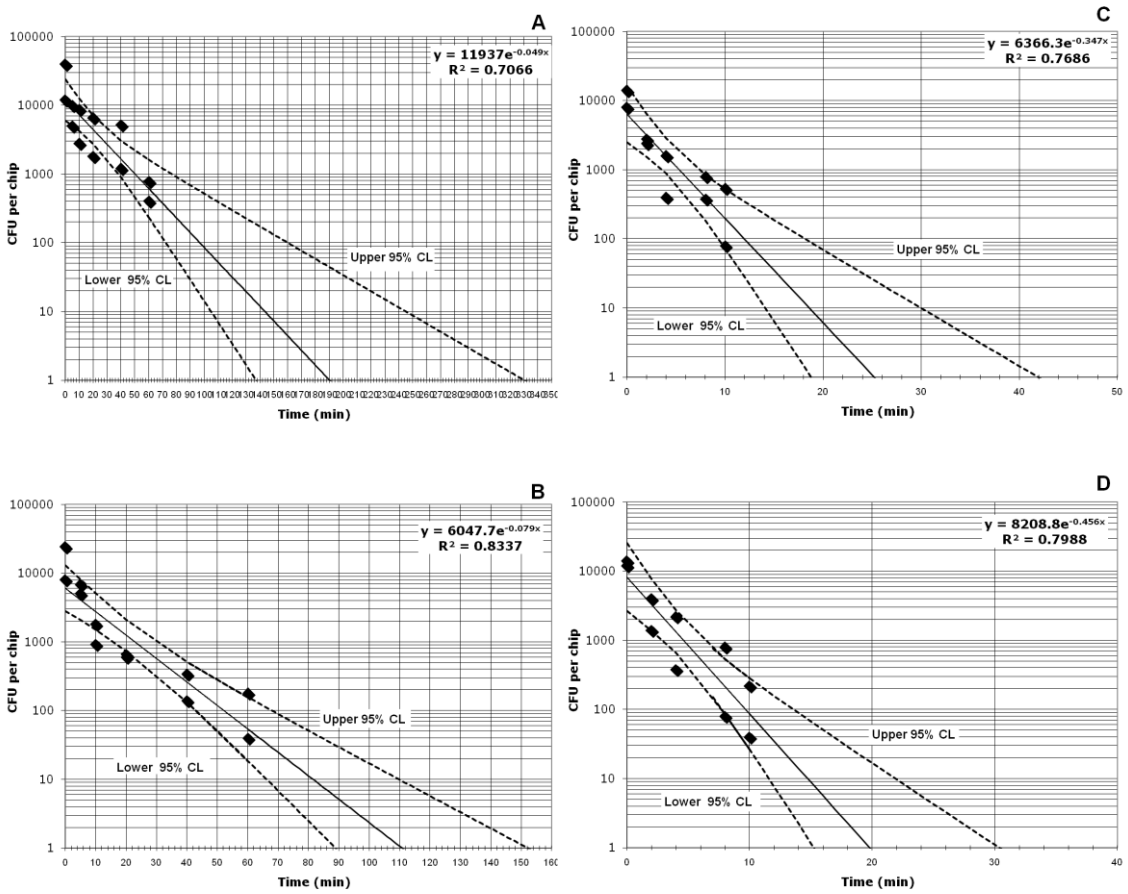
C.10. Semi-log plot of viable cell count of the three strain *E. coli* O157:H7 mixture as a function of exposure time on SSC at A) 12°C, B) 23°C, C) 30°C, and D) 37°C when inoculated in liquid form on SSC with bacteriophage cocktail BEC8 at an MOI of 100. The time at 1 CFU per chip represents the time for a 5 log reduction which is the limit of detection showing the 95% CL.



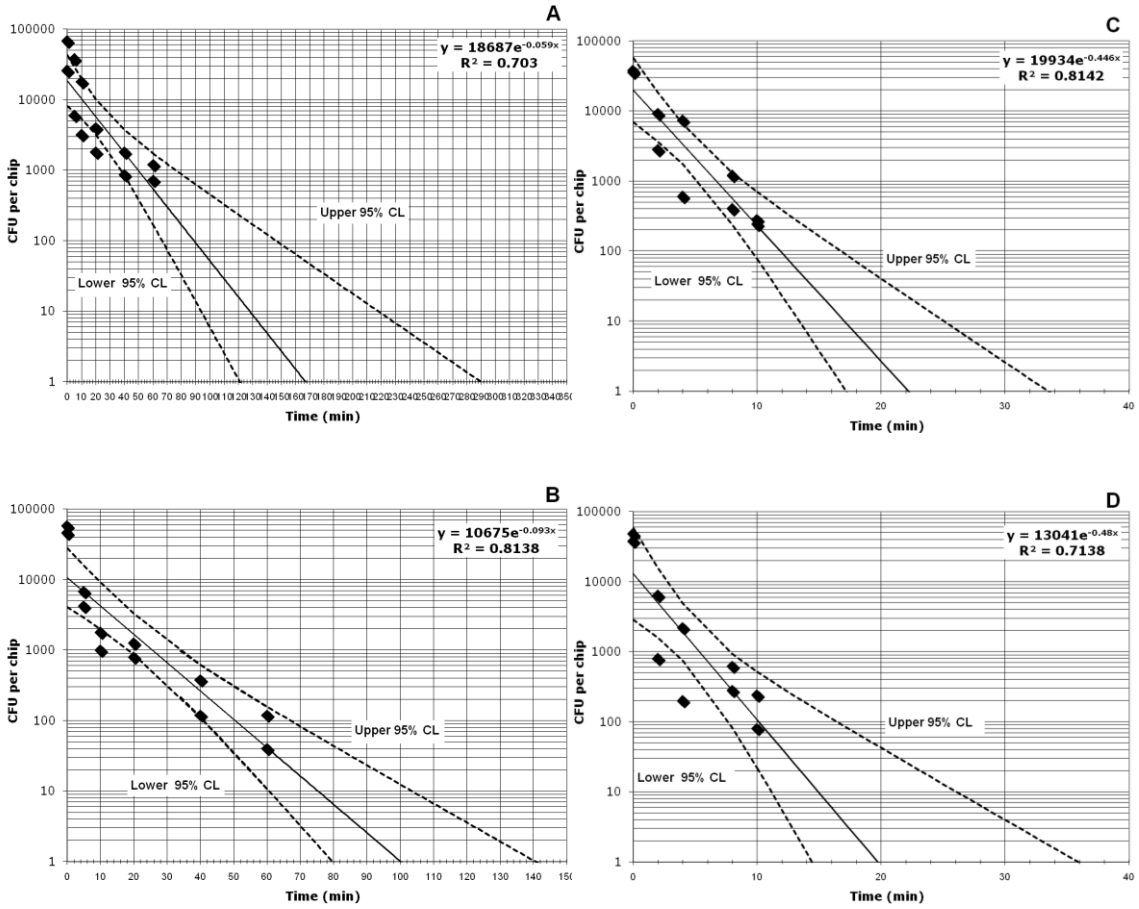
C.11. Semi-log plot of viable cell count of the three strain *E. coli* O157:H7 mixture as a function of exposure time on SSC at A) 12°C, B) 23°C, C) 30°C, and D) 37°C when inoculated in dry form on SSC with bacteriophage cocktail BEC8 at an MOI of 100. The time at 1 CFU per steel represents the time for a 5 log reduction which is the limit of detection showing the 95% CL.



