Development of a Phage Cocktail to Control the Top 7 Shiga -toxin *E. coli* Serogroups on Fresh Produce

by

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A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirement of the degree of

MASTER OF SCIENCE

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ACKNOWLEDGMENTS

First of all, I want to thank my parents, Luosi Ding and Xu Li, for their encouragements and supports in every way I can think about throughout my life. I would never have been able to achieve anything without their love and care. I also would like to thank my grandparents for believing in me and encouraging me to go through the difficult times. I owe my sincerest gratitude to my relatives back home, for staying in touch and warming my heart in the coldest winter.

I am extremely thankful to my Advisor, Dr. Narvaez, for her generous, knowledge, patience, encouragement, and inspiration for me to explore deeper into the topics. She provided me many chances to learn and triggered my passion for food and microbiology. I want to thank her for answering my silly questions all the time and reminding me there is always room for improvement. Thank you, Dr. Narvaez, for holding the light and showing the right direction on my way to food science. I would also like to thank my co-advisor, Dr. McAllister, my committee members, Dr. Holley, Dr. Nadon, and Dr. Niu for their suggestions and willingness to help me. I also want to thank Dr. Rodas of Health Canada and the Lethbridge Research Center respectively for their training and assistance throughout the project.

I want to express my appreciation to Xuan Zhang, for being not just a colleague but also my friend who helped me both inside and outside the laboratory. My way to the goal was more interesting with Xuan walking by my side. And I want to say thank you to Matthew Wells, for his kindness and patient when he helped me to improve my writing skill. I would also like to thank many of the Food Science staff; Carola, Jennifer, Pat, Denise, and Yang for your kind assistance in many ways.

Last but not least, this project was made possible by funding from Ontario Ministry of Agriculture, Food and Rural Affairs, Guelph, ON, Canada.

DEDICATION

To my parents, Luosi and Li

Organization of the thesis

This thesis is divided into five chapters and includes one manuscript, which is located in chapter three.

The first chapter gives a brief introduction about using bacteriophages as a biocontrol method against 7 STEC serogroups. General objectives are summarized at the end of the chapter.

The second chapter is the literature review including the general background information of shiga-toxigenic *E. coli* including: history, categories, symptoms, virulence factors, transmission, epidemiology, and major outbreaks caused by STEC. The current pre- and postharvest chemical and physical control methods in food, and fresh produce is also covered. Phage molecular information and current applications are also included in this chapter.

The third chapter is the methods, results, and discussion of the experimental part of this thesis including the phage lytic activity assay, spot-inoculation tests on lettuce and sprouts, and the scale-up validation study.

Chapter 4 is the appendix study of where a STEC phage cocktail was combined with the commercial phage cocktail Salmofresh™ and tested for its effectiveness against STEC. In addition, the monitoring of emergence of bacterial resistance against phage was also reported in this chapter.

Chapter 5 states the overall conclusion arising from the thesis.

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Abstract

Introduction: As the consumption of fresh produce increases worldwide, Shiga-toxigenic *Escherichia coli* (STEC) outbreaks linked to contaminated produce are becoming more frequent. Biocontrol of STEC using phages can be a safe and effective way to reduce STEC on fruits and vegetables. **Purpose:** 1) Evaluate the effectiveness of 7 bacteriophages to reduce viability of *E. coli* serogroups O26, O45, O103, O111, O121, O145, and O157 on lettuce and sprouts at 4, 10 or 25 °C and storage for 1, 24, 48, and 72 h; 2) to assess STEC phage effectiveness to reduce STEC compared to a conventional chlorinated water wash (150ppm for fresh produce, 1000 ppm for seeds); and 3) to determine if phage-insensitive mutants arise after phage treatments. **Methods:** 1) Phages either individually or as a cocktail (~10^8 log PFU/ml) were sprayed to control 10^5 log CFU/ml STEC spot-inoculated onto fresh lettuce and sprouts that were stored for 1, 24, 48, and 72 h at 2, 10, and 25°C. Samples were collected and bacteria enumerated on McConkey (STEC total numbers) and Rainbow agar (STEC individual serogroup numbers) after each storage period. STEC isolates were confirmed using latex agglutination tests specific for STEC, PCR, and Immunomagnetic Separation (IMS) to recover any surviving STEC serogroups. 2) A scale-up experiment with STEC phage cocktail and chlorinated water applied to lettuce, sprouts, and mung bean seeds (MB) was undertaken to further assess the effectiveness of STEC phage cocktail to reduce STEC on larger quantities of produce and to compare its effectiveness to chemical disinfection. Lettuce, sprouts, and MB were treated with: i) chlorinated water wash (150 ppm for lettuce/sprouts, 1000 ppm for MB); ii) STEC phage cocktail; and iii) a combination of chlorinated water and STEC phage cocktail. Phage cocktails were delivered by immersion. After treatment, lettuce and sprouts were stored for 1, 24, 48, and 72 h at 2, 10, and 25 °C. MB were stored in the dark for 24 h at 25 °C. Seeds were germinated in sterilized water and the survival of STEC isolates

was assessed by plating on Rainbow agar. 3) To determine if phage resistant mutants developed during STEC phage treatment, isolated colonies surviving after exposure to phage were randomly picked and tested for phage sensitivity using a microplate virulence assay. OD was measured after microplate incubation for 5 h at 37 °C. **Results:** 1) In spot-inoculation experiments, the highest STEC reduction (3.7 $\log_{10}$ CFU/g) caused by the phage cocktail was observed at 2 °C after 72 h of storage on lettuce; whereas on sprouts the highest reduction (2.45 $\log_{10}$ CFU/g) occurred at 25 °C after samples were stored for 1 h. All STEC O157, O26, and O103 were killed by spraying phages on both lettuce and sprouts. Overall STEC phages reduced STEC by > 2 $\log_{10}$ CFU/g on fresh produce. 2) During scale-up experiments, the combination of STEC phage cocktail and chlorinated water achieved the highest STEC reductions on produce. On MB, the highest reduction in STEC (1.69 $\log_{10}$ CFU/g) was observed after treatment with the STEC phage cocktail alone. On MB germinated sprouts, the reduction in total STEC was < 1 $\log_{10}$ CFU/g in all treatments. However, none of the treatments eliminated all 7 STEC serogroups. 3) Regarding phage resistance experiments, no STEC mutants were recovered. **Conclusion:** Results showed that the phage cocktail was able to reduce STEC O26, O45, O103, O111, O121, O145, and O157 serogroups alone, or in combination with chlorinated water on lettuce, sprouts, and MB seeds without the development of phage resistant mutants. However, for MB, the STEC phage cocktail was not effective at controlling STEC populations during germination.

Chapter 1.

1. General Introduction

Shiga-toxigenic *Escherichia coli* (STEC) especially the top 7 serogroups O26, O45, O111, O103, O121, O145, and O157 are continuously recognized as the cause of enteric disease outbreaks in North America each year (Rangel et al., 2005). The Center for Disease Control and Prevention (CDC) estimates that pathogenic *E. coli* causes approximately 73,000 foodborne illnesses and more than 60 deaths per year in the United States (Woodward et al., 2002). Based on the outbreak data base, *E. coli* O157: H7 was the cause of approximately 36% of infections, with non-O157 STEC being responsible for the remainder (CDC, 2017a). Symptoms of STEC infections include stomach cramps, diarrhea, vomiting, and in severe cases hemolytic uremic syndrome (HUS) which can lead to kidney failure, and even death (PHAC, 2015a).

Currently, studies have focused on reducing *E. coli* O157:H7 on meat and fresh produce, with few studies focusing on the development of strategies to reduce non-O157 STEC on fresh produce (Thorpe, 2004). As the demand for fresh produce increases, more enteric disease outbreaks in the U.S. have been associated with non-O157 STEC (Brooks et al., 2005). Reports indicated that the use of chemical antimicrobial agents such as chlorinated water alone is insufficient to eliminate STEC associated with fresh produce and seeds destined for sprout production such as those from mung bean and alfalfa seeds (Boyacioglu et al., 2013).

The consumption of fresh produce is steadily increasing as consumers seek to reduce the level of meat and increase the level of fiber in their diet (Tobler et al., 2011). As the demand for fresh produce grows, foodborne illness outbreaks have been increasingly linked to contaminated fresh produce over the past three decades (Lynch et al., 2009). According to the Center for Science

in the Public Interest, since 1990, fresh produce is one of the highest causes of foodborne illness with lettuce and sprouts being the most frequent source of infection (Verlinden, 1998). Other sources of contaminated produce related to foodborne illnesses include uncooked fruits, vegetables, and juice (Sivapalasingam et al., 2004). Furthermore, as consumers keep looking for safer, nutritious, and flavorful choices in their daily diet, fresh produce and organically labelled produce is increasingly being consumed (Lagasse, 2017; Verlinden, 1998).

1.1 Organic produce and food safety

According to the U.S. Department of Agriculture (USDA)'s definition; "Organic is a labeling term that indicates that the food or other agricultural product has been produced through approved methods as having no use of synthetic fertilizers, pesticides, or antibiotics during crop production (USDA, 2017c). At post-harvest, no irradiation, or biotechnology technologies can be applied during processing. The USDA set up standards including the "Organic Food Protection Act", "USDA Organic Regulations", and "National Organic Program Handbook" to standardize the handling of organic ingredients during food production. These standards must be met for both domestic and imported food to be labelled as "organic". The list of ingredients and chemicals allowed or prohibited on processed products labeled as "organic" or "made with organic" can be found in the "Organic Food Protection Act". Food that contains only organic ingredients can be labeled as "100% organic", and food that contains 95% organic ingredients by weight can be designated as "USDA ORGANIC" (USDA, 2017d). Food producers with an organic certification will be reviewed, inspected, and evaluated for certification by an agent of USDA, or a consultant (USDA, 2017b). In Canada, any product that is labelled as "organic" is regulated by the Canadian Food Inspection Agency (CFIA) (CFIA, 2009). A product can be certified organic if it meets the

Canadian Organic Standards, which is similar to the USDA standard. Procedures of organic food production must be allowed that meet all commodity-specific requirements. Overall, organic operations must demonstrate that they are protecting natural resources, conserving biodiversity, and using only approved substances. However, a previous study showed that the prevalence of STEC *E. coli* in organic vegetables was higher than conventionally produced vegetables (Mukherjee et al., 2004). The use of some conventional chemical sanitizers became a limitation of organic food to meet the expected standards for food safety. One approach to deal with this issue is to come up with more biological techniques for sanitization of produce labelled as organic, and one of these approaches can be the use phages to control pathogens. Phages are a natural intervention approved by the USDA for use with organic food products without special labelling requirement (USDA, 2017b).

Phages are viruses that invade bacterial cells and, in the case of lytic phages, disrupt bacterial metabolism causing cell lysis (Abedon, 2011a). Phages have unique advantages compared to other conventional biocontrol methods as they are species and often even strain specific so they do not affect other members of the gut microbiome (Atterbury et al., 2007; Guarner & Malagelada, 2003). Phages can have two types of life cycles: lytic or lysogenic. The lytic cycle involves phage reproduction where the phage uses the host's protein synthetic machinery to replicate and cause lysis of the host cell. In contrast, phages that enter a lysogenic cycle do not immediately kill the bacterium, but the phage genome is incorporated into the host genome and is replicated with it (Bertani, 1953). During the process of selecting phage for therapy or food decontamination, phages are first tested for lytic activity to ensure that they will kill the host. Secondly, they must be sequenced to ensure that they contain no unwanted genes (e.g. virulence, antibiotic resistance genes) within their genome. One limitation of using phages in food

production, specifically on food that needs refrigeration is that at low temperatures bacteria metabolism slows down, which in turn decreases the efficiency of phage replication, as they rely on the metabolic activity of the host to replicate. However, some phages exhibit the phenomenon known as "lysis from without" (LO), which could overcome this temperature mediated limitation. LO is a mechanism where phages interact with their specific host to induce early lysis of cell walls through high-multiplicity virion adsorption (Abedon, 2011a). Another limitation of phage therapy is the potential for phage insensitive bacterial mutants (PIM) to arise, which could reduce phage efficacy. Bacterial hosts can develop resistance to at least one phage type due to a mutation on the surface of the outer cell membrane which prevents phage attachment (Abedon, 2016). Some phage-susceptible bacteria can convert to PIM as a result of environmental stress. Since resistance is more likely to occur if only a single phage is employed, multiple phages in cocktails can be used in an effort to overcome PIM through their ability to recognize multiple receptors (Fischer et al., 2013). With the increasing linkage of STEC outbreaks to produce commodities, the potential for wider use of lytic phages as a safe, natural, and organically approved intervention to reduce foodborne pathogens in fresh produce is substantial (Sharma, 2013).

1.2 Objectives

There are 4 objectives for this project: 1) To assemble a phage cocktail against the top 7 STEC for testing on produce at 4, 10 or 25 °C. 2) To evaluate the effectiveness of the phage cocktail against STEC on lettuce, sprouts, and mung bean seeds over different storage periods. 3) To compare the effectiveness of phages in combination with chlorinated water washes; 4) To determine if phage-insensitive mutants arise after phage treatments.

Chapter 2. Literature review

2.1 *E. coli* History

Escherichia coli (*E. coli*) is a Gram-negative, oxidase-negative, rod-shaped bacterium, named after the physician Theodor Escherich, who discovered this bacterium in 1885 (Jones & Roworth, 1996). *E. coli* are commonly found in the gut of warm-blooded organisms and can help protect the intestinal tract against bacterial infection. *E. coli* grow under both aerobic and anaerobic conditions with an optimal growth temperature of 37 ˚C (Torres, 2010). *E. coli* reproduces by binary fission with cell division occurring every 20 min in warm nutrient broth with glucose (Berg, 2008). *E. coli* uses oxygen when it is available to produce pyruvic acid, formic acid, hydrogen, and amino acids (Ingledew & Poole, 1984). In an anaerobic environment, *E. coli* undergoes anaerobic fermentation which produces end products of lactate, acetate, succinate, ethanol, carbon dioxide, and hydrogen (Torres, 2010).

Most *E. coli* strains are not pathogenic, but some serogroups can cause illness in humans, particularly in children and seniors (CDC, 2017a; Singleton, 2004). *E. coli* O157: H7 and other non-O157 serogroups produce Shiga toxins with virulence factors *Stx1* and *Stx2* that cause damage to intestinal and kidney cells in humans (Jones & Roworth, 1996). STEC can also cause illness in some warm-blooded animals such as rabbits and pigs but are not harmful to most other livestock species that serve primarily as carriers of the bacterium. Research has shown that STEC do not cause disease in cattle because they lack globotriaosylceramide (Gb3) vascular receptors for *Stx* (Pruimboom-Brees et al., 2000).

Non-O157 STEC are less studied than *E. coli* O157: H7, probably because they emerged more recently (Johnson et al., 1996) and were not as frequently associated with severe foodborne

illness. The development of molecular and immunological techniques to identify *stx* gene sequences and to detect expressed *Stx* has led to an increase in the recognition of the role of non-O157 STEC in outbreaks (Karch et al., 1999). From 2000 to 2006, the number of reported non-O157 STEC associated infections have increased 4-fold, raising concerns over their impact on public health (Clark, 2015).

2.2 Classification

E. coli serogroups are classified based on three antigens; H (flagellar), K (capsular), and O (somatic lipopolysaccharide) antigens (CFSPH, 2016). The somatic (O) antigens are important virulence factors which contain repeats of an oligosaccharide unit that can be found on the lipopolysaccharide of the outer membrane on *E. coli*. Somatic (O) antigens determine the O serogroup (DebRoy, 2011) with *E. coli* being divided into 166 O serogroups based on differences in their somatic (O) antigen structures (Wang & Reeves, 1998).

There are 6 major *E. coli* pathotypes that can cause diarrhea including: enteropathogenic *E. coli* (EPEC), Shiga-toxin-producing *E. coli* (STEC) (enterohemorrhagic *E. coli* [EHEC]), *Shigella*/enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC), and enterotoxigenic *E. coli* (ETEC) (Croxen et al., 2013). The *E. coli* pathotypes are categorized based on their difference in epidemiological, phenotypic traits, disease symptoms, and virulence factors (Levine, 1987).

STEC are strains of *E. coli* which can produce Shiga toxins (*Stx*) or Shiga-like toxins (verotoxins). In humans, STEC from EHEC are found associated with hemorrhagic colitis and hemolytic uremic syndrome (HUS) (CFSPH, 2016; FDA, 2016). Serogroups known to cause human disease, include *E. coli* O157: H7, *E. coli* O157: H-, *E. coli* O26, O45, O103, O111, O121,

O145, pathogenic *E. coli*, serogroups O55, O91, O80, O104, O113, O117, O118, and others (CFSPH, 2016). Undercooked ground beef, raw milk, cold sandwiches, water, unpasteurized apple juice, sprouts, and vegetables have been commonly associated with EHEC infections (FDA, 1992). The characteristics of the other *E. coli* pathotypes are shown in Table 2.1 (FDA, 2016; Corxen, 2013).

Table 2.1. Characteristics of *E. coli* Pathotypes

Pathotypes	Infection sources	Symptoms	*E. coli* detection method	Pathogenicity mechanism
EPEC	contaminated water, meat, produce	Watery diarrhea	PCR assays	Intimin proteins and EPEC adherence factor (EAF)
EIEC	Infected person	Dysenteric form of diarrhea	Invasion assays, PCR assay	Invasive phenotype coding by a high molecular weight plasmid
EAEC	Traveler's diarrhea, contaminated water and food	Watery secretory diarrhea	DNA probe	Pet toxin, EAST-1 enterotoxin, ShET toxin, and Pic toxin
DAEC	Infected person	Watery diarrhea, chronic inflammatory intestinal diseases	Unclear	Afa/Dr adhesins
ETEC	Water, soft cheeses, raw vegetables	Watery diarrhea with little or no fever	Y-1 adrenal cell assays, PCR assay	Enterotoxins

2.2.1 Shiga-toxigenic *E. coli* (STEC)

Escherichia coli (STEC) was first recognized as a human pathogen in 1982 when *E. coli* O157:H7 caused two outbreaks of hemorrhagic colitis in the U.S. (Karch et al., 1999). STEC-infected individuals presented the following symptoms: nausea, vomiting, abdominal cramps, low fever (<38.5˚C) and diarrhea (often bloody). In some cases the illness may progress to hemolytic uremic syndrome (HUS), thrombocytopenia purpura (TTP) or pulmonary edema (AHCIP, 2011). STEC can produce one or two toxins (*Stx1, Stx2*) that affect the distal ileum and colon in both adults and children. STEC can survive over a wide range of temperatures 4- 45°C (39-113°F), and overcome hurdles such as low pH (3.6) and low water activity (0.9) (AHCIP, 2011). *E. coli* O157: H7 is the most common STEC isolated, and combined with the other non-O157 serogroups known as the "big 6" (O26, O45, O103, O111, O121, O145), are linked to the majority of STEC outbreaks worldwide. The Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture classified the 6 non-O157 STEC strains as adulterants in June 2012 due to an increase in outbreaks and the severity of the illness they cause (FSIS, 2012). FSIS began to test for the presence of serogroups O26, O45, O103, O111, O121, and O145 in beef slaughter facilities in United States in 2012. Products that yield a positive result for the "big 6" are considered adulterated and must be further processed to destroy the pathogen (IFT, 2011).

As a part of STEC surveillance, the Centers for Disease Control and Prevention (CDC) requested that all non-O157 STEC isolates from foods involved in STEC outbreaks be sent to and confirmed by public health laboratories. Results of this surveillance system have shown that the number of non-O157 isolates being sent to CDC for serotyping is increasing yearly. In the United States, records showed that on average there are approximately 113,000 illnesses and 300

hospitalizations yearly that are caused by members of the "big 6" non-O157 STEC serogroups (CDC, 2017c).

The "big 6" STEC are also considered as adulterants in Canada; outbreaks caused by *E. coli* are continuously investigated and monitored by the Public Health Agency of Canada (PHAC) with the collaboration of federal and provincial/territorial partners (PHAC, 2015b). Cases related to STEC infections were proposed to be made nationally notifiable in all provinces and territories (PHAC, 2015a). These 6 *E. coli* serogroups have been isolated from contaminated meat, watermelon, lettuce, blueberries and strawberries in North America (Lajhar et al., 2017). The Public Health Agency of Canada (PHAC, 2017) reported 28677 cases of STEC infection from 1991 to 2015. In the United States, there were 46 outbreaks caused by non-O157 STEC from 1971 to 2010, 17 (45%) of which were foodborne. The type of food responsible was unidentified in two outbreaks with the remainder being associated with: dairy (n = 3), leafy vegetables (n = 2), game meat (n = 2), beef (n = 1), pork (n = 1), and fruits or nuts (n = 1) (Luna-Gierke et al., 2014).

The infective dose of *E. coli* O157:H7 and the STEC "big 6" sufficient to cause foodborne illness has been estimated at 10-100 cells. The infective dose for *E. coli* O157 is as low as 10 cells, but for serogroup O111 is between 6.5×10^9 and 9×10^9 (Kothary &Babu, 2001).The infective dose of the other serogroups has yet to be estimated (FDA, 1992, 2016).

2.2.2 *E. coli* O157: H7 reservoirs and resistance to environmental hurdles

In healthy cattle and sheep, *E. coli* O157: H7 can reside in the gastrointestinal (GI) tract and be shed in feces. Other animals such as pigs, camelids, rabbits, horses, dogs, cats, zoo mammals, and various free-living wild species have also been shown to harbour *E. coli* O157:H7 (Kudva et al., 1998). Research has shown that *E. coli* O157:H7 can survive up to 630 days in sheep

manure on-farm under normal environmental conditions (< 23 ºC) (Rogers and Haines, 2005; Howard et al., 2017). *E. coli* O157:H7 can survive in acidic environments (pH 1.2-2.0) such as those in the digestive tract due to the presence of acid resistance systems including the acid-induced oxidative system, the acid-induced arginine-dependent system, and the glutamate-dependent system (Lin et al., 1996). The arginine-dependent system is unique to STEC strains as this system is not present in generic *E. coli*. Some pathogenic STEC serogroups may be more resistant to acidic environments than others. However, in a previous study that compared the survivability of STEC O157 with non-O157 STEC in acid environments (pH 2.5) there was no significant difference in resistance to low pH (Pihkala et al., 2012).

2.2.3. Non-O157: H7 reservoirs and resistance to environmental hurdles

At least 150 non-O157 strains of *E. coli* have been identified to cause foodborne illness, and as with *E. coli* O157, some of the non-O157 serogroups can also cause HUS. Among the "big 6", O26 and O111 are most frequently isolated from patients involved in STEC outbreaks (ATCC, 2014; Huppertz et al., 1996) and isolates from these two serogroups are most frequently associated with illness (Karch et al., 1997; Werber et al., 2002). Cattle and sheep are major reservoirs for non-O157 serogroups which have been commonly isolated from livestock, primarily cattle (Bettelheim, 2007). Non-O157 serogroups have been likely underestimated as the laboratory methods for their detection are less developed than those used to detect *E. coli* O157: H7 (Karch et al., 1997). Based on the statistical data of all the research associated with STEC in food or environment samples, more studies have been conducted on *E. coli* O157: H7 (79%) than the other non-O157 serogroups (21%) (Vimont et al., 2006).

2.3. STEC detection methods

To isolate and identify STEC O157: H7 from stool and food samples, methods have been developed based on distinguishing its unique metabolic traits. There are two phenotypic characteristics of STEC O157: H7; one is the lack of rapid sorbitol fermentation, and the other is the lack of β-glucuronidase production. A common media used for *E. coli* O157:H7 isolation and detection is sorbitol MacConkey agar (SMAC). SMAC is a selective and differential medium which contains sorbitol and it is used for the detection of non-sorbitol fermenting *E. coli* O157: H7 (March & Ratnam, 1986). During fermentation, bacteria ferment sugar and produce acids that cause a decrease in medium pH. Thus, pH indicators can be used to generate a visual color change that is indicative of fermentation (Johnson et al., 2013). In SMAC, *E. coli* O157:H7 uses peptone rather than sorbitol for growth, and the resulting increase in pH enables it to be differentiated as clear colonies from the red colonies produced by other sorbitol fermenting *E. coli*. SMAC agar can also be made more selective for *E. coli* O157:H7 by adding cefixime and potassium tellurite to create cefixime-tellurite-SMAC (CT-SMAC). These additives further inhibit the background flora that can interfere with the identification of *E. coli* O157: H7 colonies (Zadik et al., 1993). Colonies that exhibit the clear morphology on SMAC can be further confirmed as *E. coli* O157: H7 through a Vero cell cytotoxicity assay, immunomagnetic bead separation (IMS), polymerase chain reaction (PCR), and agglutination using *E. coli* O157:H7 specific antiserum or anti-O157 latex reagents (Karch et al., 1999).

Non-O157 STEC serogroups are more difficult to identify than *E. coli* O157 as they all ferment sorbitol. The use of molecular and immunological techniques to detect *Stx* and *wzx* genes in the O-antigen gene cluster in non-O157 STEC serogroups contributed to recognition of their role in outbreaks (DebRoy, et al., 2011; Caprioli et al., 1994). Screening for non-O157 STEC

includes the use of cytotoxicity assays, immunological detection of *Stx*, DNA-based assays to detect *Stx* genes and agglutination with defined anti-O -sera to determine the O serogroup of the isolate (Karch et al., 1999). Some culture methods using more specific media to identify non-O157 serogroups are available including Rainbow Agar, and CHROMagar®(Bettelheim, 2007). To detect the STEC serogroups that survived phage treatment, the present study used Rainbow agar which contains chromogenic substrates that are specific for two enzymes (β-galactosidase and β-glucuronidase). *E. coli* possessing β-galactosidase generate blue-black coloured colonies and those possessing β-glucuronidase generate a red chromogenic substrate. The degree of colour development depends on the amount of the two enzymes. *E. coli* O157:H7 is glucuronidase negative which appears as a distinctive black or gray colour on Rainbow agar; while many other non-O157 serogroups produce more β-galactosidase as compared to β-glucuronidase. As a result, they typically appear as purple, violet or blue colonies. Other bacterial species that are not *E. coli* will either not grow or appear as white or cream-coloured colonies.

Nonculture methods are also used to detect non-O157:H7 STEC, such as enzyme immunoassay [EIA] or polymerase chain reaction [PCR] (CDC, 2007). Most non-O157 STEC strains can produce Shiga-toxins (*Stx*) and thus the presence of *Stx* toxin can be one of the indicators for the presence of STEC at screening of non-O157 STEC serogroups.

2.4 STEC virulence factors

Not all *E. coli* strains encode virulence genes; strains that lack virulence factors are called commensal *E. coli*, which are different than pathogenic *E. coli* strains in both genome content and phenotypic traits (Dobrindt et al., 2013). The genes that encode virulence factors on phages, plasmids, transposons, and pathogenicity islands (PAIs) in *E. coli* can potentially cause diseases

in humans (Torres and Kaper, 2002). However, the key virulence traits of STEC are the Shiga-toxins, which are the primary factors responsible for HUS. Humans infected with STEC strains show symptoms of differing degrees of severity (Hedican et al., 2009). As a pathogen, the virulence factors in most *E. coli* O157:H7 strains result from the production of two phage-encoded toxins; Shiga toxin 1 (*Stx*1) and Shiga toxin 2 (*Stx*2). Pathogenic *E. coli* strains carry the gene(s) encoding for *Stx1* and/or *Stx2*; and these may result in the production of either toxin or both simultaneously (Döpfer et al., 2012; Tarr et al., 2005).

Stx1 is the toxin produced by *Shigella dysenteriae* type I, and it is 50% homologous with *Stx2* which has a different amino acid sequence (Jackson et al., 1987; Tesh et al., 1993). *Stx1* and *Stx2* bind to target cells through B subunits which exhibit different affinities for epitopes through a receptor called globotriaosylceramide (Gb3). Gb3 is a sphingosine-based molecule which has attached a fatty acid side chain and terminal trisaccharide. Induced expression of receptor Gb3 allows both *Stx1* and *Stx2* to bind to target endothelial cells (Kar et al., 1992). The affinity of the two toxins for the binding site differs depending on the orientation of the terminal trisaccharide (Karmali and Mohamed, 2004). *Stx1* can readily bind to and detach from Gb3, while *Stx2* binds more slowly, but once attached it exhibits a more sustained affinity for Gb3 (Noris and Remuzzi, 2005). Thus, the *Stx2* toxin is 1000-fold more toxic than *Stx1*, despite the fact that they share similar amino acid sequences (Louise and Obrig, 1995). A study of *stx* producing *E. coli* isolated from children showed that those isolates associated with the expression of *Stx2* were more likely to result in HUS. Serogroups producing only *Stx1* often resulted in children developing only diarrhea, but otherwise remained asymptomatic. Strains isolated from HUS patients showed that 82 % carried *Stx*2, and 32 % *Stx*1 (Jenkins et al., 2003; Noris and Remuzzi, 2005). The A subunit on the surface of eukaryotic cells acts as a specific N-glycosidase which can block cell protein

synthesis, as it can disrupt the ribosomal subunit in eukaryotic cells by cleaving a single adenine residue from 28S rRNA (Bray et al., 2001; Endo et al., 1988). Shiga-toxins can cause damage by binding to podocytes, mesangial cells, and glomerular endothelial cells in human tissue (Tarr et al., 2005). Most STEC are acid resistant and thus can survive the acidic environments of the stomach and reach the large intestine where they can form attaching and effacing (A/E) lesions in intestinal epithelial cells (Gyles, 2007). The risk of HUS increases as more attaching and effacing (A/E) lesions are formed. The capacity to form lesions depends on the interaction between bacterial proteins encoded on a pathogenicity island (PAI) which is a chromosomal segment that is absent in commensal *E. coli.* The PAI carry genes for hemolysin production (*hly*) and P-related fimbriae are important virulence factors in *E. coli*. The pathogenicity island also contains a region termed the locus of enterocyte effacement (LEE), composed of several components including intimin, an outer membrane protein, and an encoded receptor protein (Tir) (Paton et al., 1998; Swenson et al., 1996). Intimin and Tir are proteins that mediate adhesion between the enterocyte cell and A/E pathogens; Tir is translocated by A/E bacteria to the membrane of cells and activates additional host signaling events for lesion formation (Luo et al., 2000).

Another virulence factor produced by O-antigen *E. coli* is an endotoxin called lipopolysaccharide (LPS). It acts as the prototypical endotoxin and induces a strong response by immune systems and acts as a mediator of the inflammatory response (Currie & Poxton, 1999).

2.5 Symptoms of STEC infection

After consuming food or drinks contaminated with STEC, most people will develop symptoms such as nausea, vomiting, abdominal cramps, and in some cases bloody diarrhea for a period of 1 to 9 days, with the onset of illness usually taking 3 to 4 days (Clark, 2015; FDA, 1992).

Patients with mild symptoms usually recover within a week, but more severe cases can result in hemorrhagic colitis and kidney failure through the development of hemolytic uremic syndrome (HUS). Symptoms of patients with HUS include fever, abdominal pain, pale skin tone, fatigue, small but unexplained bleeding from the nose and mouth, and decreased urination. Children, elderly, and people with a weakened immune system are most easily infected (CDC, 2013).

Hemolytic uremic syndrome (HUS) is a non-immune hemolytic anemia disease, which causes a decline in platelet counts and renal impairment. HUS is the most severe disease that arises as a result of STEC infection. Approximately 5 to 10% of patients affected by STEC will develop HUS, which has a mortality rate as high as 25% (Secher et al., 2015).

E. coli O157: H7 is the most common serogroup that causes HUS in North America and Western Europe (Farmer and Davis, 1985). *E. coli* O157 is associated with a higher risk of HUS because of its higher affinity of colonic cells as compared to the other 6 non-O157 serogroups, resulting in greater inflammation (Pollock et al., 2010). Other STEC serogroups (O111: H8, O103: H2, O121, O145, O26, O113, and O104) (Brooks et al., 2004; McCarthy et al., 2001; Sonntag et al., 2004) have also been linked to HUS cases. In 2011, an outbreak started in Germany and the illness was transmitted to people in 17 other countries (Bucholz et al., 2011). The source of this outbreak was fenugreek sprouts contaminated by STEC O104:H4, which caused 3965 people to be sick; 878 of the patients developed HUS, and 54 died.

Mild illness caused by STEC infection usually does not require medical treatment, as most people will recover within a few weeks (CDC, 2017a). For more serious conditions such as severe bloody diarrhea and HUS, patients require hospitalization. Rapid and accurate diagnosis of STEC infections is important so that appropriate early treatment can be started to prevent more serious

complications (Gould et al., 2009). Conventional treatment for STEC infection includes using electrolyte fluid therapy. Antibiotics are not recommended as a treatment for STEC infections as they may induce more toxin production as result of the induction of prophages containing Shiga toxin-encoding genes, worsening the condition (PHAC, 2001).

2.6 Transmission of STEC infections

2.6.1 Contaminated food and drinks

STEC transmission can be foodborne, waterborne, or be from person-to-person through fecal-oral transmission. Foodborne transmission occurs by eating food or drinking water containing STEC. STEC contamination often originates from feces coming in contact with meat, dairy, or produce (Chaves et al., 2014)). Prior to 1998, the most common source of outbreaks associated with *E. coli* O157:H7 in the U.S. was undercooked beef and unpasteurized milk (Rangel et al., 2005). Other products frequently associated with outbreaks include roast beef, salami, game meat, and cheese curds (AHCIP, 2011). Beef is the most common meat product adulterated with STEC (Midgley and Desmarchelier, 2001), and the contamination of cooked meat and dairy products has also been associated with STEC infections (Karch et al., 1999).

Since 1991, fruits and vegetables have been increasingly identified as vehicles of *E. coli* O157:H7 contamination, probably due to these foods comprising an increasing proportion of the daily diet (Fairbrother & Nadeau, 2006). Some sources of fresh produce contamination by STEC are animal feces, insect pests, contaminated water, equipment, or nematode vectors during pre-harvest (Oliveira et al., 2010). *E. coli* O157: H7 is capable of survival in the soil for more than 20 months, particularly if present in manure, and it can persist even longer in crop leaves and roots (Fairbrother and Nadeau, 2006). If STEC enter the soil they can flow with rainfall into the

groundwater and if the groundwater is used to irrigate crops, produce or drinking water can act as agents of transmission to humans and livestock (Castro-Rosas et al., 2012; Fairbrother and Nadeau, 2006). Furthermore, the use of animal manure as opposed to chemical fertilizer in the production of organic produce can also increase the risk of STEC contamination (Beuchat, 2002). During postharvest, potential sources of STEC contamination include feces, harvesting, processing equipment, transport vehicles, and insects. The survival of STEC is determined to some extent by their ability to remain metabolically active in foods. Bacterial growth and survival can vary in fresh produce. For example, Gram-negative bacteria are more likely to occur in vegetables as opposed to fruits where the low pH can inhibit bacterial growth (Beuchat, 2002; Ruimy, 2010). Also, STEC strains have been reported to be less active under conditions of low water activity, but the adaptation to stressful environments can allow them to survive dehydration using mechanisms such as biofilm formation (Møretrø et al., 2010; Skandamis et al., 2009). Additionally, STEC survival is also influenced by temperature, with lower temperatures < 25 °C being more favorable for the survival of STEC as compared to higher temperatures > 55 °C (Hiramatsu et al., 2005). During processing, the surfaces of cut tissues can contain nutrient rich fluids that promote the growth of molds and may also create conditions conducive for the growth of foodborne pathogens (Beuchat, 2002; Sofos et al., 1998). Some typical examples of produce involved in STEC infections are lettuce, sprouts, cantaloupes, potatoes, berries, apple cider, and fruit/vegetable juices (Beuchat, 2002). A significant portion of fruit and vegetable outbreaks have been associated with ready-to-eat foods, such as bagged salads, which represent a greater hazard to consumers as physical disinfection treatments such as cooking are not applied (Karch et al., 1999).

Some *E. coli* O157: H7 outbreaks have originated as a result of the consumption of contaminated drinking water and beverages (Jones and Roworth, 1996). STEC infections have also

been linked to summer activities such as swimming in lakes or pools due to the accidental consumption of contaminated water (Jones and Roworth, 1996).

2.6.2 Person-to-person transmission

Secondary cases during outbreaks can easily occur in daycare facilities and indoor public environments through person-to-person transmission via the fecal-oral route, either directly (e.g., households, public facilities) or indirectly (contaminated water) (Belongia et al., 1993). The primary sources of STEC infection through person-to person transmission are cross-contamination of clean food handling areas from dirty areas (5%), contaminated equipment (3.7%), and food handling by asymptomatic or ill people (7%) (Møretrø et al., 2010). Some facilities like restaurants, day care centers, nursing homes and hospitals are at a high risk of STEC contamination unless strict hygienic measures and proper food handling techniques are practiced. Food prepared in these facilities has a higher risk of contamination as large batches are prepared that have the potential to come in contact with untrained personnel that may carry STEC. (Erickson and Doyle, 2007; Fairbrother and Nadeau, 2006; Karmali et al., 1988; Reida et al., 1994).

2.6.3 Animal Contact

Direct contact between human and animals through farm visits and petting zoos also could trigger STEC outbreaks. STEC are found in the feces of wild and domestic animals including cattle, sheep, goats, pigs, water buffalo, deer, dogs, cats, rodents, birds, insects, and horses. The sources of STEC in these animals are contaminated drinking water, feed, and the farm environment (Fairbrother and Nadeau, 2006). Inadequate handwashing facilities and a lack of information informing visitors of the importance of personal hygienic practice can also increase the risk of

STEC illness, particularly for people with compromised immune systems (Conrad et al., 2017; Shukla et al., 1995).

2.7 Epidemiology of STEC in human infection

Several surveillance networks have been established to track foodborne outbreaks in order to reduce public health risks, the Foodborne Diseases Active Surveillance Network (FoodNet) is one of the major networks in United States. FoodNet is part of the Centers for Disease Control and Prevention's (CDC's) Emerging Infections Program (EIP) which has the goals of determining and tracking the sources of foodborne illness across the U.S., monitoring the trend of foodborne diseases over time, providing information to improve public health, and developing more effective methods to reduce the number of foodborne illnesses (CDC, 2008). In Canada, cases involving STEC are monitored using different surveillance systems including FoodNet Canada, the National Enteric Surveillance Program (NESP), Provincial and Territorial Reportable Disease Surveillance System, National Studies on Acute Gastrointestinal Illness (NSAGI), and the Canadian Notifiable Disease Surveillance System (CNDSS). The surveillance systems obtain data from local public health authorities or provincial and territorial public health ministries, and estimate foodborne illness caused by STEC (PHAC, 2015a).