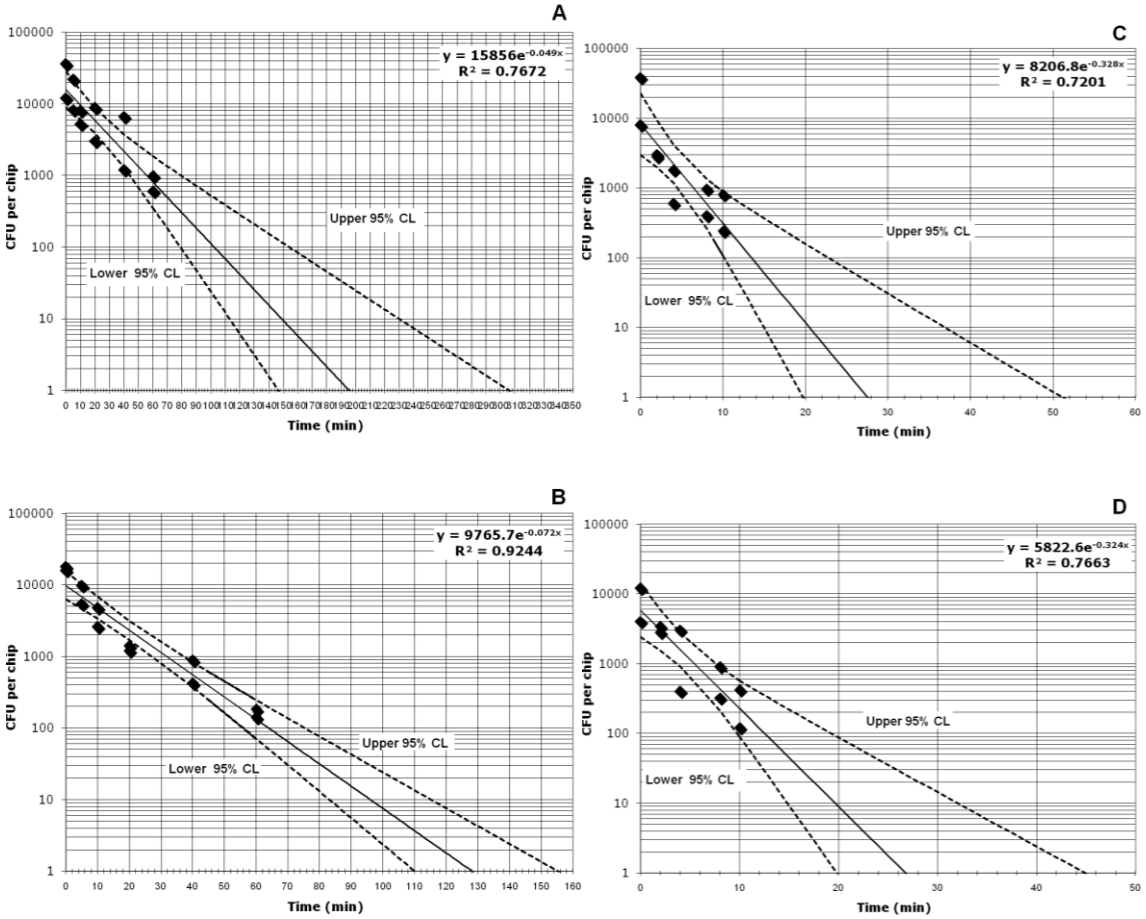
C.12. Semi-log plot of viable cell count of the three strain *E. coli* O157:H7 mixture as a function of exposure time on SSC at A) 12°C, B) 23°C, C) 30°C, and D) 37°C when inoculated in liquid form on CTC with bacteriophage cocktail BEC8 at an MOI of 100. The time at 1 CFU per chip represents the time for a 5 log reduction which is the limit of detection showing the 95% CL.