E. coli O157:H7 is the largest source of STEC infection in North America and is associated with 15%-30% of reported bloody diarrhea cases (PHAC, 2001). Hemorrhagic colitis may develop after STEC ingestion, with 10% of hemorrhagic colitis victims developing HUS (Pohlenz et al., 2005). The average rate of HUS caused by STEC is 2.1 cases per 100,000 individuals per year, while the highest peak occurs with children under 5 years old, and the lowest with adults 50-59 years old (Ruggenenti et al., 2001). *E. coli* O157: H7 tolerate a broader temperature range; from

6.7°C (44° F) to 43.8°C (111° F), as compared to generic *E. coli* strains. *E. coli* O157 can survive freezing, dry, and acidic environments (Clark, 2015). HUS caused by *E. coli* O157: H7 has reported to be seasonal with incidence peaking during the warmer months (June-September) (Ruggenenti et al., 2001). In the U.S., the prevalence of *E. coli* O157:H7 infection is low (< 1%) in individual animals, but higher in herds (10%-20%) (Hancock et al., 1994). The prevalence of non-O157 strains in cattle depends on the detection method ranging from 10% to 20% or even higher. For example, research has shown a prevalence of 94% and 51% for serogroups O26 and O103 in cattle feces, respectively (Armstrong et al., 1996). The percentage of animals that carry STEC is higher in calves compared with mature cattle and is influenced by feed type, antimicrobials added to feed, and transportation stress (USDA, 2014).

Non-O157 serogroups are more likely to cause uncomplicated diarrhea; approximately 80% of STEC-related diarrhea cases are caused by non-O157 STEC (Rangel et al., 2005; Hughes et al., 2006). STEC serogroups O26, O103, O111, and O145 have been isolated from a variety of animals in the U.S., the United Kingdom, Canada, Europe, Australia, Argentina, and Hong Kong. *E. coli* O157 has been found in many other countries including Japan, Korea, China, Argentina, and Brazil (Fairbrother and Nadeau, 2006).

The largest *E. coli* O157: H7 outbreak in the U.S. occurred from December 1, 1992, through February 28, 1993, among 4 western states: Washington, Idaho, Nevada, and California. This outbreak linked hamburgers consumed at a fast-food restaurant chain to 501 cases with 151 hospitalizations, 45 cases of HUS, and 4 deaths. Most HUS patients associated with this outbreak were children (Bell et al., 1994).

In Europe, the largest outbreak associated with *E. coli* O157: H7 occurred in Central Scotland in 1996, which involved 120 patients, 34 of whom developed HUS, resulting in 16 deaths. The source of the pathogen was identified as contaminated cooked meat (Dundas et al., 2001).

Due to the public health and financial threats of STEC, the meat industry has adopted many pathogen reduction interventions including physical disinfection methods, chemical sanitizers, and biological control methods. Other food commodities are also now being linked to outbreaks such as fruit and vegetables, flour, and dairy products, and as result interventions that are suitable for these food industries are also urgently needed. Data on the largest outbreaks reported worldwide are summarized in Table 2.2 (Rangel et al., 2005; Yoon and Hovde, 2013).

Table 2.2. Major Outbreaks Caused by STEC O157: H7 and non-O157 (>50 cases and ≥1 fatality)

Year	Strain	Total No. cases	Food	Country	References
2016	O26 and O121	63	Flour	US	(CDC, 2016)
2015	O26	55	Chipotle Mexican Grill Restaurants	US	(CDC, 2015)
2009	O157:H7	80	Raw prepackaged cookie dough	US	(CDC, 2009)
2007	O157	Recall	Topp's Brand ground beef patties	US	(CDC, 2007)
2006	O157:H7	71	Taco Bell	US	(CDC, 2006)
1996	O157:H7	490	Cooked meat	Scotland	(Ahmed & Donaghy 1998)
1995	O111:H-	161	Uncooked fermented mettwurst	Australia	(Hanna et al., 1996)
1993	O157:H7	731	Hamburgers	US	(Meng et al. 2007)

2.7.1 Outbreaks related to produce commodities

Vegetable and fruits can become contaminated with STEC originating from sources such as manure, wild animals, contaminated water, or during post-harvest processes. In the U.S., fresh produce is a major food safety concern as it is one of the leading causes of foodborne illness (López-Gálvez et al., 2009). From 2006 to 2016, there were 27 outbreaks associated with STEC. Eight of them were caused by fresh produce; 3 from sprouts, 3 from raw lettuce and ready-to-eat salad, and 2 from spinach and spring mix vegetables. Among all the outbreaks, *E. coli* O157: H7 caused 5 of them, and the other 3 were associated with STEC O26, O121, and O145 (CDC, 2017b).

In 2012, 58 patients were reported to be infected by *E. coli* O157: H7 linked to cut romaine lettuce from bagged salad. This outbreak involved 9 states in the U.S. with patients ranging from 1 to 94 years of age. A total of 33 of the patients were hospitalized and 3 developed HUS, but there were no fatalities (CDC, 2017b).

Increasing numbers of foodborne illness outbreaks linked to produce is probably related to the increased per capita consumption of fresh fruits and vegetables in North America during the last decade, which is due to healthy eating campaigns, their year-round availability, and the convenience of having entre-sized portions pre-washed and ready to eat products (Taban et al. 2011; Sagoo et al., 2003). When vegetables are cut or shredded, they release plant cellular fluids which provides nutrient for microorganisms to grow. Cut vegetables can also provide shelter to foodborne pathogens; it has been showed that the cut edges can allows pathogens internalization in the plant tissue. Impropriate post-harvesting processing and poor hygiene also increased the likelihood of cross-contamination (CFIA, 2014).

Outbreaks related to fresh produce commodities are included in Table 2.3 (CDC, 2017b). The consumption of raw sprouted seed products has become popular because of their nutritional value and health benefits. However, sprouts are considered to be a minimally processed food and the lack of a significant intervention to reduce foodborne pathogens makes it a high-risk product. Thus, sprouts and sprouted seeds have also become a growing food safety concern. In the past two decades, sprouts have been associated with 55 foodborne outbreaks and these affected over 15,000 people (Erdozain et al., 2013). Results from previous studies have shown that most of the pathogens in sprouts likely originated from contaminated seeds. Contamination of seeds can occur during pre-, and post-harvesting. As temperatures and nutrient availability typically increases during germination, low pathogen numbers in seeds can increase and reach levels that are sufficient to cause disease (FAO, 2011). In July 1996, approximately 8000 people in Sakai City, Japan were infected with *E. coli* O157:H7. The majority of the patients were school children and teachers as the school lunch system provided the same food items to all schools in the area in which the outbreak occurred. In this incident, more than 4000 students became ill, 398 were hospitalized and 4 died. Uncooked white radish sprouts served in the school lunches were identified as the most probable source of the outbreak (Buck et al., 2003; Michino et al., 1999).

Table 2.3. STEC outbreaks related to fresh produce between 2006 to 2017 in North America (CDC, 2017b; PHAC, 2018)

Year	Source	STEC serogroups	Location	Cases	Hospitalized	HUS
2006	Fresh Spinach	*E. coli* O157:H7	Multi-state (26), US	200	102	31
2010	Shredded romaine lettuce	*E. coli* O145	Multi-state (5), US	30	12	3
2011	Romaine lettuce	*E. coli* O157:H7	Multi-state (9), US	58	33	3
2012	Organic spinach and spring mix	*E. coli* O157:H7	Multi-state (5), US	33	15	2
2012	Raw clover sprouts at Jimmy John's restaurants	*E. coli* O26	Multi-state (11), US	29	7	0
2013	Ready-to-eat salad	*E. coli* O157:H7	Multi-state (4), US	33	10	2
2014	Raw clover sprouts	*E. coli* O121	Multi-state (6), US	19	8	0
2015	Costco rotisserie chicken salad	*E. coli* O157:H7	Multi-state (7), US	19	5	2
2016	Alfalfa sprouts	*E. coli* O157	Multi-state (2), US	11	2	1
2017	Romaine lettuce	*E. coli* O157:H7	Multi-province (5), Canada	42	17	1

2.8 Current antimicrobial interventions on fresh produce to reduce STEC

2.8.1. Government regulations on food safety

Pathogens may contaminate fresh produce before or after harvesting and they can be difficult to remove once established. Proper management at pre- and post-harvest stages is essential if preventive measures are to reduce the risk of microbial contamination of fresh produce. Standards and regulations have been developed to promote the safe and hygienic harvesting, processing, packaging, and transportation of produce in the U.S. and Canada. However, despite all efforts, epidemiological studies have shown that there has been a significant increase in the number of foodborne illnesses associated with produce (CSPI, 2015).

The U.S. Food and Drug Administration encourages fruit and vegetable manufacturers to follow Good Agricultural Practices (GAP) and Good Handling Practices (GHP) during production, packaging, handling, and transportation to minimize the risk of food safety hazards (FDA, 1998). At pre-harvest, it is important to follow GAP, which includes avoiding the overuse of nitrogen fertilizer, applying compost, and ensuring proper human and animal hygiene. High nitrogen levels can increase the risk of pathogen outbreaks (FAO, 2011). Composting is a bio-oxidative process that frequently achieves temperatures above 55 °C which can reduce the risk of pathogens and phytotoxins coming into contact with agriculture products (Bernal et al., 2009). During manufacturing, there are a variety of steps where potential hazards could be introduced such as slicing and cutting which weakens the natural barriers in fruits and vegetables that deter or prevent pathogens from contaminating sources. Thus, the principles of Hazard Analysis Critical Control Point (HACCP), and Good Hygiene Practices (GHP) are essential for postharvest processes to reduce the risk of produce contamination (James, 2006; Lynch et al., 2009).

2.8.2. Chemical interventions

The limited number of antimicrobial treatments that can be applied to fresh cut produce makes it difficult to ensure that it is unadulterated. A previous study showed that water alone resulted in less than a 1 log CFU/g reduction in coliforms on fresh produce (Allende et al., 2008). The most common way to reduce microbial counts on produce is to wash it with chemical disinfectants (López-Gálvez et al., 2009).

Currently, there are a variety of disinfectants applied to fresh produce in an effort to reduce the risk of pathogens entering the food chain. The most common commercial disinfectants currently used with fresh produce is chlorine prepared from sodium hypochlorite. It is approved for use with certified organic produce and is added into processing wash water at ≤ 200 ppm (GPO, 2017). However, no sanitizing agent can be applied at a level that will effectively kill all pathogens in produce without having a negative impact on produce quality.

Chlorine compounds has antimicrobial action against bacteria, such as the chloramines that arise from sodium hypochlorite. Chloramines can disrupt phospholipids, inactivate enzymes, and promote the degradation of lipids and fatty acids (Allende et al., 2008; Estrela et al., 2002). Sodium hypochlorite degrades fatty acids in cell wall and transform them into fatty acid salts and glycerol. The hypochlorous acid present in sodium hypochlorite solution will releases chlorine when it contacts with organic tissue and forms chloramines that degrades the amino acid which is required for cell metabolism (Estrela et al., 2002). Sodium hypochlorite chlorination action produce hydroxyl ions which serves as the bacteria inactivation mechanism, high concentration of hydroxyl ions can promote irreversible oxidation on bacterial essential enzymatic sites by alteration of proteins located in the cytoplasmic membrane which lead to enzymatic inhibition, biosynthetic

alterations, phospholipid degradation, and eventually cause the death of the bacteria. (Estrela et al., 1995). There are many advantages of using sodium hypochlorite, as it is readily soluble in water, relatively non-toxic at approved concentrations, leaves no toxic residuals and does not alter the colour of the produce. Hypochlorite is effective against a broad range of microorganisms including; viruses, bacteria, fungi, and protozoa (Rutala and Weber, 1997). The most common concentration used with fresh produce is 50-200 ppm with a contact time of 30 min. Used in this manner hypochlorite can usually achieve 1 to 2 log reduction in bacterial numbers (Allende et al., 2008; Sapers and Gerald, 2001). There are health concerns associated with chlorinated by-products if the chlorine is not completely removed post-processing or if it is used above recommended limits. Accidental consumption of chlorinated by-products may cause negative effects in humans. For example, halo acetic acids can irritate mucous membranes, and toxic chlorine gas may also be formed when chlorine reacts with organic compounds in water. Residual levels of chloroform have also been detected in chlorine-treated water (Rutala and Weber, 1997). Consumption of sodium hypochlorite can cause gastric damage, diarrhea, and vomiting, (Kaufman and Keila, 1989; Lenntech, 2017; Zock et al., 2009). In some instances, allergic reactions caused by accidental consumption of hypochlorite have also been reported in both humans and pets (Habets et al., 1986). Additionally, the emergence of pathogens that exhibit resistance to hypochlorite is also cause for concern as they become more difficult to eliminate (Tomás-Callejas et al., 2012). Moreover, maintenance of targeted concentrations of chlorine with sodium hypochlorite can also pose a challenge as it can be inactivated by exposure to UV light, high pH (> 7.5), high temperature, and organic materials (López-Gálvez et al., 2009). Therefore, during processing, chlorine rapidly becomes inactive (0.75g/day) especially with the presence of organic materials from fresh produce,

and as a result the level of free chlorine must be monitored to ensure that concentrations are sufficient to ensure disinfection.

Acidified sodium chlorite is another commonly used sanitizer which has been reported to be more effective than hypochlorite as it is less affected by organic matter and does not react with phenolics to produce a foul smell (Singh et al., 2002). Sodium chlorite generates chlorine dioxide when added to water, and is approved by the FDA for disinfecting fruits, vegetables, and poultry. Chlorine dioxide produces fewer toxic chlorinated by-products, exhibits higher antimicrobial activity at neutral pH and is most effective when an equilibrium is reached between hypochlorous acid and hypochlorite at a pH of 7.5 (Tuthill et al., 1998). Sodium chlorite targets the bacterial cell membrane where it produces lethal lesions and can negatively affect protein synthesis (Gómez-López et al., 2009). Researchers have shown that more than 90% of the microorganisms (e.g., bacteria, yeast, and mold) on lettuce and baby carrots are killed after exposure to 34 mg/L of sodium chlorite for 30 min (Singh et al., 2002). Sodium chlorite has also been used to disinfect produce seeds prior to planting, and others have explored the ability of chlorine dioxide to kill *E. coli* O157: H7 on seeds. Application of 100mg/L chlorine dioxide resulted in a 2.7 $\log_{10}$ CFU/g reduction of *E. coli* O157:H7 on alfalfa seeds (Taormina and Beuchat, 1999).

Other treatments such as bromine, iodine, trisodium phosphate, quaternary ammonia compounds, acid and hydrogen peroxide have also been investigated for their ability to eliminate pathogens on produce (Table 2.4), but none are capable of completely annihilating pathogens. Pathogens in the seed can grow rapidly during germination due the increase of temperature and humidity and become the source of contamination in plants. Consequently, more efficient methods of disinfecting seeds and germinated produce are needed (Fan et al., 2008; Parish et al., 2003;

Singh, 2003; USDA, 2017b). More detailed information on the chemical sanitizers current used as well as their advantages and disadvantages are shown in Table 2.4 (Rutala and Weber, 1997; Allende et al., 2008; Sapers and Gerald, 2001).

Table 2.4. Chemical disinfection methods used in fruits and vegetable production

Method	Current use	Advantage	Disadvantage	Research comments	Use in organic food
Chlorine dioxide	5 ppm is allowed in whole fruit and vegetables 1 ppm in peeled potatoes	Less reactive with organic material than hypochlorite Less chlorinated by-product More effectiveness against microbial activity at neutral pH	Not allowed to use on cut produce	Studies relate to fresh produce commodity with concentration 1 to 500 ppm Achieved few logs reduction against *E. coli*, *E. coli* O157:H7, *Salmonella*, *Cryptosporidium parvum* oocycts	Yes
Bromine	Not used as sanitizer	Enhance its effectiveness when combining with chlorine compounds	Lack of information on its by-products	Effective against *Salmonella, E. coli, and Staphylococcus aureus*	No
Iodine	Commonly used on equipment and food contact surface, not on product	Less corrosive than chlorine Broad antimicrobial spectrum	Corrosive above 50°C Changes product colour	May effective on spore-forming microorganisms	No
Trisodium phosphate	Raw poultry	Less corrosive	Ineffective on *Listeria*	1-15% concentration can	No

		than other methods	High pH (11-12)	reduce pathogen population from 0 to 6 logs	
Quaternary ammonium compounds	Commonly used on equipment and food contact surface	No colour, no odour Stable to heat Noncorrosive Good penetrating ability Stable to organic material	Not effective at low pH High cost	Reduced 95% of native orange-surface microflora	No
Acids	Acidification for food preservation Spray on meat carcasses On citrus at 200ppm	Economical	Low pH Effectiveness variable, depends on acid type	Effective on control of native populations and specific pathogens (*Salmonella spp. Campylobacter spp, Yersinia spp, Shigella spp, Listeria spp)* 200 ppm peracetic acid is effectively used in fruits and vegetables	Depends on the type of acids used
Hydrogen peroxide	On package and food contact surfaces	Effective for spore-forming bacteria Rapid effects	changes colour of products	Vapour and aqueous dips (1-5% range)	Yes

2.8.3 Non-chemical disinfection methods

Some other disinfection methods used to reduce pathogens in fruit and vegetable production involve both physical and biological control methods (Table 2.5). Physical methods such as ozone, irradiation, heating, and canning can decrease the risk of foodborne illness, and extend produce shelf-life while at the same time reducing pathogens and spoilage microorganisms. Other novel technologies like plasma processing, high-pressure processing, electric field and ultrasound methods may also contribute to pathogen reduction but are often prohibitively expensive. Once products leave the production line, cold chain storage and management are essential to reduce the risk of microbial contamination (Lynch et al., 2009).

The use of biocontrol methods as a natural antagonist to control bacterial contamination during food processing may overcome some of the limitations of other disinfection methods (Kazi and Annapure, 2016). Tom Unruh, an entomologist from the University of Washington, defined biological control as "a means of keeping pests below damaging levels through the activities of predators and parasitoids." An effective natural enemy should be adapted and exhibit a preference to target the desired pest without interfering with other microorganisms. The biocontrol agent should be able to reproduce rapidly and remain viable over a range of changing environments (Unruh, 1993). The use of phage to control target pathogens in food is a classic example of a biological control method that could have beneficial applications in the produce industry.

Table 2.5. Non-chemical disinfection methods commonly used in fruits and vegetable production (Kazi and Annapure, 2016; Gómez-López et al., 2009)

Method	Current use	Advantage	Disadvantage	Research comments	Use in organic food
Ozone	Water treatment	Requires low concentration and short contact time Broad antimicrobial spectrum Good penetrating ability Effective on nontoxic products	Possible injury to products Corrosive Possible effect on flavour and colour Unstable Possible damage to employees in process facilities	Effective against pathogens in fruits and vegetables 3 to 4 log/g reduction of *Salmonella* and *E. coli* populations	Yes
Irradiation	1-10 kGy used to reduce pathogens in food < 1 kGy used to inhibit sprouting in products	No chemical used Extend product shelf-life Can be applied after packaged	Public concern of irradiation Possible effect on sensory	Effectiveness depends on type of pathogen	No
Thermal	Used on ready-to-eat products	No chemical used Good penetrating ability	Possible effect on flavour and colour	Effective against most of the pathogens except thermophilic bacteria.	Yes

	Canning products	Low cost Extent product shelf-life	Not suitable for some products		
Phage	In apples to control pathogen from post-harvest In poultry for intestinal pathogen control Fermentation in meat and dairy products	No chemical used	Narrow antimicrobial spectrum Public concern of consuming live microorganisms	Effectiveness depends on application method, phage type, and bacterial specie	Yes

2.9 Phages

Phages are nature's counterbalance to bacteria as these bacterial viruses have an estimated population of 10^{13} on earth (FDA, 2015). Most phages are safe to add to food products due to their high specificity for their bacterial host, which means that they do not harm other members of the bacterial community or infect eukaryotic cells in animals, plants, or humans (Lone et al., 2016).

Cofactors (e.g., Ca^{+2} or Mg^{+2}) are required for the absorption of phage to bacterial cells and subsequent infection (Clokie and Kropinski, 2008). These cations can enhance phage absorption by neutralizing the negative electrostatic forces between phage and the host and cause subtle alterations in receptor sites (Landry and Zsigray, 1980). Temperature is another important factor which affects phage efficacy through altering attachment, penetration, lytic activity, and multiplication. Most phages remain active at temperatures ranging from 5 - 35 °C, but some can remain infective at 40 - 90 °C. However, most phages are inactivated by low pH and an acid environment such as in the stomach (Sawant, 2015). Under conditions of low pH bacteria often exhibit a reduced growth rate which may also inhibit the lytic cycle of phage.

Recently, phage therapy has been seen as a possible alternative to antibiotic therapy (Lone et al., 2016). Phage therapy is the application of phage to reduce the presence of pathogens or nuisance bacteria. It has potential applications in veterinary medicine, agriculture, food microbiology, and the treatment and prevention of infection in humans (Kutter et al., 2010).

2.9.1 Phage taxonomic classification

There are 19 phage families that were classified by the International Committee on Taxonomy of Viruses (ICTV) based on their virion type, genome, morphology, and nucleic acid

(Ackermann, 2009; Grath and Sinderen, 2007). Among all phage families, 17 have either double-stranded (dsDNA) or single-stranded (ssDNA) DNA genomes, 2 have RNA genomes; 5 families are enveloped. More details of DNA phage classification are summarized in Table 2.6.

Table 2.6. ICTV classification of phage (Grath and Sinderen, 2007)

Family	Enveloped	Morphology	Nucleic acid	Host
Myoviridae	No	Contractile tail	Linear dsDNA	Bacteria and archaea
Siphoviridae	No	Noncontractile tail	Linear dsDNA	Bacteria and archaea
Podoviridea	No	Noncontractile tail	Linear dsDNA	Bacteria
Lipothrixviridae	Yes	Rod shape	Linear dsDNA	Thermophilic archaea
Rudiciridae	No	Rod shape	Linear dsDNA	Hyperthermophilic archaea
Ampullaviridae	Yes	Bottle shape	Linear dsDNA	Acidianus archaea
Bicaudaviridae	No	Lemon shape	Circular dsDNA	Acidianus archaea
Clavaviridae	No	Rod shape	Circular dsDNA	Archaea
Corticoviridae	No	Isometric	Circular dsDNA	Bacteria
Cystoviridae	Yes	Spherical	Segmented dsRNA	*Pseudomonas* bacteria
Fuselloviridae	No	Lemon shape	Circular dsDNA	Thermophilic archaea
Globuloviridae	Yes	Isometric	Linear dsDNA	*Pyrobaculum* and *Thermoproteus* archaea
Guttaviridae	No	Ovoid	Circular ssDNA	*Sulfolobus* newzealandicus archaea
Inoviridae	No	Filamentous	Circular dsDNA	Bacteria
Leviviridae	No	Isometric	Linear ssDNA	Enterobacteria
Microviridae	No	Isometric	Circular dsDNA	Enterobacteria, intracellular parasitic bacteria, and spiroplasma
Plasmaviridae	Yes	Plemomorphic	Circular dsDNA	Mycoplasma bacteria
Tectiviridae	No	Isometric	Linear dsDNA	Bacteria and Archaea

2.9.2. Lytic and lysogenic pathway

2.9.2.1. Lytic pathway

Phage are composed of a nucleic acid molecule (DNA or RNA) that is surrounded by a protein shell. Most phages inject their genetic material in the host and use its energy and biochemical machinery to replicate through a lytic cycle. Infection by temperate phages may also lead to the propagation of phage during lysis if the lytic pathway is induced. At the first stage phages attach to specific receptors located on the surface of bacterial cells followed by injection of their genomes into the host. Phages contact the bacterial host through phage tail fibers which interact with a variety of bacterial surface receptors including flagella, capsules, proteins, teichoic acids, and lipopolysaccharides (Lindberg, 1973). The first stage of phage infection involves a non-specific collision with the host bacterium in which the tail comes in contact with bacterial cell surface receptors. This interaction becomes more intimate as the tail fiber proteins bind to cell surface receptors. Once the phage has bound to its receptor, the injection step can be initiated as the phage penetrates the outer membrane and cell wall of the bacterium. Most phages can degrade and create pores in the bacterial cell wall through the production of holins. Genetic material is then injected into the cytoplasm through these pores helped by the mature phage tail (Molineux, 2006). After entrance into the bacterial cell, the phage DNA/RNA uses the host's chemical energy and biosynthetic machinery to produce nucleases that degrade the bacterial host DNA to nucleotides which are then used to synthesize phage DNA (Kropinski, 2006; Scholl et al., 2005). Depending on the strain, the lytic cycle of most phages normally requires 20 to 40 min to complete (Todar, 2012). In an attempt to resist phage infection, some bacteria secrete polysaccharides which serve as a physical barrier against phage attachment to the cell membrane. However, some phages can produce polysaccharides depolymerases that degrade these polysaccharide, enabling them to

contact outer membrane receptors on the surface of the bacterial cell (Hughes et al., 1998; Molineux, 2006).

2.9.2.2. Lysogenic pathway

The lysogenic cycle can also result in the replication of phage within bacterial cells. The main difference between lytic and lysogenic cycles is that the lysogenic cycle does not result in destruction of the host cell. Instead, phage DNA is integrated with the host chromosome to form a prophage and its lytic functions are repressed (Kropinski, 2006). Prophages reproduce along with the host chromosomes and are transferred into daughter cells during cell division (Jiang and Paul, 1998). The lysogenic cycle will continue unless environmental signals trigger the phage to return to the lytic cycle (Trinh et al., 2017). Both lytic and lysogenic cycles can occur in conjunction with the growth of the host bacterium. Unlike lytic phages, lysogenic phages have a symbiotic relationship with their host as they may carry genes that offer a metabolic advantage to the host and protect it from lytic phages (Weinbauer et al., 1999).

2.9.3. Advantages using phage technology as a biocontrol method

There are many advantages of using phages for biocontrol as they are present in nature, can be combined with other pathogen reduction interventions, and are harmless to humans and animals. Phages have been previously used for clinical applications with success in the Soviet Union, Eastern Europe, France, Switzerland and Egypt as a safe treatment against a broad spectrum of bacterial infections in humans (Garcia et al., 2008; Sulakvelidze, 2005). Furthermore, as phages replicate in the presence of bacterial hosts, they offer the possibility of ongoing control of the bacterial population. Phage also exhibit activity against biofilms and owing to their high degree of specificity, have application in pathogen detection (FDA, 2015; Garcia et al., 2008).

2.9.4. Limitations

Although there are many advantages of using phage as a biocontrol method in food, there are some limitations and factors which influence the effectiveness of phage. For example, phage efficiency is influenced by phage characteristics (e.g., initial dose, absorption rate), physical and chemical properties (e.g., temperature, water activity or a_w), properties of food (i.e. phages are more effective in liquid as compared to solid matrices), bacterial host population, carriage of undesirable genes, and phage insensitive mutants (PIM) (Sawant, 2015).

One limitation of using phage in food production is that low temperatures limit the metabolic activity of bacteria, which may also decrease the effectiveness of phage therapy as phages use their host's metabolic machinery to replicate. One indicator of phage activity is the measurement of the multiplicity of infection (MOI). The MOI is the ratio of the number of phage particles introduced to the number of infected target cells present. A previous study showed that a minimal MOI is required for some phages to efficiently kill *E. coli* O157 (Kudva et al., 1999) as only a minor decline in O157 population was observed when phages were used with MOIs at 10^2 PFU/CFU. To achieve a significant reduction in the *E. coli* O157 population, phage with an MOI of at least 10^3 PFU/CFU was required.

The efficacy of phages can also be affected by environmental factors such as oxygen content, and food matrices. From a previous study, a cocktail of 4 coliphages was more effective against *E. coli* under anaerobic conditions within the intestine than under aerobic conditions in the laboratory (Chibani-Chennoufi et al., 2004). Phage effectiveness can be influenced by food surface structure as food with irregular surfaces can make it more difficult for phage to come in contact with host bacteria (Badawy et al., 1985; Stine et al., 2005). Also, phages exhibit greater efficacy

in liquid compared to solid foods as phage can more readily access bacteria as the thermal motion-drive of particles occurs more readily in fluids than in solids (Ramiasa et al., 2013).

The other concern of using phage as a biocontrol method to against pathogens is that phages may carry undesirable genes (i.e. virulence and antimicrobial resistance) which may be unsafe when phages are used as a biocontrol method on foods (FDA, 2005; Lancu, 2016). To assess the risk associated with phage acquisition and carriage of undesirable genetic information from the environment, Jiang and Paul (1998) studied the potential for gene transfer between bacteria isolated from different water samples with the present of phages, the results showed that the genetic material from a previous host (40.5%) were transferred to a new host if it is infected by a phage. Both lytic and temperate phages can transduce chromosomal and plasmid DNAs which may be undesired from the microorganisms present in the surrounding environment into their host. These data indicate that phages may serve as reservoirs and source of exogenous genes from the other microorganisms (Miller et al., 1991; Morrison et al., 1978; Saye et al., 1987; Stotzky, 1989). However, the phage transduction frequency is low; often below the detectable limit, and phage products approved for its use on food are required to have their whole genome sequenced to ensure there are not undesirable genes present (Jiang and Paul, 1998; FDA, 2015).

2.9.5. Lysis from without

Research data indicates that phages are very active and effective against growing pathogens but less effective against bacterial cells that are in a static state (Oliveira et al., 2014). Ideally, the most appropriate temperature range for phage activity is between 30 and 45 °C with lytic activity declining as the temperature drops below this range (Obeso et al., 2008). Thus, for effective control at low temperatures, some phages that exhibit high titers can eliminate pathogens through "lysis

from without" mechanisms (Oliveira et al., 2014). Lysis from without (LO) occurs when phages are applied with a high MOI, therefore, a substantial number of phages are adsorbed to the host and cause bacterial lysis due to cell-wall destruction, without phage progeny being released (Abedon, 2011a; Molineux, 2006). Not all phages can cause lysis from without as it depends on the presence and expression of specific genes and is most frequently associated with T-even phages (T1, 4, 6). In T4 phage, gene 5 encodes a baseplate protein called gp5 which affects cell envelope penetration (Young, 1992). The gp5 protein alone is not sufficient to induce cell lysis when only a few phages are attached, but when a substantial number of phages become attached to the bacteria, lysis of the cell envelope can occur (Abedon, 2011a). Phages that exhibit the potential to lyse bacterial cells but for which the LO mechanism has not been confirmed, are termed to have undergone "abortive infection", a process in which the bacterium is killed without phage multiplication (Schlesinger, 1969).

At low temperatures < 25 °C, bacterial cell division can be inhibited, and the normal lytic cycle of phages cannot be completed. The LO mechanism may enable phages to be exploited by the food industry as a biocontrol method to reduce foodborne pathogens at refrigeration temperatures where produce is typically processed and stored (Lone et al., 2016).

2.9.6 Bacterial resistance and phage evasion tactics

Phage resistant bacterial mutants are another limitation for the use of phages as a biocontrol method. Phages are tested before they are employed either in the medical or the food industry to assure all phages applied undergo a lytic cycle. In this scenario, lytic phage DNA replicates separately from the host DNA. With lysogenic phage, the phage DNA integrates into the bacterial DNA in the form of a prophage. Phage attach to bacterial cells through receptors on the bacterial

cell membrane and mutations in the host genome that alter the structure or form of the receptors, can result in the bacteria becoming resistant to phage (Skurnik & Strauch, 2006). A summary of bacterial resistance mechanisms of phage tactics can be found in Table 2.6.

Some *E. coli* strains can produce extracellular polysaccharide capsules (K antigens) that contribute to their pathogenesis by increasing invasiveness, protecting against phagocytosis, and avoiding host immune responses. These same polysaccharides may also prevent phage adsorption. The bacterial capsule is composed of long carbohydrate chains known as the capsular polysaccharides (CPS), that form an extensive layer that surrounds the bacterial cell (Willis and Whitfield, 2013). As the receptors of some phages, capsular polysaccharides also act to exclude some phages as a bacterial defense mechanism. For example, phages can fail to recognize the structures below the capsule, which blocks adsorption (Scholl et al., 2005). Other mechanisms that offer a measure of defense against phages include lipopolysaccharide O-antigen chains that have been shown to block phages that bind to outer membrane proteins (Scholl et al., 2005; Scholl and Merril, 2005). Some phages have evolved to produce specific capsule components that have enzymatic activities that degrade the peptidoglycan layer in the bacterium host and enable the phage capsid to inject DNA into the interior of the bacterial cell. Enzymes such as "holins", and a murein hydrolase or "lysins" can create lesions in the cytoplasmic membrane that allow endolysins to escape and finally lead to osmotic lysis of the host cell (Young, 1992). As potential antimicrobial agents, endolysins can also kill other susceptible organisms when applied as recombinant proteins, especially for bacteria which are resistant to classical antibiotics (Schmelcher et al., 2012). Purified endolysins are effective against bacteria through lysis from without (Nelson et al., 2001).

There are other bacterial anti-phage mechanisms that aid bacteria in resisting phages, but phages also have counter-mechanisms that can overcome bacterial defenses (Table 2.6). Since

resistance in the host bacterium is more likely to occur if only a single phage is employed, cocktails containing multiple, highly active, lytic phages are often formulated to ensure that multiple host receptors are targeted by the preparation (Fischer et al., 2013). However, even with these steps obtaining regulatory approval for the use of phages in both medicine or in the food industry can prove challenging (Kropinski, 2006). Additionally, one of the benefits of using a phage cocktail with two or more phages is to decrease the likelihood of successful resistance development in the bacterial host. The chance of having two or more resistance mutations that confer resistance in a single bacterium is lower than if only a single mutation is needed to confer resistance (Chan et al., 2012).

Prokaryotes have an adaptive immune system that is capable of functioning in cells as a defense for phage genetic elements known as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and associated (cas) gene system (Rath et al., 2015). The CRISPR, which are found in both chromosomal and plasmid DNA, are short repeated sequences separated by unique spacers. The phage DNA can be recognized and cut by Cas proteins when new spacers are added. Spacers are the recognition elements used to find and destroy the virus genomes should further attacks to the bacterial cell by similar viruses occur. Phages have evolved simple and complex mechanisms to overcome the bacterial CRISPR-Cas system. Phage can develop a simple point mutation that can delete the pro-spacer to avoid the spacer acquisition by Cas proteins. Some phages harbor complex anti-CRISPR proteins which prevent Cas-crRNA complex formation and inhibit cleavage of Cas proteins. Furthermore, phages have evolved the CRISPR-Cas system themselves which can form Cas-crRNA in a manner similar to that in bacteria. The Cas-crRNA produced by phage can then deactivate the bacterial CRISPR system (Samson et al., 2013)

Table 2.7. Bacterial resistance and phage functionality (FDA, 2015; Sawant, 2015)

Bacterial antiviral mechanisms		Phage counter-mechanisms
Preventing phage adsorption	Block receptors	Use other cell surface moieties as receptors or apply multiple phages
	Producing an extracellular matrix; expression of surface molecules at the receptor site	Produce depolymerase which degrades secreted substances and unmasks the receptors
	Producing competitive inhibitors	Phages can recognize multiple receptors
Preventing phage DNA injection	Superinfection exclusion systems are used by both phages and host to prevent other infections	
Cutting phage nucleic acid	Restriction modification (RM) systems block other antivirus defense systems to increase the overall resistance to phage infection	Phages initiate anti-restriction strategies
	CRISPR-Cas systems target and destroy invading foreign phage DNA	Phages can mutate. For example, encode an anti-CRISPR protein that prevents the formation or blocks the action of the CRISPR-Cas complexes
Abortive infection systems (Abi)	Secret toxins that inhibit bacterial growth	Phage mutant genes involved in nucleotide metabolism counteract toxin activity and avoid host death

2.9.7. Previous phage interventions in food

In recent years, phages have been used as biocontrol agents against foodborne pathogens in the food industry. The high host specificity of phage ensures that only the target bacterium is eliminated. Most phages have a narrow host range, which means they can only lyse one or at most a few host serogroups (Kropinski, 2006). As described in section 2.9.3, there are disadvantages of using chemical, antibiotic, and physical disinfection methods in food production. Phages have the potential to control foodborne pathogens in food products. Interventions using lytic phages to control foodborne pathogens such as *E. coli* O157:H7, *Salmonella* spp., and *Listeria monocytogenes* have been approved by regulatory agencies for use on fresh and fresh-cut produce (Judy, 2001). The use of phages against *Campylobacter*, *Enterobacter sakazakii,* and *Staphylococcus aureus* are also being studied. There are 10 companies that have products that employ phage to control targeted bacteria in water, food, agricultural environments and animal health (Garcia et al., 2008).

Agencies such as the USDA and its Food Safety and Inspection Service (FSIS) review new technology or ingredients to ensure that they will not adversely affect consumer safety. They implement inspection procedures, review inspector safety, and enforce agency regulations. Phage products approved must not pose any safety concern and be deemed suitable for use in food. FSIS defined "suitability" as "effectiveness of the substance in performing the intended technical purpose of use, at the lowest level necessary, and with the assurance that the conditions of use will not result in an adulterated product or one that misleads consumers." (FDA, 2014; USDA, 2017a). For example, a full lethality treatment in beef production to control *E. coli* O157:H7 has to achieve a 5 log reduction of this pathogen. Thus, the bar required for approval of such a product is high

since the infectious dose of STEC is so low (CFIA, 2015). Commercial phage products used as biocontrol treatments on food are shown in Table 2.7. Studies show that the effectiveness of phage is enhanced when they are combined with other antimicrobial treatments such as modified atmosphere packaging, cetylpyridinium chloride (CPC), lauric arginate (LAE), chlorine and peracetic acid treatments (Theradiyil, 2015).

Table 2.8. Commercial phage products used for pathogen biocontrol in food (FDA, 2015)

Commercial phage product	Application on food type	Target bacterium	Effective temperature (°C)	# of phages in cocktail	Effectiveness	Company	Initial concentration (PFU/ml)	Amount of product used on food
EcoShield™	Red meat parts, and ground trims	*E. coli* O157:H7	2 °C – 42 °C	3	Eliminate 95 to 100% of *E. coli* O157: H7	Intralytix, Inc., USA	1×10^{10}	1 mL per 250 cm^2 of food surface area
Finalyse™	On live animals	*E. coli* O157:H7	24 °C – 38 °C	Information not available	1 to 2 log reductions of *E. coli* O157:H7	Passport Food Safety Solutions, Inc., USA	3×10^{10}	3×10^{10} phage/head of cattle in approximately one (1) gallon of water/head
ShigaShield™	Deli meat, smoked salmon, pre-cooked chicken, lettuce, melon and yogurt	*Shigella*	2 °C-42 °C	5	at least 1 log (90%) reduction of *Shigella* in all the food types	Intralytix, Inc., USA	≥10.0 log10	$2x10^7$ or $9x10^7$ PFU/g
Salmofresh™	Poultry, fish and shellfish, and fresh and processed fruit and vegetable	*Salmonella enterica*	2 °C – 42 °C	6	Reduction up to 2.5 log CFU/cm^2 when combined with peracetic acid (400ppm) treatment	Intralytix, Inc., USA	1×10^9	1 - 4 mL per pound of food product
SalmoPro®	Poultry products	*Salmonella* spp.	4 °C – 25 °C	2	Lytic activity was demonstrated on over 95% of the tested *Salmonella*	Phagelux Inc.	1×10^9	$1x10^8$ PFU/g per gram of food

					enterica strains and non-serogroupd strains.			
Salmonelex™	Pork and poultry products	*Salmonella* spp.	15 °C - 20 °C	2	3-5 log *Salmonella* reduction	MICREOS B.V., Netherlands	2 x 10^{11}	1x10^{8} PFU/g of food
ListShield™	Ready-to-eat food, fresh produce, daily products, meat and poultry products	*L. monocytogenes*	2 °C-42 °C	6	Reduce 90% to 99% of *L. monocytogenes.* Reduction differently depend on the types of product	Intralytix, Inc., USA	1 x 10^{9}	1 - 2 mL per 250 cm^2. of food product surface
Listex™ P100	Ready-to-eat meat, deli products, poultry products	*L. monocytogenes*	1°C – 35 °C	1	It is capable of infecting 95% of common *L. monocytogenes* strains	Micreos Food Safety, formerly EBI Food Safety, The Netherlands	2 x 10^{11}	Up to levels of 10^{9} PFU/g of food

2.9.8. Conclusion

Shiga-toxigenic *E. coli* are one of the most concerning foodborne pathogens which cause serious enteric diseases worldwide. Serogroups including O157:H7 and the "big 6" (O26, O45, O103, O111, O121, and O145) brought public attention to STEC as they are frequently involved in food outbreaks. STEC have been linked with several outbreaks in a variety of food products, and among these, fresh produce is one of the major sources. Currently, the most commonly used disinfection method is washing fresh produce with chlorinated water. However, chlorinated water alone is not sufficient to reduce STEC on produce, thus other methods must be investigated that could enhance current interventions or that could be more efficient and safer for use on fresh produce in both conventional and organic production systems.