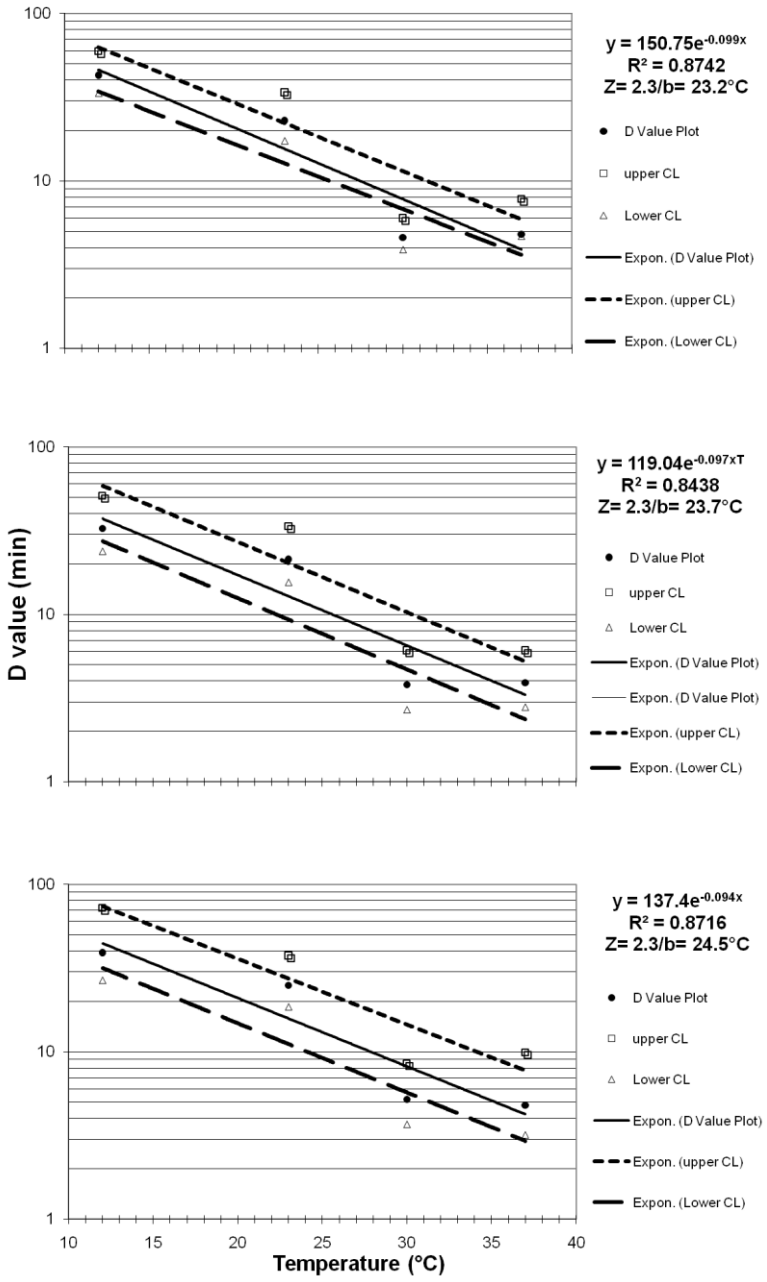
C.13. Semi-log plot of viable cell count of the three strain *E. coli* O157:H7 mixture as a function of exposure time on SSC at A) 12°C, B) 23°C, C) 30°C, and D) 37°C when inoculated in dry form on CTC with bacteriophage cocktail BEC8 at an MOI of 100. The time at 1 CFU per chip represents the time for a 5 log reduction which is the limit of detection showing the 95% CL.



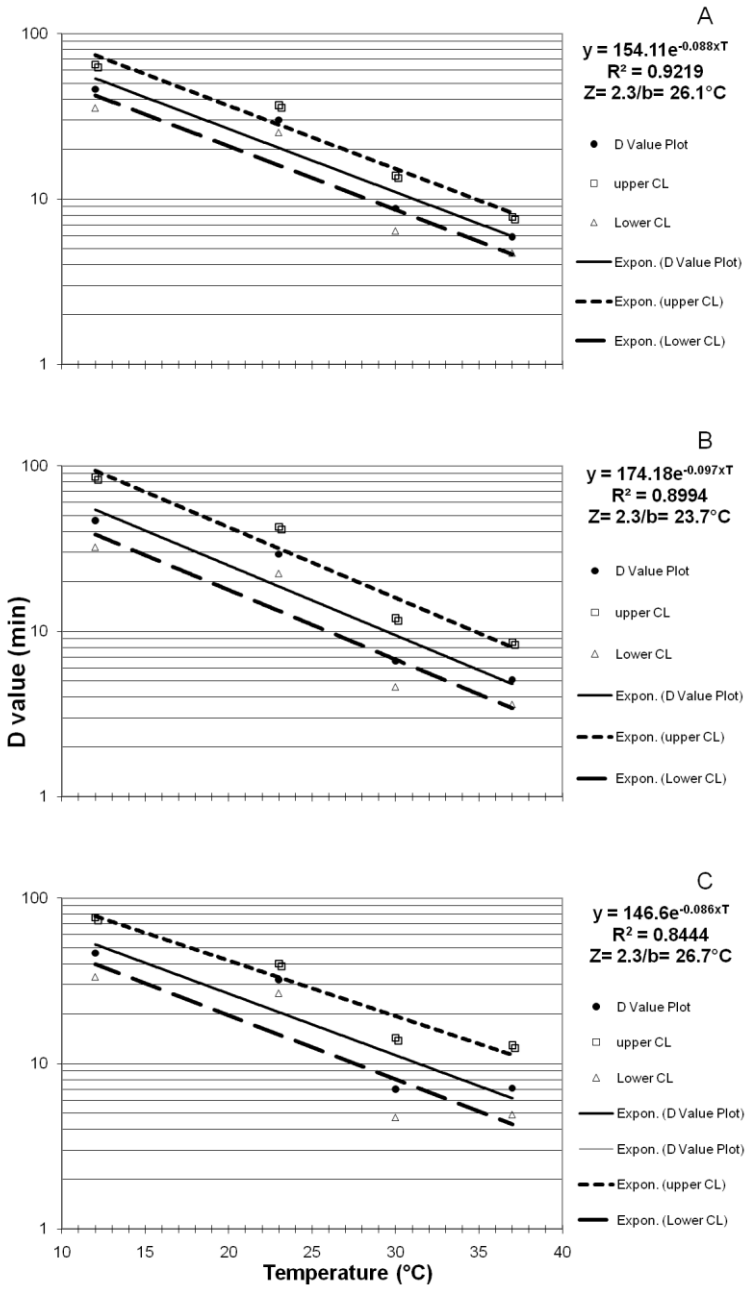
C.14. Semi-log plot of viable cell count of the three strain *E. coli* O157:H7 mixture as a function of exposure time on SSC at A) 12°C, B) 23°C, C) 30°C, and D) 37°C when inoculated in liquid form on HDPEC with bacteriophage cocktail BEC8 at an MOI of 100. The time at 1 CFU per chip represents the time for a 5 log reduction which is the limit of detection showing the 95% CL.



C.15. Semi-log plot of viable cell count of the three strain *E. coli* O157:H7 mixture as a function of exposure time on SSC at A) 12°C, B) 23°C, C) 30°C, and D) 37°C when inoculated in dry form on HDPEC with bacteriophage cocktail BEC8 at an MOI of 100. The time at 1 CFU per chip represents the time for a 5 log reduction which is the limit of detection showing the 95% CL.



C.16. D-plots showing the Z-values of the inactivation of a mixture of liquid cells of *E. coli* O157:H7 by BEC8 on A) SSC, B) CTC, and C) HDPEC.



C.17. D-plots showing the Z-values of the inactivation of a mixture of dry cells of *E. coli* O157:H7 by BEC8 on A) SSC, B) CTC, and C) HDPEC.

C.18. Effect of BEC8 against dry and liquid cells of three *E. coli* O157:H7 strains inoculated on stainless steel chips at 23 and 37°C.

Temperature (°C)	Treatment	Inoculum level	Time	Inactivation (log CFU)
23	CN+	H	10 min	1.26±0.46
			1 h	1.86±0.30
			24 h	3.09±0.75
		M	10 min	2.27±0.29
			1 h	2.62±0.11
			24 h	2.84±0.13
		L	10 min	2.37±0.27
			1 h	3.34±0.37
			24 h	3.22±0.11
	BEC8	H	10 min	0.52±0.04
			1 h	0.68±0.01
			24 h	1.17±0.44
		M	10 min	1.04±0.14
			1 h	1.55±0.13
			24 h	2.06±0.69
		L	10 min	2.43±0.06
			1 h	3.34±0.37
			24 h	3.22±0.11
37	CN+	H	10 min	1.90±0.77
			1 h	3.57±-/64
			24 h	5.25±0.24
		M	10 min	2.34±0.20
			1 h	3.21±0.05
			24 h	4.31±0.23
		L	10 min	3.26±0.26
			1 h	3.35±0.14
			24 h	3.28±0.03
	BEC8	H	10 min	0.83±0.04
			1 h	0.99±0.001
			24 h	2.70±0.43
		M	10 min	1.04±0.31
			1 h	1.55±0.27
			24 h	2.06±0.23
		L	10 min	3.26±0.26
			1 h	3.35±0.14
			24 h	3.28±0.03

C.19. Effect of BEC8 against dry and liquid cells of three *E. coli* O157:H7 strains inoculated on stainless steel chips at 4 and 12°C.

Temperature (°C)	Treatment	Inoculum level	Time	Inactivation (log CFU)
4	CN+	H	10 min	0.52±0.10
			1 h	0.62±0.27
			24 h	0.52±0.22
		M	10 min	1.33±0.27
			1 h	2.00±0.15
			24 h	2.05±0.07
		L	10 min	1.40±0.18
			1 h	1.25±0.04
			24 h	1.95±0.07
	BEC8	H	10 min	0.84±0.05
			1 h	0.90±0.34
			24 h	1.09±0.55
		M	10 min	0.13±0.34
			1 h	0.87±0.33
			24 h	1.42±0.12
		L	10 min	1.40±0.28
			1 h	1.75±0.18
			24 h	1.82±0.08
12	CN+	H	10 min	0.70±0.49
			1 h	1.17±0.80
			24 h	1.43±1.08
		M	10 min	1.86±0.38
			1 h	1.60±0.06
			24 h	2.12±0.36
		L	10 min	1.70±0.51
			1 h	2.91±0.13
			24 h	3.30±0.06
	BEC8	H	10 min	1.14±0.30
			1 h	1.11±0.18
			24 h	0.98±0.31
		M	10 min	0.75±0.19
			1 h	1.20±0.11
			24 h	1.89±0.31
		L	10 min	2.98±0.70
			1 h	3.30±0.43
			24 h	3.30±0.06

C.20. Effect of BEC8 against dry and liquid cells of three *E. coli* O157:H7 strains inoculated on ceramic tile chips at 23 and 37°C.

Temperature (°C)	Treatment	Inoculum level	Time	Inactivation (log CFU)
23	CN+	H	10 min	0.30±0.06
			1 h	-.58±0.04
			24 h	1.93±0.58
		M	10 min	1.92±0.04
			1 h	2.94±0.62
			24 h	3.69±0.72
		L	10 min	1.71±0.57
			1 h	2.54±0.51
			24 h	2.69±0.12
	BEC8	H	10 min	0.54±0.45
			1 h	0.45±0.11
			24 h	0.92±0.43
		M	10 min	1.27±0.04
			1 h	1.70±0.82
			24 h	2.29±0.23
		L	10 min	1.06±0.14
			1 h	2.60±0.42
			24 h	2.69±0.12
37	CN+	H	10 min	0.37±0.08
			1 h	2.70±0.32
			24 h	3.36±0.64
		M	10 min	2.36±0.23
			1 h	2.81±0.16
			24 h	4.3±0.09
		L	10 min	3.66±0.15
			1 h	3.66±0.05
			24 h	3.10±0.28
	BEC8	H	10 min	0.47±0.34
			1 h	1.09±0.46
			24 h	2.65±0.64
		M	10 min	1.37±0.60
			1 h	3.02±0.62
			24 h	4.32±0.09
		L	10 min	1.95±0.18
			1 h	3.66±0.05
			24 h	3.10±0.28

C.21. Effect of BEC8 against dry and liquid cells of three *E. coli* O157:H7 strains inoculated on ceramic tile chips at 4 and 12°C.