Lytic STEC phages have the potential to reduce STEC in food commodities and are considered Generally Recognized As Safe (GRAS), making them an excellent alternative to chemical sanitizers for use in organic food production. There is also evidence that some phages may be effective under refrigerated temperatures through the LO mechanism.

There are a variety of phage products which have gained regulatory approval and are commercially available to control pathogens in food including *E. coli* O157:H7. However, there are no commercial phage products targeting non-O157 STEC that have been approved for use in the food industry. Furthermore, there is little information on using phage to control pathogens in sprouts or seeds.

Chapter 3. *In vitro* and *in vivo* phage cocktail effectiveness against the top 7 STEC.

3.1 Introduction

Foodborne outbreaks involving O157 and non-O157 STEC contaminated fresh produce, among other food commodities, such as meats and grains, have become increasingly common (Rangel et al., 2005; CDC, 2016). The Center for Disease Control and Prevention (CDC) reported that pathogenic *E. coli* causes an average of 73,000 foodborne illness and 60 deaths in the U.S. annually (CDC, 2017c). Seven STEC serogroups (O26, O45, O103, O111, O121, O145, and O157:H7) have been frequently found to be the cause of many of these outbreaks (CFSPH, 2016). STEC can produce one or two toxins (*Stx1, Stx2*) that affect the distal ileum and colon and cause illness in humans. People with weakened immune systems such as the elderly and children are more sensitive to STEC infections and therefore more likely to develop complications (Ruggenenti et al., 2001). The symptoms of STEC infection include stomach cramps, diarrhea, vomiting, and hemolytic uremic syndrome (HUS) which can lead to kidney failure (PHAC, 2015a).

STEC can be transmitted by the fecal-oral route with one of the major routes of STEC acquisition being the consumption of contaminated food and drinks. As consumers have increased the substitution of high fiber foods for meat in their diets, the number of outbreaks caused by STEC in fresh produce has increased in the past three decades (Mukherjee et al., 2004; Verlinden, 1998; CDC, 2017c). Lettuce, sprouts, cantaloupes, potatoes, berries, apple cider, and juices are among the most common plant-based foods contaminated with STEC (Karch et al., 1999). Multiple disinfection methods have been used during fresh produce production to reduce the risk of infection (Oliveira et al., 2010). Currently, the most common method used for fresh produce at the processing plant is chlorine prepared from sodium hypochlorite (GPO, 2017), which can result in

1-2 log reductions in STEC when applied at 50-200 ppm with a contact time of 30 min (Allende et al., 2008). Unfortunately, some chemical disinfectants such as chlorine dioxide and bromine that are effective against STEC are not allowed to be used with organic products (Parish et al., 2003). The limitation of using some conventional chemical sanitizers has made it challenging for the organic produce industry to meet the expected standards for food safety. More biological approaches to sanitize produce are needed to reduce the risk of STEC infection in organic fresh produce.

Lytic phages have been used as a safe and natural method to reduce pathogens in fresh produce. There are some commercial phage products approved by regulatory agencies for use in food commodities. Lytic phages are viruses that kill bacteria by using bacterial metabolism to replicate and finally lyse the host cell to release phage progeny (Abedon, 2011b). Outer membrane bound lipopolysaccharides and proteins in bacteria serve as unique receptors for phage which are specific to the target host strain, rendering them harmless to humans when used for antimicrobial therapy or to decontaminate food (Sharma et al., 2008). Fresh produce is commonly processed and stored at low temperature for the purposes of maintaining freshness and extending shelf-life. Low temperature inhibits bacterial growth and also limits phage activity since phages replicate only when the host is metabolically active, resulting in an optimal temperature for phage activity of between 30 to 45°C (Obeso et al., 2008). Some phages which cause lysis from without (LO) can overcome the low temperature issue and remain effective during cold processing and storage. LO is an outcome where a high-multiplicity of infection by phage causes lysis of the bacterial cell without producing phage progeny (Abedon, 2011a). A substantial number of phages capable of LO can produce gp5 proteins that damage the host cell wall and cause cell lysis (Abedon, 2011a; Molineux, 2006). There are commercial phage cocktails (EcoShield™ and Finalyse™) approved

for use on food which can reduce the *E. coli* O157:H7 population in meat by 95 to 100% (FDA, 2015). However, phage have only been applied as an antimicrobial agent to reduce *E. coli* O157:H7 in the food industry and there are no commercial phage products targeting non-O157 STEC on the market. The objectives of this project are 1) To assemble a phage cocktail to reduce the top 7 STEC at 4, 10 or 25 °C; 2) To evaluate the effectiveness of the phage cocktail against STEC on lettuce, sprouts, and mung bean seeds at different temperatures and storage times; 3) To compare the effectiveness of phages to reduce STEC with chlorinated water wash; and 4) to determine if phage-insensitive mutants arise after phage treatments.

3.2 Materials and methods

3.2.1. STEC strains and phages

All STEC strains used in this study were obtained from the Agriculture and Agri-food Canada Lethbridge research center, Lethbridge, AB. All STEC strains were isolated from cattle (Table 3.1). Cultures were stored at -80 °C in glycerol-supplemented media, and streaked for growth on MacConkey agar (Criterion Dehydrated Media Inc., Santa Barbara, CA, U.S.) and incubated at 37 °C for 24 h.

Phages used for this study were isolated from cattle feces from previous study in AAFC, Lethbridge research center (Table 3.2). Briefly, from May 2013 to August 2013, fecal samples were collected from livestock transport trailers (47 cattle per trailer) used to transport feedlot cattle to slaughter plants in western Canada as previously described (Wang et al, 2015). Five 100 g composite fecal samples were randomly collected from fresh fecal pats in each trailer and transferred to the laboratory within 12 h for phage isolation and purification. A total of 37 lytic

phages against non-O157 (O26, O45, O103, O111, O121, O145) and O157:H7 were isolated. Phage genome size was measured by pulsed-field gel electrophoresis and ranged from 38 to 177 kb, and phages were assigned to phage families based on morphology as identified using transmission electron microscopy and genotyping using genomic sequencing. Host range and lytic capability of the phages were tested using a microplate phage virulence assay at 2, 10, and 25 °C (Wang et al., 2015). The best lytic phages with titers of $\geq 10^8$ PFU/ml were selected for use in the present study. Phage candidates were selected based on their 1) lytic activity, 2) effectiveness over a broad temperature range, 3) cross-infectivity against multiple STEC serogroups and the absence of unwanted genes (antimicrobial resistance and virulence genes). Phage stocks were stored at 4°C prior to use.

Table 3.1. STEC Bacterial Isolates

Serogroup	Strain
O26	EC19960464
O45	EC19940040
O103	EC20010670
O111	EC20030053
O121	EC20040083
O145	EC20020231
O157	R508

Table 3.2 STEC Phage isolate master stocks

Serogroup	Phage official designation	Phage label	Phage Titer
O26	vB_EcoM_AYO26A	PRWS-9I(O26)-VIAL 005 Nov.28.2013-ID5	7.55×10^{8}PFU/ml
O45	-	PRWS-13I(O45) – ID14-V02 Oct.26.14	5.7×10^{9}PFU/ml
O103	vB_EcoS_AXO103A	PRWS-1M(O103)-01-VIAL 04 Nov.28.2013-ID11	2.3×10^{9}PFU/ml
O111	vB_EcoM_AXO111A	PRWS-10I(O111)-01-VIAL 04 Nov.28.2013-ID10	3.5×10^{8}PFU/ml
O121	vB_EcoM_AXO121A	PRWS-1M(O121)-01-VIAL05 Nov.28.2013-ID1	1.7×10^{10}PFU/ml
O145	AYO145A	PRWS-17I(O145-22)-ID34-v03 Nov.15.14	2.5×10^{10}PFU/ml
O157	vB_EcoS_AKFV33	PRWS-33(R508)-03-VIAL01 31-Oct-13(JP)	1.6×10^{11}PFU/ml

3.2.2. Fresh produce and seeds

Lettuce and sprouts were purchased from a Winnipeg local market. There was approx. 1400g lettuce (6 heads) per package. Outer leaves and the stems 3 cm from the base of the head were removed. Lettuce leaves were peeled from the plants and washed individually prior to use. Whole mung bean seeds were obtained from a commercial source (Mumm's seeds, Shellbrook, Saskatchewan, Canada). Lettuce and sprouts were rinsed three times with 10 °C tap water in a salad spinner. Seeds were washed three times with tap water at room temperature.

3.2.3. Phage propagation

All 7 STEC serogroups (O26, O45, O103, O111, O121, O145, O157) were grown on MacConkey agar. An isolated colony from each serogroup was selected and then enriched in 10 ml Tryptic soy broth (TSB) (Difco, Becton Dickinson Co., Franklin Lakes, NJ, U.S.) at 37 °C for 24 h. After growth, cultures were diluted with TSB to achieve an optical density of OD_{600}= ~0.5 (4 log CFU/ml). The OD was measured using a spectrophotometer (Thermo Scientific, Genesys 20, Waltham, MA, U.S.). Phage master stocks were serially diluted using Lambda diluent (60.56 g Tris base, 58 g NaCl, 10 g $MgSO_4$, 1000 ml dH_2O) based on the MOI of the master stock (e.g. serially diluted 2 times if MOI = 0.01). Later, 500 µl of TSB, 100µl of the diluted phage and 1 ml bacterial culture (4 log CFU/ml) were transferred to a 10 ml sterilized, capped tube (Thermo Scientific, Waltham, MA, U.S.). The tubes were incubated at 37°C, in a shaking incubator at 130 rpm for 15 min to enhance the interaction between phages and the host. Then, 1600 µl of the pre-mixed bacteria and phage were aseptically transferred from centrifuge tubes into a 500 ml glass bottle containing 100 ml TSB mixed with 1 ml of 10mM/L $MgSO_4 \cdot 7H_2O$ to enhance phage absorption. The bottles were then incubated at 37 °C on an orbital shaker (VWR, Radnor, PA,

U.S.) at 140 rpm for up to 5 h and then transferred into a 500 ml sterile centrifuge bottle and centrifuged (Beckman Coulter™, 22R, Mississauga, ON, Canada) for 15 min at 4 °C and 5590 ×g. Lysates were filtered through a bottle-top filter (Nalgene Fitration 0.45 µm SFCA membrane filter, Darmstadt, Germany) into a sterile bottle to remove viable bacteria and cellular debris. The sterilized phages were stored in plastic bottles (Nalgene®, Darmstadt, Germany) at 4 °C for future use. Phage working stocks stored more than two months were tested to ensure their effectiveness against 7 STEC serogroups with microplate virulence assay prior applied on fresh produce. Propagated phages exhibiting titers ranging from 10^8 -10^{10} log PFU/ml were used in this study (Table 3.3).

Table 3.3. Phage titer after propagation

Phage Sample ID	Phage Titer (log PFU/ml)
AKFV33	$3.8x10^{10}$
AYO26A	$6.8x10^{9}$
O45	$1.5x10^{9}$
AXO103A	$3.2x10^{8}$
AXO111A	$1.2x10^{9}$
AXO121A	$3.2x10^{9}$
AYO145A	$3.2x10^{8}$

3.2.4. Double agar overlay plaque assay

The titer of the propagated phages was determined using the double agar overlay plaque assay as described by Kropinski et al. (2009). Newly propagated phages were serially diluted from 10^{-5} to 10^{-8} using Lambda diluent. A single colony of the particular bacterial host was inoculated in 10 ml TSB and grown overnight at 37 °C. Cultures were then diluted to an $OD_{600=}$ ~ 0.5 which corresponded to 4 log CFU/ml. Then, 100 µl of the diluted bacterial host was mixed with 100 µl

of the phage (dilution factor: -5 to -8) and 3 ml of 0.6% ultrapure agarose (Invitrogen, Waltham, MA, U.S.) supplemented with $MgSO_4$ (2.46g/L) was added. The tubes were inverted twice and the agarose was rapidly poured on the surface of a nutrient agar (MNA) plate. Duplicate samples were made for each dilution. Plates were left undisturbed for at least 20 min for the top agarose to solidify before being inverted and placed into an incubator at 37 °C for 24 h. Two positive controls a) 200 µl host culture only, and b) 100 µl host culture +100 µl Lambda diluent), one negative control (200 µl Lambda diluent), and one blank control (3 ml of agarose only) were included. The number of plaques formed by phage were counted after 24 h and the titer was estimated as:

$$\text{Phage titer } (\log_{10} \text{PFU/ml}) = \frac{\text{Average of plaques count} \times 10}{\text{Counted dilution}}$$

3.2.5. *In vitro* microplate phage virulence assay

The microplate phage virulence assay was used to determine the lytic activity of STEC phages. One isolated STEC colony from each serogroup was transferred into 10 ml TSB at 37°C for 24 h and then diluted to 7 log CFU/ml (OD_{600}=1.1-1.2). Twenty microliters of each lytic phage candidate were serially diluted (10^{-1}-10^{-8}) individually in a 96-well microplate (Thermo Scientific, Waltham, U.S.) containing 180 µl TSB supplemented with 10 mM/L $MgSO_4$. (1%) (mTSB) per well. Wells were inoculated with 20 µl of bacterial culture (7 log CFU/ml) of each STEC serogroup. Appropriate positive controls with STEC cultures and a negative control with only mTSB were included. The microplate assay was incubated at 2, 10 and 25 °C for 5 h. The bacterial culture used in the microplate method previously described was diluted (10^{-1}-10^{-5}) and plated on Tryptic Soy Agar (TSA) (Difco) in duplicate to determine host concentration. After incubation, the microplate was visually observed for clear wells, and then a laser microplate reader (BioTek,

Winooski, VT, U.S.) was used to measure the optical density of each well. Clear wells were considered to indicate phage lytic activity.

Shiga toxigenic *E. coli* is a mesophilic bacterium which grows between 20 and 45 °C and therefore its growth is inhibited at low temperatures. For microplates that were incubated at 2 and 10°C, results obtained from the laser reader may not accurately reflect phage lytic activity, since clear wells could also be the result of a lack of STEC growth at low temperature. Therefore, the lytic activity at 2 and 10 °C was assessed using the spot plate technique as described in section 3.2.6. For microplates incubated at 25 °C, both techniques (optical density and spot plate) were followed. Phage concentrations used for MOI calculations were based on the spot plate results at 2 and 10°C and the observation of the highest phage dilution showing clearness in wells as confirmed by the OD at 25 °C. The MOI for each phage-host assay was calculated based on three repetitions with 6 observations in total (MOI = phage concentration of the highest dilution showing lytic activity/host concentration).

The phage cocktail was prepared by mixing 20 ml of each phage filtrate (10^8 -10^{10} log PFU/ml) in a sterile bottle prior to use. The microplate assay as described above was then used to measure the MOI of the phage cocktail.

3.2.6. Spot plate technique

Microplates prepared in section 3.2.5 were examined to check for survival from the microplate phage virulence assay using the spot plate technique. For this purpose, 2 µl of sample from each well from the microplate phage virulence assay was transferred to MacConkey agar plates using a multichannel pipette. Plates were incubated at 37 °C for 24 h. Growth of colonies

on MacConkey agar that exhibited the morphology of *E. coli* was considered indicative of STEC survival.

3.2.7. Effectiveness of phage cocktail against 7 STEC serogroups on fresh produce

The effectiveness of a 7 phage cocktail against STEC on lettuce and sprouts stored at 2, 10, 25 °C for 1, 24, 48, and 72 h, was examined using a 2×3×4 factorial design.

Produce (lettuce and sprouts) experiments were performed as independent trials and replicated in triplicate. A positive control containing only the STEC bacterial cocktail and a negative control with only growth media were included. All produce samples were handled with sterile tweezers. For lettuce, a sterilized blade was used to cut lettuce into 3×3 cm pieces. Lettuce leaves and sprouts were rinsed with tap water and then placed into sterile Petri dishes and sprayed with 70% ethanol using a 100 ml plastic sprayer (Fisher Scientific, Waltham, MA, U.S.) to reduce background flora (Kampf and Kramer, 2004). To allow residual ethanol to evaporate, produce was allowed to air dry within a biosafety cabinet for at least 30 min. Prior to inoculation with STEC, two pieces of lettuce/sprouts were collected as negative controls. Produce samples were spot-inoculated with the mixture of 7 STEC bacterial strains. Five spots of 2 µl of each STEC serogroup (5 log CFU/ml) were applied to each piece of lettuce with individual spots applied to the cotyledon, stem, and root of each sprout. After inoculation, samples were air dry for 1-3 h in a biosafety cabinet. Prior to inoculation with STEC phages, two pieces of lettuce/sprouts inoculated with each serogroup were collected and separately packed as positive controls. Adulterated lettuce and sprouts were separated into treatment groups and treated with the STEC phage cocktail using a 50 ml sanitized plastic sprayer (0.21 ml/spray) (Fisher Scientific, Waltham, MA, U.S.). Each lettuce/sprout piece was sprayed with the phage cocktail once with 10 cm distance so as to fully

cover the sample. Following a 10 min incubation at room temperature, two pieces of lettuce/sprouts were placed into sterile bags and stored at 2, 10, and 25 °C for up to 72 h. At each storage time, bags stored at 2, 10, and 25 °C were removed for STEC enumeration. Ten-fold dilutions were prepared for each sample by adding 9 ml buffered peptone water (BPW) (Criterion Dehydrated Media Inc., CA, U.S.). Each dilution (100 µl) was plated on MacConkey and Rainbow agar plates (Biolog Inc. Hayward, CA, U.S.) containing Novobiocin (10 mg/L) (Alfa Aesar, Haverhill, MA, U.S.) and Cefixime (20 mg/L) (Oxoid, Nepean, ON, Canada). Plates were incubated at 37 °C for 24 h. Colonies were counted from MacConkey plates to represent the total reduction in STEC which estimated the degree of reduction in the STEC population as a result of phage treatment as compared to the number of STEC enumerated in the positive control (log_{10} CFU/ml). Rainbow agar was used to differentiate STEC serogroups based on colony colour, with colonies exhibiting the same colour providing an estimate of the survivability of a particular STEC serogroup. Up to 5 colonies were picked based on colour and isolates were tested with latex agglutination to confirm their specific serogroup (O157 latex Test kit Oxoid, Hampshire, U.K., O45, O103, O121, O145 antisera SSI®, Statens Serum Institute, Denmark; and O26, and O111 antiserum Denka Seiken Co, Ltd, Fisher Scientific, Tokyo, Japan). The serogroup of the isolates was also confirmed using a multiplex PCR assay as described below. Samples in which not all 7 STEC were isolated on agar plates were screened using immunomagnetic separation (IMS) following manufacturer recommendations (Dynabead; Dynal Wirral, U.K.). Briefly, samples were enriched by adding 1 ml of the sample to 9 ml of modified TSB (mTSB) broth with Novobiocin (10 mg/L) and incubated at 37°C overnight. One milliliter of enriched, modified TSB (mTSB) broth from each sample was then mixed with 10 µl of anti-STEC beads corresponding to the absent STEC serogroup. Beads possessed surface-adsorbed and affinity-purified antibodies against *E. coli*

strains O157, O26, O45, O103, O111, O121, and O145. Beads were washed three times in phosphate buffered saline (PBS)-Tween 20 (Invitrogen), and 100 µl of the bead suspension was spread plated onto Rainbow agar using sterilized cotton swabs. Plates were incubated at 37 °C for 24 h.