Temperature (°C)	Treatment	Inoculum level	Time	Inactivation (log CFU)
4	CN+	H	10 min	0.29±0.20
			1 h	0.49±0.21
			24 h	0.45±0.11
		M	10 min	0.92±0.63
			1 h	1.31±0.01
			24 h	2.16±0.38
		L	10 min	1.02±0.47
			1 h	0.88±0.41
			24 h	1.14±0.08
	BEC8	H	10 min	0.69±0.30
			1 h	0.81±0.15
			24 h	1.03±0.30
		M	10 min	.92±0.52
			1 h	1.14±0.16
			24 h	1.22±0.14
		L	10 min	0.91±0.44
			1 h	0.90±0.32
			24 h	2.01±0.08
12	CN+	H	10 min	0.57±0.08
			1 h	0.55±0.04
			24 h	0.66±0.49
		M	10 min	0.77±1.02
			1 h	1.66±0.31
			24 h	1.48±0.52
		L	10 min	1.37±0.27
			1 h	1.78±0.21
			24 h	2.92±0.48
	BEC8	H	10 min	0.69±0.47
			1 h	0.63±0.31
			24 h	0.57±0.39
		M	10 min	0.79±0.14
			1 h	1.13±0.12
			24 h	1.44±0.37
		L	10 min	1.07±0.03
			1 h	1.59±0.28
			24 h	2.97±0.41

C.22. Effect of BEC8 against dry and liquid cells of three *E. coli* O157:H7 strains inoculated on high density polyethylene chips at 23 and 37°C.

Temperature (°C)	Treatment	Inoculum level	Time	Inactivation (log CFU)
23	CN+	H	10 min	0.93±0.01
			1 h	1.53±0.27
			24 h	1.23±0.20
		M	10 min	0.95±0.67
			1 h	2.15±0.47
			24 h	3.22±0.22
		L	10 min	1.73±0.38
			1 h	2.89±0.44
			24 h	2.72±0.21
	BEC8	H	10 min	0.46±0.29
			1 h	0.56±0.31
			24 h	0.40±0.16
		M	10 min	1.08±0.56
			1 h	1.94±0.51
			24 h	2.09±0.22
		L	10 min	1.46±0.38
			1 h	2.17±0.15
			24 h	2.36±0.76
37	CN+	H	10 min	0.97±0.22
			1 h	1.95±0.46
			24 h	3.75±0.53
		M	10 min	3.16±0.51
			1 h	4.11±0.47
			24 h	3.84±0.09
		L	10 min	3.12±0.69
			1 h	3.45±0.15
			24 h	3.08±0.25
	BEC8	H	10 min	0.69±0.23
			1 h	0.96±0.26
			24 h	3.18±0.40
		M	10 min	1.92±0.67
			1 h	2.91±0.23
			24 h	3.84±0.09
		L	10 min	1.93±0.31
			1 h	3.45±0.15
			24 h	3.08±0.25

C.23. Effect of BEC8 against dry and liquid cells of three *E. coli* O157:H7 strains inoculated on high density polyethylene chips at 4 and 12°C.

Temperature (°C)	Treatment	Inoculum level	Time	Inactivation (log CFU)
4	CN+	H	10 min	0.75±0.67
			1 h	0.73±0.31
			24 h	0.65±0.34
		M	10 min	0.67±0.61
			1 h	1.26±0.63
			24 h	1.58±0.97
		L	10 min	0.29±0.30
			1 h	0.67±0.33
			24 h	1.79±0.69
	BEC8	H	10 min	0.23±0.01
			1 h	0.61±0.33
			24 h	0.17±0.13
		M	10 min	0.86±0.58
			1 h	1.05±0.52
			24 h	0.89±0.60
		L	10 min	0.36±0.06
			1 h	0.71±0.07
			24 h	1.32±0.45
12	CN+	H	10 min	0.57±0.10
			1 h	1.05±0.29
			24 h	0.90±0.66
		M	10 min	1.56±0.68
			1 h	2.49±0.12
			24 h	1.40±0.18
		L	10 min	0.72±0.25
			1 h	1.56±0.47
			24 h	2.72±0.26
	BEC8	H	10 min	0.40±0.04
			1 h	0.51±0.42
			24 h	0.33±0.31
		M	10 min	1.51±0.35
			1 h	1.84±0.70
			24 h	1.43±0.43
		L	10 min	0.53±0.12
			1 h	0.88±0.17
			24 h	1.57±0.47

C.24. Effect of BEC8 against dry and liquid cells of three *E. coli* O157:H7 strains inoculated on baby spinach leaves at 23 and 37°C.

Temperature (°C)	Treatment	Inoculum level	Time	Inactivation (log CFU)
23	CN+	H	10 min	1.00±0.49
			1 h	1.28±0.40
			24 h	1.32±0.03
		M	10 min	0.80±0.11
			1 h	2.32±0.07
			24 h	3.00±0.07
		L	10 min	2.01±0.26
			1 h	2.43±0.43
			24 h	3.53±0.07
	BEC8	H	10 min	1.28±0.53
			1 h	1.43±0.19
			24 h	1.69±0.67
		M	10 min	1.89±0.65
			1 h	2.12±0.21
			24 h	2.5±0.04
		L	10 min	1.98±0.03
			1 h	2.36±0.34
			24 h	3.53±0.07
37	CN+	H	10 min	0.81±0.36
			1 h	1.94±0.42
			24 h	2.86±0.07
		M	10 min	1.64±0.38
			1 h	2.47±0.07
			24 h	2.53±0.57
		L	10 min	1.73±0.56
			1 h	3.12±0.31
			24 h	3.08±0.00
	BEC8	H	10 min	0.66±0.33
			1 h	1.82±0.25
			24 h	2.83±0.15
		M	10 min	1.67±0.24
			1 h	2.04±0.68
			24 h	2.54±0.53
		L	10 min	1.79±0.12
			1 h	2.82±0.12
			24 h	3.08±0.00

C.25. Effect of BEC8 against dry and liquid cells of three *E. coli* O157:H7 strains inoculated on baby spinach leaves at 4 and 8°C.

Temperature (°C)	Treatment	Inoculum level	Time	Inactivation (log CFU)
4	CN+	H	10 min	0.26±0.06
			1 h	0.16±0.05
			24 h	0.40±0.58
		M	10 min	0.56±0.48
			1 h	0.98±0.63
			24 h	1.66±0.76
		L	10 min	0.41±0.41
			1 h	1.79±0.29
			24 h	1.51±0.03
	BEC8	H	10 min	0.16±0.07
			1 h	0.14±0.06
			24 h	0.92±0.05
		M	10 min	1.07±0.46
			1 h	1.44±0.62
			24 h	1.25±0.41
		L	10 min	1.80±0.57
			1 h	1.81±0.31
			24 h	1.42±0.57
8	CN+	H	10 min	0.39±0.08
			1 h	0.68±0.23
			24 h	1.21±0.54
		M	10 min	0.84±0.19
			1 h	1.30±0.53
			24 h	2.45±0.35
		L	10 min	1.37±0.73
			1 h	2.18±0.04
			24 h	2.81±0.05
	BEC8	H	10 min	0.53±0.26
			1 h	0.64±0.17
			24 h	1.01±0.68
		M	10 min	1.34±0.30
			1 h	1.36±0.62
			24 h	2.04±0.62
		L	10 min	1.60±0.14
			1 h	2.09±0.17
			24 h	2.96±0.69

C.26. Effect of TC against dry and liquid cells of three *E. coli* O157:H7 strains inoculated on baby spinach leaves at 23 and 37°C.

Temperature (°C)	Treatment	Inoculum level	Time	Inactivation (log CFU)
23	CN+	H	10 min	3.26±0.30
			1 h	3.61±0.73
			24 h	5.08±0.70
		M	10 min	1.65±0.75
			1 h	1.52±0.17
			24 h	3.72±1.5
		L	10 min	1.51±0.17
			1 h	2.22±0.41
			24 h	3.74±0.08
	TC	H	10 min	2.30±0.34
			1 h	3.67±0.51
			24 h	5.11±0.96
		M	10 min	2.97±0.77
			1 h	2.15±0.63
			24 h	3.68±0.32
		L	10 min	1.45±0.07
			1 h	2.39±0.07
			24 h	3.74±0.08
37	CN+	H	10 min	3.90±0.42
			1 h	4.24±0.51
			24 h	5.38±0.43
		M	10 min	1.64±0.69
			1 h	4.01±0.21
			24 h	4.62±0.24
		L	10 min	3.75±0.34
			1 h	3.83±0.21
			24 h	3.43±0.32
	TC	H	10 min	3.95±0.56
			1 h	4.41±0.45
			24 h	5.38±0.43
		M	10 min	1.58±0.52
			1 h	4.01±0.21
			24 h	4.62±0.24
		L	10 min	3.12±0.54
			1 h	3.83±0.21
			24 h	3.43±0.32

C.27. Effect of TC against dry and liquid cells of three *E. coli* O157:H7 strains inoculated on baby spinach leaves at 4 and 8°C.

Temperature (°C)	Treatment	Inoculum level	Time	Inactivation (log CFU)
4	CN+	H	10 min	1.62±0.71
			1 h	3.28±0.99
			24 h	3.96±0.12
		M	10 min	2.59±0.02
			1 h	2.26±0.62
			24 h	2.79±0.82
		L	10 min	2.49±0.84
			1 h	2.58±0.75
			24 h	2.45±0.64
	TC	H	10 min	2.30±0.43
			1 h	3.02±0.65
			24 h	3.58±0.90
		M	10 min	1.77±0.14
			1 h	1.61±0.01
			24 h	2.66±0.37
		L	10 min	1.73±0.28
			1 h	2.65±0.66
			24 h	2.99±0.13
8	CN+	H	10 min	3.19±0.16
			1 h	3.99±0.54
			24 h	4.02±0.03
		M	10 min	1.96±0.27
			1 h	2.51±0.20
			24 h	2.69±0.97
		L	10 min	2.60±0.07
			1 h	1.76±0.76
			24 h	2.99±0.13
	TC	H	10 min	2.55±0.79
			1 h	2.97±0.35
			24 h	3.11±0.16
		M	10 min	1.99±0.02
			1 h	2.13±0.61
			24 h	2.78±0.12
		L	10 min	2.41±0.58
			1 h	1.44±0.37
			24 h	2.99±0.12

C.28. Effect of BEC8/TC against dry and liquid cells of three *E. coli* O157:H7 strains inoculated on baby spinach leaves at 23 and 37°C.