3.2.8. Multiplex polymerase chain reaction (mPCR)

Genomic DNA was extracted from STEC isolates recovered from Rainbow agar at 10^{-1} dilution by adding 20 µl of alkaline lysis buffer (0.25% SDS, 0.05M NaOH) followed by boiling at 100 °C for 15 min on a heating block (VWR, Radnor, PA, U.S.) followed by placement on ice for 5 min. The precipitated DNA was re-suspended in 180 µl of RNase-free water and centrifuged for 5 min at 4,800 ×g. The multiplex PCR assay the *wzx* gene in the O-antigen gene cluster, which is a unique gene of the non-O157 serogroups. The isolated STEC will be amplified and identified using PCR assay (DebRoy, et al., 2011). The forward (F) and reverse (R) primer sets for O-group STEC are summarized in Table 3.3 (DebRoy, et al., 2011). Primers were diluted 10-fold from the original stock with RNase-free water and kept on ice throughout the experiment. Multiplex PCR reactions were prepared according to Qiagen multiplex master mix kit instructions (Qiagen, Valencia, CA, U.S.). Briefly, PCR master mix solution for each reaction was made with 25 µl of 2x Mutiplex PCR Master mix, 10 µl of Q-solution, 4.8 µl of nuclease-free water, and 0.3 µl of each primer. Three microliters of extracted DNA from bacteria that survived phage treatment were mixed with the PCR master mix reaction solution with a total volume of 47 µl used for each reaction. PCR amplifications were conducted by initial denaturation at 95 °C for 15 min followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 57 °C for 1.5 min, extension at 72 °C for 1.5 min, and with a final extension for 10 min at 72 °C. The reactions were held at 4 °C

until electrophoresed. The amplified DNA was electrophoresed in 1.5% agarose (CulGene, Summit, NJ, U.S.) stained with 1 µl gel red dye (Sigma, St. Louis, MO, U.S.) and ran for 90 min at 100 V. The PCR DNA ladder (100 bp) (Fisher Scientific, Waltham, MA, U.S.) and 10x loading buffer (Invitrogen) were used for gel loading. The gel was observed under ultraviolet light for visualizing bands, and the gel images were captured using a gel imaging system (Syngene; Cambridge, U.K.)

Table 3.4 STEC primers used in multiplex PCR to determine STEC survival after phage treatment

Primer	Sequence
O26F	caatgggcggaaattttaga
O26R	ataattttctctgccgtcgc
O45F	tgcagtaacctgcacgggcg
O45R	agcaggcacaacagccactact
O103F	ttggagcgttaactggacct
O103R	gctcccgagcacgtataaag
O111F	tgtttcttcgatgttgcgag
O111R	gcaagggacataagaagcca
O121F	tccaacaattggtcgtgaaa
O121R	agaaagtgtgaaatgcccgt
O145F	ttcattgttttgcttgct
O145R	ggcaagctttggaaatga
O157F	tcgaggtacctgaatctt
O157R	accagtcttggtgctgctctgaca

Note: Source of primers (DebRoy, et al., 2011)

3.2.9. Scaled-up validation study

To further examine the effectiveness of the STEC phage at reducing the 7 STEC on fresh produce, a scaled-up experiment was performed. All experiments were performed as independent trials and replicated in duplicate. Fresh produce and mung bean seeds (MB) were washed three times with tap water (10 °C for lettuce and sprouts, room temperature for seeds). Excess water was removed using a salad spinner. Prior to inoculation with STEC, 10 g of lettuce/sprouts/seeds were collected as negative control. Produce was inoculated by immersion in a 10^5 CFU/ml STEC cocktail (OD_{600} = 0.7) for 30 min and excess liquid was removed using a salad spinner. Lettuce (600g), 300 g sprouts, and 30 g of seeds were selected for one trial. Samples were then assigned in duplicate to one of three treatments: 1) T1 lettuce leaves/sprouts/seeds were inoculated with the STEC cocktail plus washed in chlorinated water (150 ppm for lettuce and sprouts, 1000 ppm for seeds); 2) T2 lettuce leaves/sprouts/seeds inoculated with STEC cocktail and STEC phage cocktail; 3) T3 lettuce leaves/sprouts/seeds inoculated with STEC cocktail and STEC phage cocktail plus washed in chlorinated water. Prior treatment with chlorinated water or the phage cocktail, 10 g of lettuce/sprouts/seeds that were inoculated with STEC bacterial cocktail only were collected as a positive control. Commercial liquid bleach containing sodium hypochlorite (Old Dutch®, Montreal, QC, Canada) was used to prepare chlorinated water at concentration of 150 and 1000 ppm. The concentration of the chlorinated water was measured using water quality test strips (HACH; Elkhart, IN, U.S.) for high-range chlorine after preparation and before application to produce/seeds. The washing of lettuce and sprouts in 10 °C 150 ppm chlorinated water was conducted over 3 min, followed by rinsing three times with 10 °C water to remove residual chlorine, mimicking current industry practices. Seeds were immersed in 1000 ppm chlorinated water for 3 min and rinsed with chlorine-free water at room temperature. Lettuce and sprouts were

immersed in the STEC phage cocktail for 15 min while seeds were immersed in the phage cocktail for 1 h. After treatment, 10 g of produce from each treatment were collected and placed in in sterile bags (Seward, Bohemia, NY, U.S.) and stored at 2, 10, and 25 °C for 1, 24, 48, and 72 h. Seeds were dried at room temperature in a biosafety cabinet for at least 1 h until no liquid appeared on the surface of seeds and then stored at 25 °C for 72 h in a dark room. At the end of each storage period, leaves/sprouts/seeds were mixed 90 ml of BPW and homogenized using a stomacher (Intersciences Inc., Markham, ON, Canada). For enumeration, 10-fold serial dilutions (10^{-1}-10^{-8}) were prepared and plated onto Rainbow and MacConkey agar plates. For treatments with chlorinated water, 9 ml of D/E Neutralizing Broth (Becton Dickinson, East Rutherford, NJ, U.S.) were substituted for BPW to neutralize the chemical. Plates were incubated at 37 °C for 24 h to determine the *E. coli* reduction and to estimate the survivability for each serogroup. Each of the colonies was tested with latex agglutination and confirmed by specific multiplex PCR as described above. For those samples where not all 7 STEC were detected on plates, IMS was used to recover STEC strains within the enumerable range as previously explained.

3.2.10. Statistical analysis

All statistical analyses were conducted using SAS, version 9.2. The MOIs for *in vitro* experiments were analyzed following the mixed linear procedure (for fixed and random effects). Data on the effectiveness of the phage cocktail against the 7 STEC serogroups used in the spot-inoculation and scaled-up validation experiments were analysed using SAS. Each produce type was analyzed separately. The model used was a completely random design with a CRD factorial arrangement where the replications of treatments were assigned at random to independent

experimental subjects. Treatments, storage time, temperature, and their interactions were considered as fixed effects.

3.3 Results

3.3.1 Phage effectiveness on STEC at 2, 10, and 25 °C *in vitro* through microplate virulence assay

Data analysis showed an interaction ($p = 0.003$) between phage lytic activity and temperature, meaning that phage lytic activity against STEC improved as temperature increased. At 25 °C, all STEC phages were effective against their targeted host with an overall 5 $\log_{10}$ CFU/ml reduction in STEC (Figure 3.1). When applied individually, only 5 phages (AKFV33, AYO26A, phage O45, AXO103A, AXO121A) were able to completely eliminate their targeted STEC host in the spot plate assay at 25 °C (Table 3.5). Although microplate wells appeared clear, a few (5-10) colonies of *E. coli* O145 and O111 were observed when the samples from microplate wells were spotted onto agar plates, indicating that some bacteria remained viable even after exposure to the phages. At 2 and 10 °C, AKFV33, AYO26A, AXO103A, and AYO145A phages were able to achieve a 5 $\log_{10}$ CFU/ml reduction for STEC serotypes O26, O103, O145 and O157, respectively.

The OD_{600} measurement for all STEC serogroups with the purpose to assess the lytic activity of both phage cocktail and individual phage at 25 °C can be found in Fig. 3.1 and 3.2. Similar to individual phage results, the STEC cocktail was also effective against all 7 STEC serogroups when tested in TSB at different temperatures as clear wells were observed in all cases. The phage cocktail was able to eliminate STEC serotypes O26, O103, and O157 at all temperatures

(Table 3.5). *E. coli* serogroup O145 was also eliminated at 2 and 10 °C using the STEC phage cocktail but a few colonies were found at 25°C.

The MOI results indicated the ratio of phages needed to kill their specific host at 2, 10, and 25 °C. It was found that the MOI was higher (P = 0.001) at 2 and 10 °C than 25 °C (Table 3.6). The higher MOI indicated that a larger number of phages were needed to eliminate STEC (10^5 CFU/ml) at 2 and 10 °C than at 25 °C. For example, the results in Table 3.6 indicated that 8×10^4 PFU/ml phage were required at 2 °C (MOI= 8×10^{-1} PFU/CFU), while 4×10^1 PFU/ml phage were needed at 25 °C (MOI= 4×10^{-4} PFU/CFU) to kill 10^5 CFU/ml STEC O157.

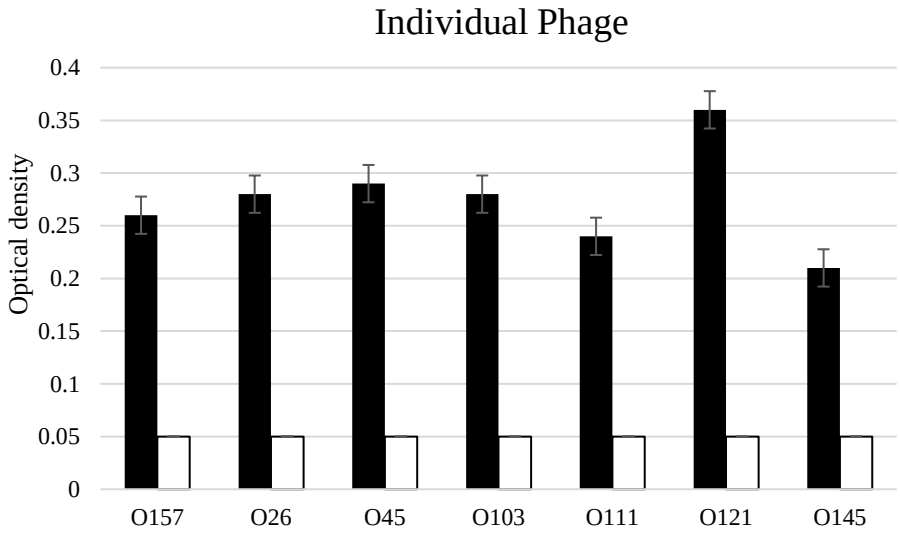

Figure 3.1. Microplate assay to assess lytic activity of STEC phages against 10^5 CFU/ml STEC after treatment with a single phage at 25 °C.

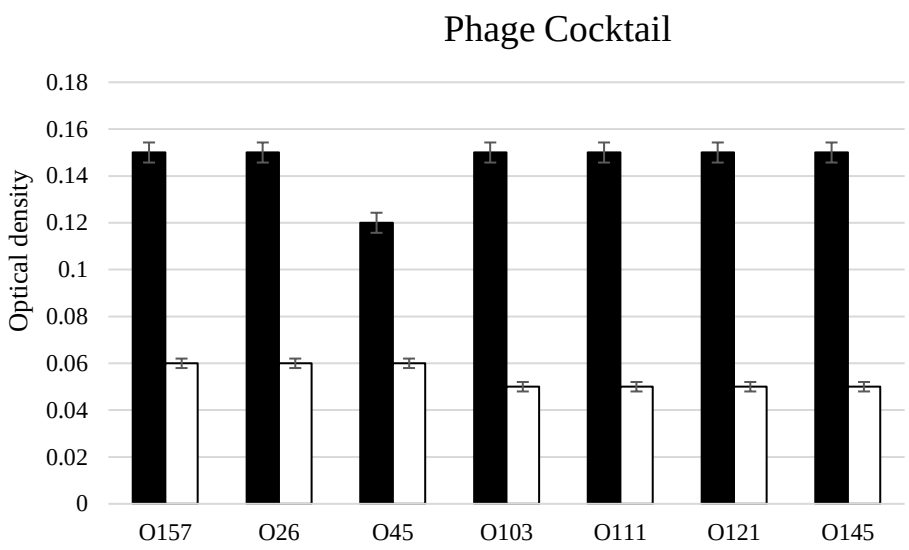

Figure 3.2. Microplate assay to assess lytic activity of STEC phages against 10^5 CFU/ml STEC after treatment with a cocktail of STEC phage at 25 °C.

Table 3.5 STEC survival as detected using a spot plate method after treatment with individual phage and a STEC phage cocktail at 2, 10, and 25 °C for 5 h

	Temp (°C)	STEC strains						
		O26	O45	O103	O111	O121	O145	O157
Individual phage	2°C	0	1	0	1	1	0	0
	10°C	0	1	0	1	1	0	0
	25°C	0	0	0	1	0	1	0
Phage cocktail	2°C	0	1	0	1	1	0	0
	10°C	0	1	0	1	1	0	0
	25°C	0	0	0	1	1	1	0

[1]: STEC survival detected from spot plating

[0]: No STEC survival detected from spot plating

[Note]: One phage was used against it's specific serogroup at each temperature for treatment with individual phage.

STEC phage cocktail was mixed with 20 µl of phage AKFV33, AYO26A, phage O45, AXO103A, AXO111A, AXO121A, and AYO145A at high titer.

Table 3.6. Temperature effects on individual multiplicity of infection (MOI) of STEC phages against the specific STEC strains (PFU/CFU)

	Temperature (˚C)		
STEC strains	2	10	25
O157	8×10^{-1} a	7×10^{-1}a	4×10^{-4} b
O26	3.4×10^{1} a	3.4×10^{1}a	2×10^{-2} b
O45	4×10^{-2} a	4×10^{-2} a	4×10^{-2} b
O103	8×10^{-2} a	8×10^{-2} a	9×10^{-2} b
O111	2.9 a	2.9 a	2×10^{-2} b
O121	5×10^{-1} a	5×10^{-1}a	5×10^{-4} b
O145	4×10^{-1} a	8×10^{-1}a	4×10^{-4} b

[a,b] : Different letters mean comparison of the same strain at different temperatures was different, P = 0.01

3.3.2. Spot-inoculation experiment on lettuce and sprouts

3.3.2.1. Lettuce

Based on the total STEC numbers, the highest reduction as result of exposure to the phage cocktail was 1.41 $\log_{10}$ CFU/g on lettuce at 2 °C after 1 h of storage (Fig. 3.3). A reduction in STEC total numbers was observed throughout the 72 h storage period at both 2 and 10°C in samples treated with the STEC phage cocktail. At 25 °C, the highest reduction (0.45 $\log_{10}$ CFU/g) in STEC total numbers caused by a phage cocktail occurred at 1 h (Fig. 3.3), but after 24 h, the number of STEC in phage-treated lettuce increased to the same level as the control lettuce.

Based on STEC numbers on Rainbow agar, the STEC phage cocktail was the least effective against serogroups O111, O121, and O45. However, 4 (O157, O26, O103, and O145) of 7 STEC serogroups were reduced by as much as 5.2 to 8.8 $\log_{10}$ CFU/g on lettuce treated with the STEC phage cocktail. STEC O157, O26, and O103 were not recovered from lettuce nor were they detected by PCR/ IMS, regardless of the storage temperature or period (Table 3.7). Serogroups O45 and O145 were not detected from plates in any of the storage temperatures or times, but they were recovered from samples after screening using IMS. STEC O45 and O145 were detected at 1, and 24 h but not at 48 h. Isolates from O111 and O121 appeared to be the most robust as they were detected on lettuce at all temperatures throughout storage even with phage treatment (Fig. 3.5).

3.3.2.2. Sprouts

The phage cocktail showed limited ability to reduce STEC on sprouts as compared to lettuce as the reduction was < 1 log CFU/g at all of the temperatures and storage times. The phage cocktail reduced STEC total numbers at 2 and 10 °C with a total reduction of 0.54 log CFU/g and

0.38 log CFU/g at 72 h (Fig. 3.5). While at 25 °C, the highest reduction was 0.64 $\log_{10}$ CFU/g at 1 h after being treated by the phage cocktail, the STEC population recovered to the same concentration as the positive control (8.5 log CFU/g) after 24 h (Fig. 3.5). A time by temperature interaction (P = 0.001) was found for spot-inoculated sprouts, indicating that the reduction in STEC was influenced by storage temperature and storage time. In general, the effectiveness of the phage cocktail on total *E. coli* numbers declined over time at all tested temperatures on sprouts, most notably at 25°C (Fig. 3.5).

Isolates from serogroups O45, O111, O121, and O145 exhibited the greatest persistence (Fig. 3.6). As in the lettuce trials and *in vitro* assay, *E. coli* serogroups O26, O103, and O157 were not detected either on Rainbow agar or by PCR/IMS, indicating that they were eliminated by the STEC phage cocktail (Table 3.7). Phages O45, AXO111A, AXO121A, and AYO145A resulted in a 5 $\log_{10}$ CFU/ml reduction in their targeted serogroups in the microplate assay, but they were less effective when applied to fresh produce.