Temperature (°C)	Treatment	Inoculum level	Time	Inactivation (log CFU)
23	CN+	H	10 min	5.29±0.03
			1 h	5.26±0.17
			24 h	5.33±0.35
		M	10 min	4.31±0.58
			1 h	4.25±0.24
			24 h	4.14±0.51
		L	10 min	3.31±0.32
			1 h	3.27±0.70
			24 h	3.38±0.34
	TC	H	10 min	5.29±0.30
			1 h	5.26±0.17
			24 h	5.33±0.36
		M	10 min	4.31±0.58
			1 h	4.25±0.24
			24 h	4.14±0.51
		L	10 min	3.31±0.32
			1 h	3.27±0.70
			24 h	3.38±0.34
37	CN+	H	10 min	5.25±0.24
			1 h	5.34±0.11
			24 h	5.23±0.04
		M	10 min	4.39±0.27
			1 h	4.21±0.18
			24 h	4.22±0.44
		L	10 min	3.42±0.74
			1 h	3.20±0.08
			24 h	3.36±0.03
	TC	H	10 min	5.25±0.24
			1 h	5.33±0.11
			24 h	5.23±0.04
		M	10 min	4.39±0.27
			1 h	4.21±0.18
			24 h	4.22±0.44
		L	10 min	3.42±0.73
			1 h	3.20±0.08
			24 h	3.36±0.03

C.29. Effect of BEC8/TC against dry and liquid cells of three *E. coli* O157:H7 strains inoculated on baby spinach leaves at 4 and 8°C.

Temperature (°C)	Treatment	Inoculum level	Time	Inactivation (log CFU)
4	CN+	H	10 min	5.61±0.11
			1 h	5.31±0.58
			24 h	5.29±0.30
		M	10 min	4.33±0.61
			1 h	4.29±0.30
			24 h	4.35±0.14
		L	10 min	3.31±0.15
			1 h	3.12±0.31
			24 h	3.19±0.16
	TC	H	10 min	5.61±0.87
			1 h	5.31±0.57
			24 h	5.29±0.30
		M	10 min	3.79±0.15
			1 h	4.29±0.30
			24 h	4.35±0.14
		L	10 min	3.31±0.15
			1 h	3.12±0.31
			24 h	3.19±0.16
8	CN+	H	10 min	5.14±0.34
			1 h	5.21±0.19
			24 h	5.20±0.43
		M	10 min	4.25±0.66
			1 h	4.30±0.56
			24 h	4.32±0.34
		L	10 min	3.35±0.38
			1 h	3.08±0.25
			24 h	3.24±0.14
	TC	H	10 min	5.14±0.34
			1 h	5.21±0.19
			24 h	5.20±0.43
		M	10 min	4.25±0.66
			1 h	4.30±0.56
			24 h	4.32±0.34
		L	10 min	3.35±0.38
			1 h	3.08±0.25
			24 h	3.24±0.14

C.30. Effect of BEC8 against dry and liquid cells of three *E. coli* O157:H7 strains inoculated on baby romaine lettuce leaves at 23 and 37°C.

Temperature (°C)	Treatment	Inoculum level	Time	Inactivation (log CFU)
23	CN+	H	10 min	0.37±0.38
			1 h	0.59±0.13
			24 h	1.68±0.40
		M	10 min	1.40±0.17
			1 h	1.52±0.10
			24 h	2.05±0.35
		L	10 min	1.99±0.21
			1 h	2.17±0.28
			24 h	3.82±0.74
	BEC8	H	10 min	0.48±0.09
			1 h	1.03±0.58
			24 h	1.44±0.44
		M	10 min	1.20±0.03
			1 h	1.28±0.03
			24 h	1.87±0.28
		L	10 min	1.69±0.10
			1 h	1.82±0.13
			24 h	3.73±0.24
37	CN+	H	10 min	0.22±0.13
			1 h	0.94±1.30
			24 h	3.68±0.88
		M	10 min	1.66±0.15
			1 h	2.09±0.30
			24 h	3.21±0.05
		L	10 min	2.77±0.96
			1 h	3.49±0.10
			24 h	3.57±0.27
	BEC8	H	10 min	0.32±0.29
			1 h	0.91±0.04
			24 h	2.44±0.19
		M	10 min	1.54±0.11
			1 h	1.83±0.57
			24 h	2.74±0.06
		L	10 min	2.29±0.37
			1 h	3.49±0.10
			24 h	3.57±0.27

C.31. Effect of BEC8 against dry and liquid cells of three *E. coli* O157:H7 strains inoculated on baby romaine lettuce leaves at 4 and 8°C.

Temperature (°C)	Treatment	Inoculum level	Time	Inactivation (log CFU)
4	CN+	H	10 min	0.22±0.62
			1 h	0.51±0.43
			24 h	0.67±0.21
		M	10 min	0.99±0.02
			1 h	1.32±0.53
			24 h	1.83±0.26
		L	10 min	1.51±0.15
			1 h	1.77±0.19
			24 h	2.02±0.14
	BEC8	H	10 min	0.05±0.00
			1 h	0.54±0.51
			24 h	0.35±0.28
		M	10 min	0.93±0.07
			1 h	1.65±0.49
			24 h	1.41±0.70
		L	10 min	1.34±0.24
			1 h	1.59±0.35
			24 h	1.89±0.41
8	CN+	H	10 min	0.37±0.42
			1 h	0.81±0.10
			24 h	0.87±0.36
		M	10 min	0.77±0.27
			1 h	1.16±0.49
			24 h	1.51±0.24
		L	10 min	1.81±0.62
			1 h	1.92±0.34
			24 h	1.94±0.24
	BEC8	H	10 min	0.35±0.27
			1 h	0.68±0.04
			24 h	0.58±0.36
		M	10 min	1.11±0.27
			1 h	0.85±0.49
			24 h	1.81±0.11
		L	10 min	1.26±0.62
			1 h	1.63±0.34
			24 h	1.87±0.24

C.32. Effect of TC against dry and liquid cells of three *E. coli* O157:H7 strains inoculated on baby romaine lettuce leaves at 23 and 37°C.

Temperature (°C)	Treatment	Inoculum level	Time	Inactivation (log CFU)
23	CN+	H	10 min	2.16±0.77
			1 h	3.22±0.68
			24 h	3.51±0.24
		M	10 min	1.85±0.96
			1 h	3.38±0.02
			24 h	3.60±0.25
		L	10 min	1.92±0.57
			1 h	2.81±0.38
			24 h	3.08±0.25
	TC	H	10 min	2.37±0.75
			1 h	2.65±0.67
			24 h	3.14±0.06
		M	10 min	1.71±0.84
			1 h	3.53±0.44
			24 h	3.09±0.30
		L	10 min	1.88±0.85
			1 h	2.96±0.17
			24 h	3.08±0.25
37	CN+	H	10 min	1.87±0.48
			1 h	3.15±0.03
			24 h	4.85±0.85
		M	10 min	2.35±0.12
			1 h	4.15±0.33
			24 h	3.85±0.85
		L	10 min	3.26±0.26
			1 h	3.35±0.29
			24 h	3.26±0.51
	TC	H	10 min	1.30±0.38
			1 h	2.91±0.17
			24 h	5.00±0.64
		M	10 min	2.11±0.41
			1 h	4.09±0.41
			24 h	3.76±0.98
		L	10 min	3.26±0.26
			1 h	3.35±0.29
			24 h	3.26±0.51

C.33. Effect of TC against dry and liquid cells of three *E. coli* O157:H7 strains inoculated on baby romaine lettuce leaves at 4 and 8°C.

Temperature (°C)	Treatment	Inoculum level	Time	Inactivation (log CFU)
4	CN+	H	10 min	2.38±0.14
			1 h	2.28±0.90
			24 h	3.67±1.46
		M	10 min	1.71±0.45
			1 h	2.23±0.01
			24 h	2.71±0.16
		L	10 min	1.09±0.40
			1 h	2.77±0.53
			24 h	3.31±0.15
	TC	H	10 min	2.07±0.32
			1 h	2.67±0.78
			24 h	2.98±0.77
		M	10 min	1.50±0.48
			1 h	2.24±0.09
			24 h	2.50±0.28
		L	10 min	0.99±0.30
			1 h	2.74±0.58
			24 h	2.86±0.49
8	CN+	H	10 min	2.61±1.29
			1 h	2.60±0.47
			24 h	3.33±0.21
		M	10 min	1.59±0.16
			1 h	2.46±0.36
			24 h	3.79±0.01
		L	10 min	1.89±0.78
			1 h	2.82±0.37
			24 h	3.35±0.14
	TC	H	10 min	2.46±0.65
			1 h	2.56±0.14
			24 h	2.76±0.34
		M	10 min	1.04±0.31
			1 h	2.29±0.05
			24 h	3.64±0.20
		L	10 min	1.62±0.66
			1 h	2.75±0.47
			24 h	3.35±0.14

C.34. Effect of BEC8/TC against dry and liquid cells of three *E. coli* O157:H7 strains inoculated on baby romaine lettuce leaves at 23 and 37°C.

Temperature (°C)	Treatment	Inoculum level	Time	Inactivation (log CFU)
23	CN+	H	10 min	3.78±0.07
			1 h	5.03±0.60
			24 h	5.21±0.86
		M	10 min	3.34±0.83
			1 h	4.31±0.32
			24 h	4.32±0.03
		L	10 min	3.19±0.58
			1 h	3.37±0.16
			24 h	3.19±0.58
	TC	H	10 min	3.95±0.25
			1 h	5.03±0.60
			24 h	5.21±0.86
		M	10 min	3.23±0.83
			1 h	4.31±0.32
			24 h	4.32±0.03
		L	10 min	3.19±0.58
			1 h	3.36±0.16
			24 h	3.19±0.58
37	CN+	H	10 min	5.38±0.18
			1 h	5.46±0.17
			24 h	5.47±0.24
		M	10 min	4.14±0.34
			1 h	4.03±0.60
			24 h	4.25±0.24
		L	10 min	3.35±0.38
			1 h	3.42±0.48
			24 h	3.30±0.56
	TC	H	10 min	4.03±0.46
			1 h	5.46±0.17
			24 h	5.47±0.24
		M	10 min	2.81±0.02
			1 h	4.03±0.60
			24 h	4.25±0.24
		L	10 min	3.35±0.38
			1 h	3.42±0.48
			24 h	3.30±0.56

C.35. Effect of BEC8/TC against dry and liquid cells of three *E. coli* O157:H7 strains inoculated on baby romaine lettuce leaves at 4 and 8°C.

Temperature (°C)	Treatment	Inoculum level	Time	Inactivation (log CFU)
4	CN+	H	10 min	3.90±0.11
			1 h	5.16±0.36
			24 h	5.36±0.03
		M	10 min	2.53±0.38
			1 h	4.19±0.41
			24 h	4.22±0.11
		L	10 min	3.11±0.47
			1 h	3.39±0.27
			24 h	3.33±0.25
	TC	H	10 min	3.77±0.04
			1 h	5.16±0.36
			24 h	5.36±0.03
		M	10 min	2.36±0.44
			1 h	4.19±0.41
			24 h	4.22±0.11
		L	10 min	3.11±0.47
			1 h	3.39±0.27
			24 h	3.33±0.25
8	CN+	H	10 min	4.71±0.53
			1 h	5.24±0.48
			24 h	5.26±0.17
		M	10 min	3.99±0.45
			1 h	4.38±0.05
			24 h	4.29±0.30
		L	10 min	3.31±0.23
			1 h	3.34±0.27
			24 h	3.23±0.46
	TC	H	10 min	3.74±0.12
			1 h	5.24±0.48
			24 h	5.26±0.17
		M	10 min	3.83±0.87
			1 h	3.99±0.50
			24 h	4.29±0.30
		L	10 min	3.31±0.23
			1 h	3.34±0.27
			24 h	3.23±0.46