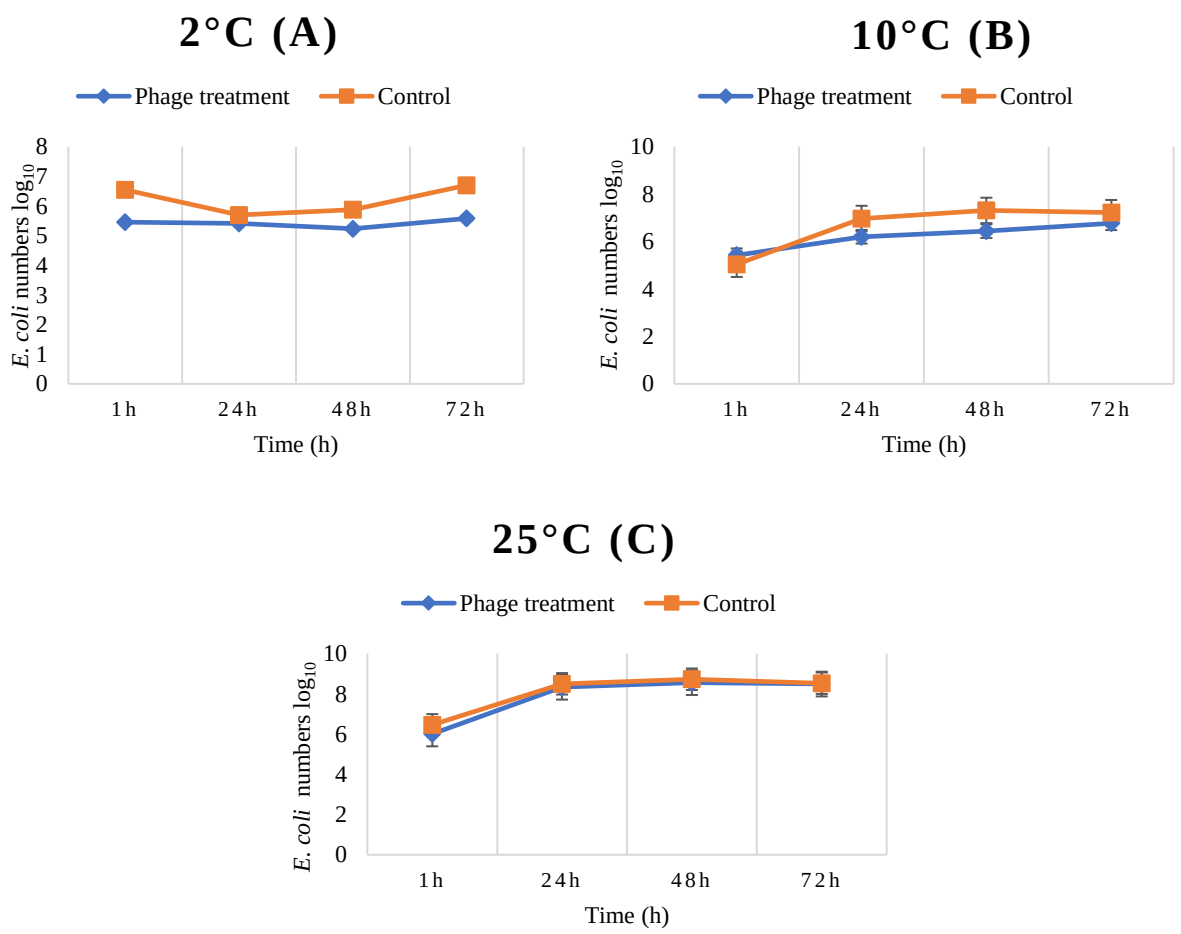

Figure 3.3. A, B, C. STEC numbers (log_{10}) in lettuce spot-inoculated with 10^5 CFU/ml STEC, followed by phage cocktail spray treatment and stored at 2, 10, and 25 °C for 72 h.

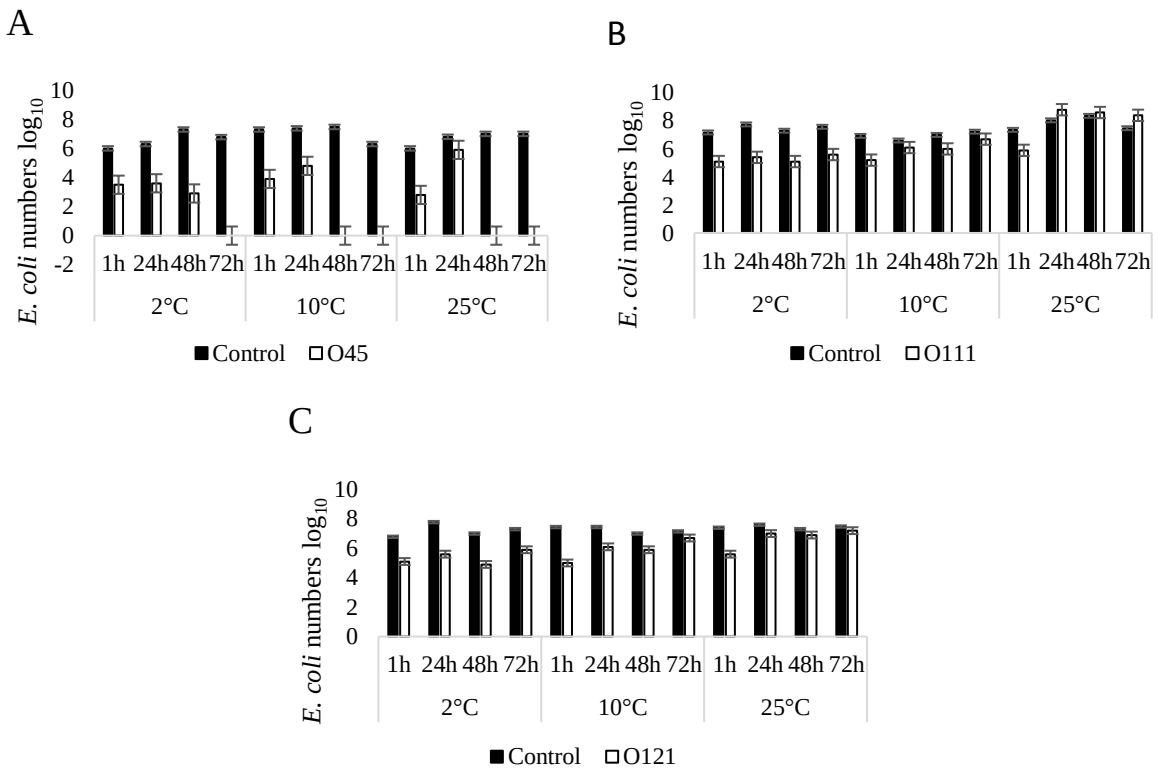

Figure 3.4. A, B, C. STEC O45, O111, and O121 numbers in lettuce spot-inoculated with 10^5 CFU/ml STEC bacteria followed by spraying with a phage cocktail and storage at 2, 10, and 25 °C for 72 h. Serogroups O157, O26, O103, and O145 were not detected on plates.

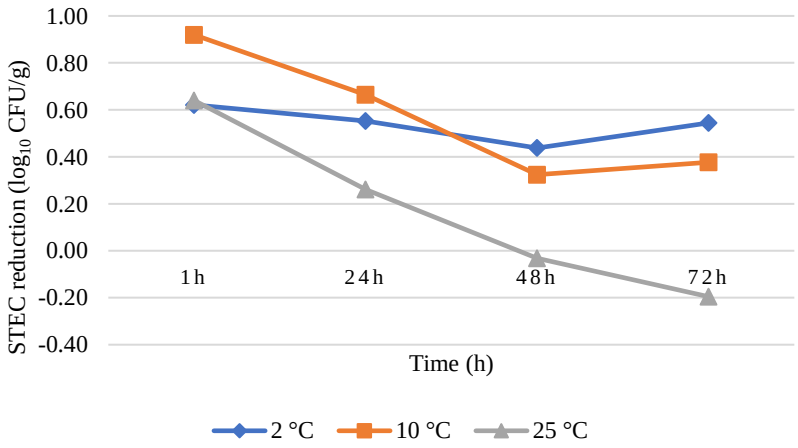

Figure 3.5. Reduction of total STEC numbers in sprouts spot-inoculated with 10^5 CFU/ml STEC bacteria followed by spraying a phage cocktail and storage at 2, 10, and 25 °C for 72 h

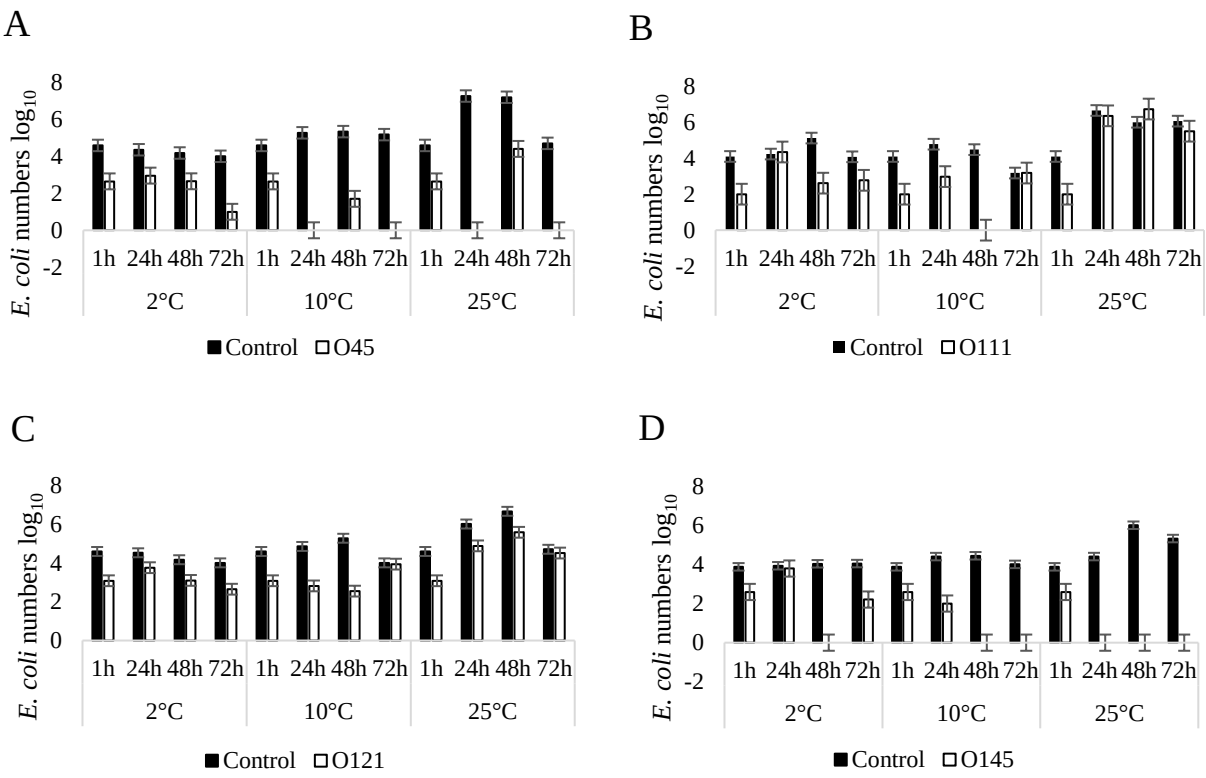

Figure 3.6. A, B, C, D. STEC O45, O111, O121, and O145 numbers in sprouts spot-inoculated with 10^5 CFU/ml STEC bacteria followed by spraying with a phage cocktail and storage at 2, 10, and 25 °C for 72 h. Serogroups O157, O26, and O103, were not detected on plates.

Table 3.7. Survival of STEC serogroups spot inoculated onto lettuce with 10^5 CFU/ml bacteria, treated with a phage cocktail and stored at 2, 10, and 25 °C for 72 h

Time (h)	Temperature (°C)	Serogroups						
		O26	O45	O103	O111	O121	O145	O157
1	2	0	1	0	1	1	1	0
1	10	0	1	0	1	1	1	0
1	25	0	1	0	1	1	1	0
24	2	0	1	0	1	1	1	0
24	10	0	1	0	1	1	1	0
24	25	0	1	0	1	1	1	0
48	2	0	0	0	1	1	0	0
48	10	0	0	0	1	1	0	0
48	25	0	0	0	1	1	0	0
72	2	0	0	0	1	1	0	0
72	10	0	0	0	1	1	0	0
72	25	0	0	0	1	1	0	0

*0 Indicates no detection of isolates by PCR or IMS

**1 Indicates that isolates were detected by PCR or IMS

Table 3.8. Survival of STEC serogroups spot-inoculated onto sprouts with 10^5 CFU/ml bacteria and treated with a phage cocktail and stored at 2, 10, and 25 °C for 72 h

Time (h)	Temperature (°C)	Serogroups						
		O26	O45	O103	O111	O121	O145	O157
1	2	0	1	0	1	1	1	0
1	10	0	1	0	1	1	1	0
1	25	0	1	0	1	1	1	0
24	2	0	1	0	1	1	1	0
24	10	0	1	0	1	1	1	0
24	25	0	1	0	1	1	1	0
48	2	0	1	0	1	1	0	0
48	10	0	1	0	1	1	1	0
48	25	0	1	0	1	1	1	0
72	2	0	1	0	1	1	0	0
72	10	0	0	0	1	1	1	0
72	25	0	0	0	1	1	1	0

*0 Indicates no detection of isolates by PCR or IMS

**1 Indicates that isolates were detected by PCR or IMS

3.3.3. Effectiveness of a phage cocktail at controlling STEC on produce stored for different periods and temperatures under scaled-up conditions

3.3.3.1. Lettuce

A significant temperature by storage time interaction ($P < 0.0001$) was found in lettuce; which indicated the phage cocktail was more active as the temperature decreased. There was no bacterial growth from any of the negative controls in any of the experiments. The phage cocktail was more effective at controlling STEC at 2 and 10 °C than 25 °C (Table 3.9). The highest reduction in STEC was 2.8 $\log_{10}$ CFU/g at 2 °C after 72 h. During storage, the reduction achieved by the STEC phage cocktail was increased at 2 °C and remained relatively stable at 10 °C. At 25 °C, the phage cocktail caused a 2.5 $\log_{10}$ CFU/g reduction in total STEC within 1 h, but the population returned to levels that were similar to the control sample after 24 h (Table 3.9).

Isolates from all 7 STEC serogroups remained viable after phage treatment; particularly those associated with serogroup O111, O121, and O145, while only a few colonies of serogroup O26, O103, O45, and O157 were detected. The phage cocktail caused a similar reduction in STEC on lettuce at 2 and 10 °C (Fig. 3.7). The phage cocktail exhibited high activity against 6 (O157, O26, O45, O103, O121, and O145) of the 7 serogroups at both 2 and 10 °C, causing at least a 2 $\log_{10}$ CFU/g reduction after 72 h of storage. The phage cocktail had less effectiveness against isolates from serogroup O111, as there was no noticeable reduction in populations at any temperature over the entire storage period. At 25 °C, STEC numbers increased after 1 h in all serogroups except for STEC O103 where the cocktail controlled the STEC O103 population over 72 h of storage.

In summary, treatment with STEC phage cocktail was effective at reducing STEC on lettuce over 72 h of storage at 2 and 10 °C against all serogroups except for O111, but it did not decrease the number of *E. coli* O157:H7 or the "big 6" STEC at 25°C after 1 h.

3.3.3.2. Sprouts

An interaction was found between treatment and temperature, as well as between temperature and time. These interactions indicated that the reduction in STEC increased as temperature decreased over time. There was no STEC growth in any of the negative controls from any of the trials. At temperatures ≤10°C, the phage cocktail caused a similar reduction in STEC during storage and consistently controlled STEC at low temperatures (Table 3.10). The highest reduction of total STEC numbers as a result of treatment with the phage cocktail was 2.4 $\log_{10}$ CFU/g both at 2 and 10 °C within 1 h after treatment. At 25 °C, the cocktail caused a 2.45 $\log_{10}$ CFU/g reduction in STEC within 1 h, but it was only effective in the short term (≤ 1 h).

The effectiveness of the phage cocktail at lowering STEC on sprouts varied depending on the storage temperature. After 1 h of storage, the phage cocktail was equally effective across all temperatures ($P > 0.05$), but after 24 h the efficacy of phage was greater at 2 and 10 °C than 25 °C ($P < 0.05$). After 48 h, this same reduction in efficacy occurred at 10°C, but the phages were still effective at 2 °C. Similar to lettuce, 25 °C was not the optimal temperature for phage interventions in sprouts stored longer than 1 h.

As with lettuce, isolates from all 7 serogroups could be recovered after phage treatment, particularly from serogroups O103, O111, and O121, while isolates from O26, O45, O145, and O157 were recovered in lower numbers. At 2 °C, an increasing reduction in all 7 STEC serogroups was observed, with the exception of O121 (Fig. 3.8). *E. coli* O26 and O45 were not isolated from

Rainbow agar after 72 h, suggesting that the phage cocktail achieved a ≥ 5 log CFU/g reduction in these two serogroups in sprouts. The phage cocktail resulted in a > 2 log_{10} CFU/g in serogroups O157, O103, O111, and O145 over 72 h storage at 2 °C. The reduction in O121 was 0.88 log_{10} CFU/g after 72 h. At 10 °C, the phage cocktail remained efficacious against serogroups O157, O45, O103, O145, and O111 during storage, but it was less effective against O26 (3.8 to 2.13 log_{10} CFU/g) and O121 (3.53 to 1.78 log_{10} CFU/g). At 25 °C, the phage cocktail caused a >5 log_{10} CFU/g reduction in STEC O26, O45, 103, and O145 over 72 h of storage. The reduction of STEC O111 and O121 declined at 25 °C after 1 h of storage, even when treated with phage cocktail (Fig. 3.8 c).

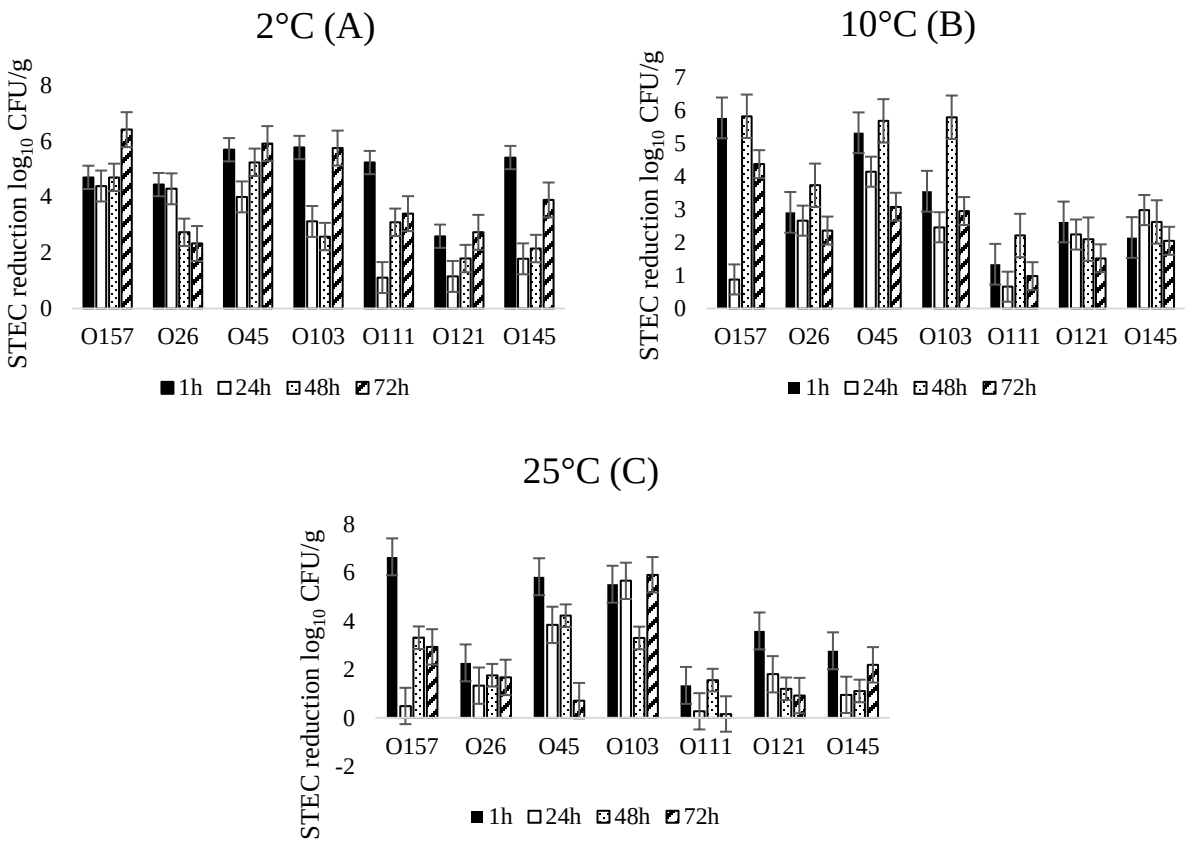

Figure 3.7. A, B, C. Reduction of individual STEC serogroups on lettuce immersed in 10^5 CFU/ml STEC bacteria, treated with a phage cocktail and stored at 2, 10, and 25 °C for 72 h.

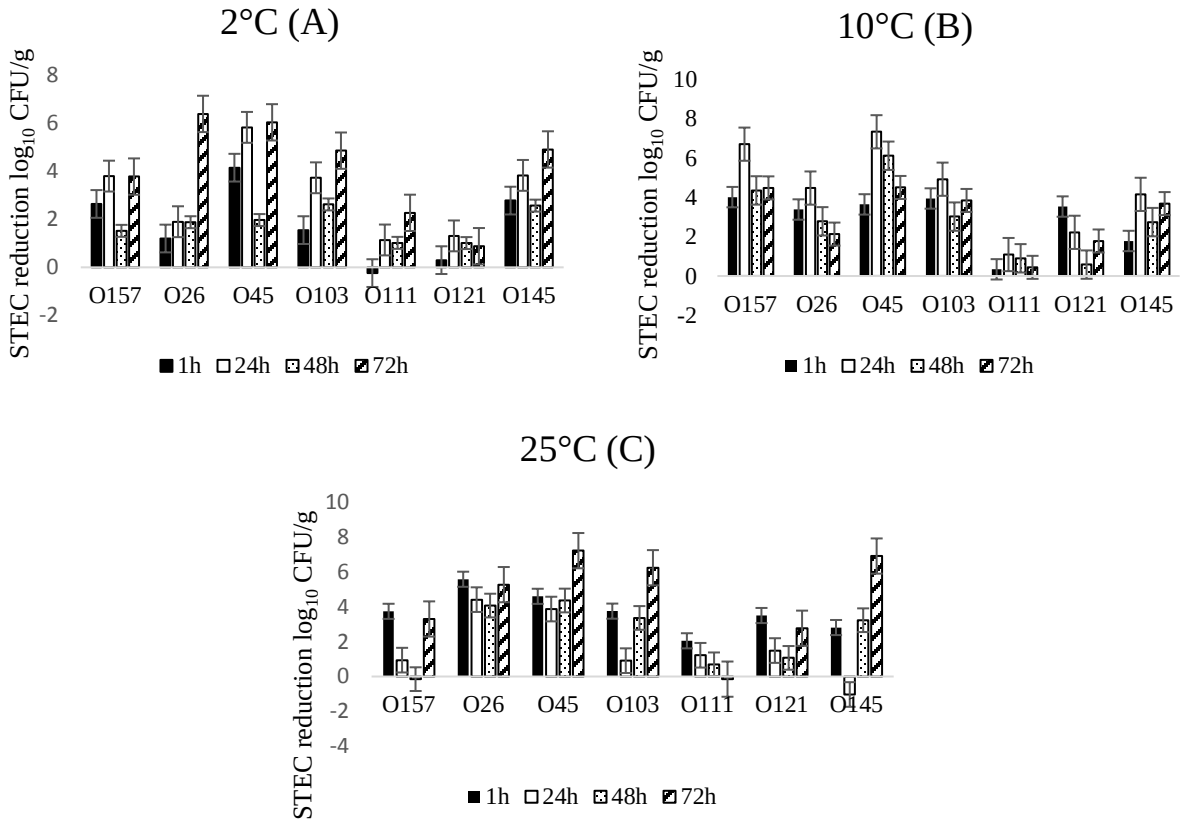

Figure 3.8. A, B, C. Reduction of individual STEC serogroups on sprouts immersed in 10^5 CFU/ml STEC bacteria, treated with a phage cocktail and stored at 2, 10, and 25 °C for 72 h.

Table 3.9 Effect of temperature x time interaction (SEM 0.25; P value < 0.0001) on reduction of total numbers of STEC as a result of immersion of lettuce in a phage cocktail

	2°C	10°C	25°C
1h	2.3 a/c	2.3 a/c	2.5 a/c
24h	2.4 a/c	2.4 a/c	0.9 b/d
48h	2.6 a/c	2.2 a/c	0.8 b/d
72h	2.8 a/c	2.3 a/c	0.4 b/d

[a,b]. Least square means within a row lacking a common letter differ (P < 0.0001)

[c,d]. Least square means within a column lacking a common letter differ (P < 0.0001)

Table 3.10. Effect of temperature x time interaction (SEM 0.25; P value < 0.0001) on reduction of total numbers of STEC as a result of immersion of sprouts in a phage cocktail

	Temperature (°C)		
Time (h)	2	10	25
1	2.4 a/d	2.35 a/d	2.45 a/d
24	2.3 a/d	2.09 ab/e	1.2 b/ef
48	2.4 a/d	1.98 b/e	1.11 b/ef
72	2.27 a/d	1.35 b/e	0.59 bc/ef

[a,b,c]. Least square means within a row lacking a common letter differ (P < 0.0001)

[d,e,f]. Least square means within a column lacking a common letter differ (P < 0.0001)

3.3.4 Phage cocktail effectiveness compared to chlorinated water on fresh produce and seeds under scaled-up conditions

3.3.4.1. Lettuce

In lettuce, the combination treatment of chlorinated water with phage cocktail (T3) (2.1 $\log_{10}$ CFU/g) achieved the highest reductions in STEC, followed by the phage cocktail (T2) (1.6 $\log_{10}$ CFU/g) and chlorinated water (T1) (1.7 $\log_{10}$ CFU/g) (Fig. 3.9). Treatment with the combination of both STEC phage cocktail and chlorinated water (T3) was more effective in reducing STEC than only the phage cocktail (T2) or chlorinated water wash (T1) on lettuce (Table 3.10). However, T1, T2, and T3 were not significantly different from each other ($P>0.05$), all treatments had similar effectiveness in reducing STEC on lettuce.

It is important to seek out the most suitable disinfection method for each individual STEC serogroup to efficiently reduce the risk of getting an STEC infection from different sources. STEC phage cocktail alone (T2) was the most suitable treatment for reducing serogroups O157, O45, and O103 numbers on lettuce, as the reduction was 0.5-1 $\log_{10}$ CFU/g higher than in T1, and T3 (Fig. 3.10). In contrast, chlorinated water wash enhanced the effectiveness of the phage cocktail against isolates from serogroup O26, O121, and O145 as the combination treatment resulted in the highest reduction of these serogroups. Washing with the chlorinated water alone (T1) was the most effective method at reducing STEC O111, indicating that the STEC phage cocktail was not effective against this specific serogroup in fresh produce.

3.3.4.2. Sprouts

As with the results observed in lettuce, the application of the STEC phage cocktail only (T2) was not as effective at reducing STEC (1.7 $\log_{10}$ CFU/g) on sprouts as compared to T1 and

T3 (~ 2 $\log_{10}$ CFU/g) (Table 3.13). The highest total reduction was found with T3 (2.77 $\log_{10}$ CFU/g) in 1 h at 2 °C, while reductions were lower for T1 and T2 (1.4 to 2.4 $\log_{10}$ CFU/g) under the same storage conditions (Table 3.13).

All 7 serogroups remained viable on sprouts after treatment, particularly isolates from serogroups O103, O111, and O121. STEC O26, O45, O145, and O157 were found in relatively lower numbers. The combination treatment was the most effective, reducing 4 of the 7 serogroups (O26, O45, O111, O145) in sprouts (Fig. 3.11). Serogroups O157, O103, and O121 were reduced more by washing only with chlorinated water, indicating that the phage cocktail was less effective against isolates from serogroups O157, O103, and O121.

3.3.4.3. Mung beans (MB)

The STEC phage cocktail alone (T2) showed the highest reduction (1.69 $\log_{10}$ CFU/g) of STEC on MB, while the reductions were lower in beans treated with 1000 ppm chlorinated water (T1) (1.22 $\log_{10}$ CFU/g) and the combination of phage cocktail and chlorinated water (T3) (1.45 $\log_{10}$ CFU/g) (Fig. 3.9).

The STEC phage cocktail showed different lytic activities against isolates from the 7 STEC serogroups on MB. All 7 serogroups remained viable after phage treatment particularly isolates from serogroups O111 and O121 (Fig. 3.12. a). Chlorinated water alone was the most effective at reducing serogroup O121, with a 3.7 $\log_{10}$ CFU/g reduction. The highest reduction of serogroup O111 was 2.74 $\log_{10}$ CFU/g in T3. The STEC phage cocktail was the most effective treatment for reduction of STEC O26 and O157. There was no significant difference ($P > 0.05$) between treatments in their ability to reduce isolates from serogroups O45 and O103. Isolates from those

serogroups were effectively controlled by using 1000 ppm chlorinated water and STEC phage cocktail either alone or as a combination.

The STEC phage cocktail was effective on seeds against all 7 serogroups either by itself or combined with 1000 ppm chlorinated water washing, but was still not able to prevent STEC proliferation during germination. After germination STEC isolates were recovered from all 7 serogroups.

3.3.4.4. Germinated sprouts

The reductions in total STEC population were less than 1 $\log_{10}$ CFU/g after germination regardless of the treatment (Fig. 3.9). Serogroup O26, O103, O121, O145, and O157 isolates were the principal survivors; particularly for serogroups O26 and O103 where there was no reduction either by the STEC phage cocktail, chlorinated water, or the treatment combination (Fig. 3.12. b). Serogroups O45 and O111 were not enumerable on the plates in any of the treatments, but they were detected through PCR/IMS, indicating that a 5 $\log_{10}$ CFU/g reduction was achieved.

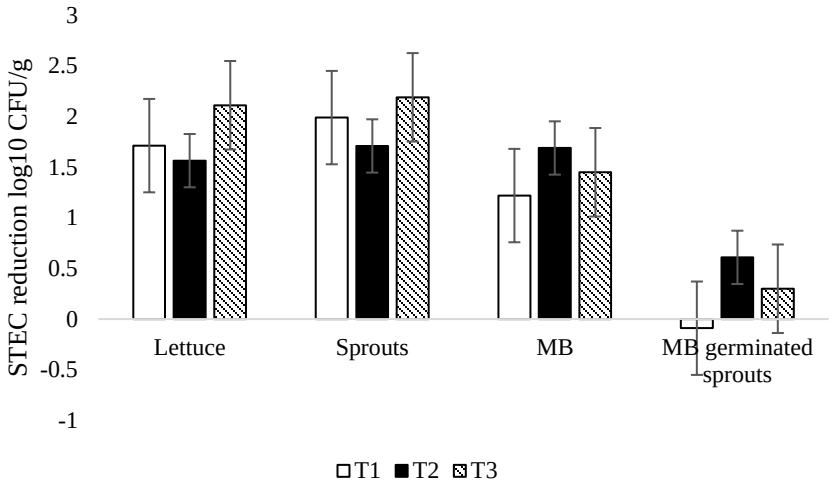

Figure 3.9. STEC reduction (log_{10} CFU/g) on lettuce, sprouts, MB, and MB germinated sprouts immersed in 10^5 CFU/ml STEC after treatment with chlorinated water (T1); STEC phage cocktail (T2); STEC phage cocktail plus chlorinated water (T3) (P<0.0001), 150 ppm chlorinated water was used for lettuce and sprouts, 1000 ppm chlorinated water was used for MB.

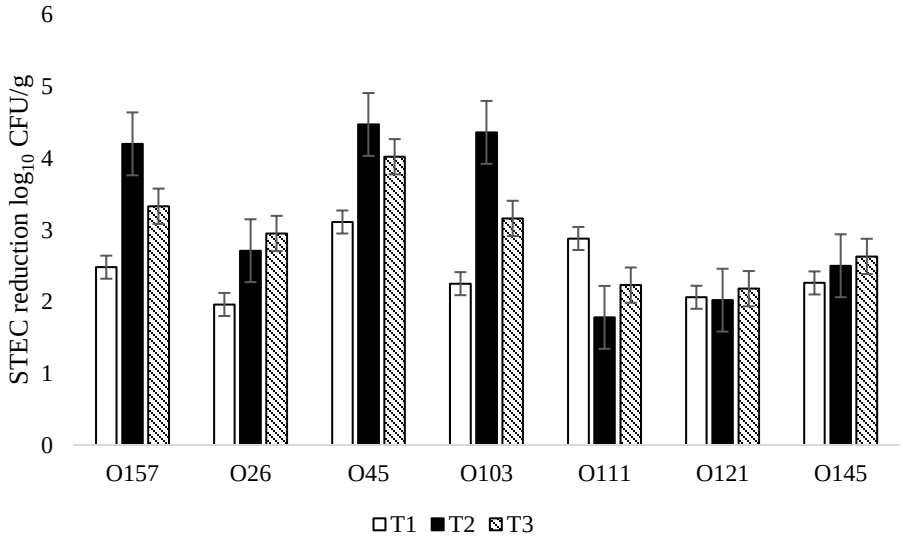

Figure 3.10. STEC serogroup reductions on lettuce adulterated through immersion with 10^5 CFU/ml STEC and treated with 150 ppm chlorinated water (T1); STEC phage cocktail (T2); STEC phage cocktail plus 150 ppm chlorinated water (T3) (P = 0.8209).

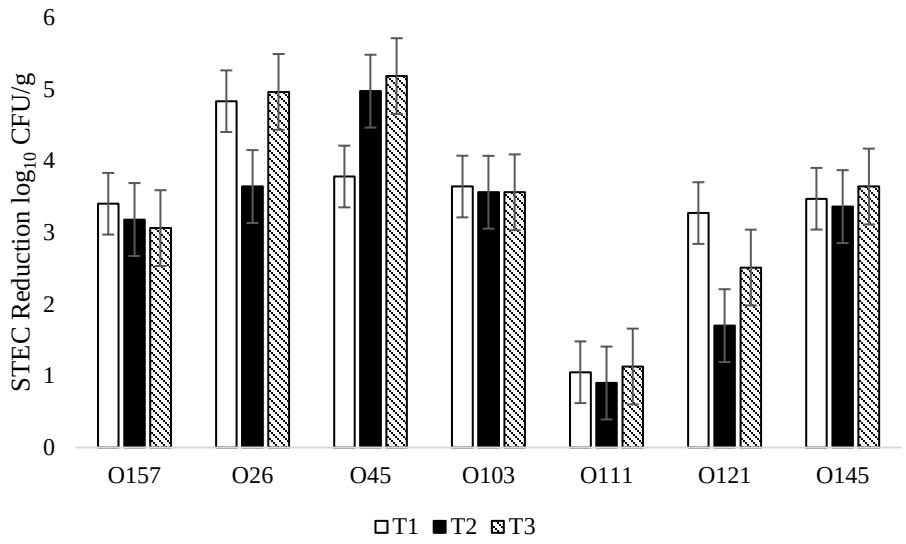

Figure 3.11. STEC serogroup reductions on sprouts adulterated through immersion with 10^5 CFU/ml STEC and treated with 150 ppm chlorinated water (T1); STEC phage cocktail (T2); STEC phage cocktail plus 150 ppm chlorinated water (T3) (P<0.001).

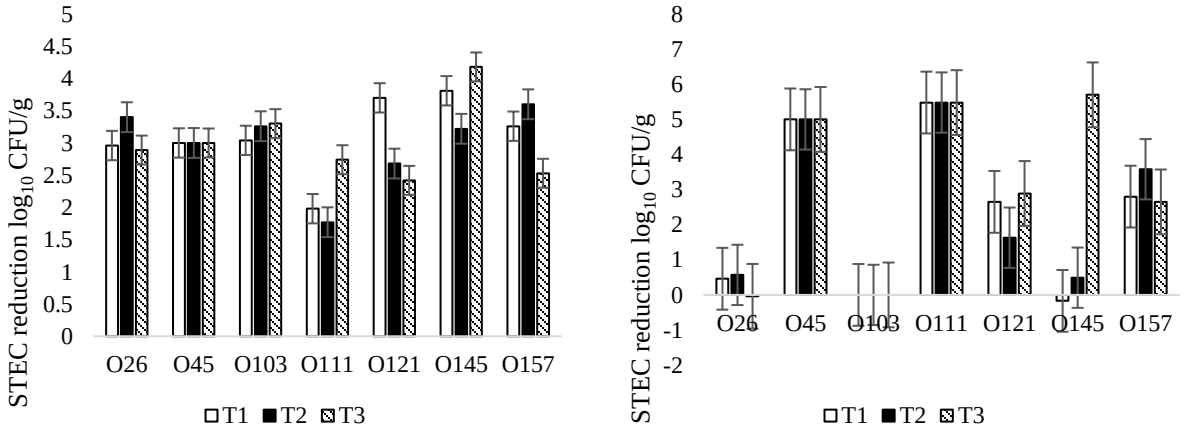

Figure 3.12. A, B. STEC serogroup reductions on MB and MB germinated sprouts adulterated through immersion with 10^5 CFU/ml STEC and treated with 150 ppm chlorinated water (T1); STEC phage cocktail (T2); STEC phage cocktail plus 150 ppm chlorinated water (T3) (P<0.05).

Table 3.11. STEC total numbers reduction (log CFU/g) on adulterated lettuce and sprouts as a result of treatment with phage cocktail by immersion

Produce Type	T1	T2	T3	SEM	P value
Lettuce	1.71a	1.56 a	2.11a	0.141	< 0.0001
Sprouts	1.99 a	1.71 b	2.19 a	0.08	< 0.0001

[a,b,·] Least square means within a row lacking a common letter differ (P < 0.0001)

[T1], Fresh produce and MB immersed in chlorinated water

[T2], Fresh produce and MB immersed in phage cocktail

[T3], Fresh produce and MB immersed in chlorinated water and phage cocktail

Table 3.12. Effect of temperature x treatment interaction (SEM 0.14; P value = 0.0069) on reduction of total numbers of STEC as a result of immersion of sprouts in a phage cocktail

	Temperature (°C)		
Treatment	2	10	25
1	2.48 a/c	2.09 a/c	1.42 a/d
2	1.92 a/c	1.63 a/d	1.59 b/e
3	2.77 a/c	2.37 a/d	1.43 ab/e

a,b. Least square means within a row lacking a common letter differ (P=0.0069)

[T1], Fresh produce and MB immersed in chlorinated water

[T2], Fresh produce and MB immersed in phage cocktail

[T3], Fresh produce and MB immersed in chlorinated water and phage cocktail

c, d, e. Least squares means within a column lacking a common superscript letter differ (P=0.0069)

3.3.5. Delivery of STEC phage cocktail in lettuce and sprouts through immersion and spraying

3.3.5.1. Lettuce

When phages were applied to reduce STEC total numbers on lettuce, the higher reductions were obtained from lettuce immersed in STEC phage cocktail than when sprayed, and was most notable at 2 and 10 °C. The highest reduction was 2.07 $\log_{10}$ CFU/g after 72 h storage at 2 °C in scaled-up experiments, which was 0.5 $\log_{10}$ CFU/g higher than when the STEC cocktail was sprayed onto produce. The reduction in STEC steadily increased over 72 h in scaled-up experiments at 2 and 10 °C, indicating that the STEC phage cocktail remained efficacious over the storage period (Fig. 3.13). Delivery of the phage through immersion as compared to spraying was more effective at controlling STEC on produce stored longer periods at lower temperatures. Based on observations here, the highest reduction occurred in 1 h in phage immersed samples (1.96 $\log_{10}$ CFU/g) at 25 °C, but declined thereafter and contrasted with the samples immersed in the phage cocktail in the scaled-up experiment (Fig. 3.13). In the spot-inoculation experiment, lettuce treated by spraying the phage cocktail had the highest reduction, which was 1.09 $\log_{10}$ CFU/g after 72 h at 25 °C, suggesting that spraying was a more effective phage delivery method than immersion if lettuce is stored at 25 °C for longer periods.

Among the STEC serogroups examined in spot-inoculated experiments survivors from serogroups O45, O111, O121, and O145 were detected, but no viable cells from O26, O103, and O157 were detected after treatment (Table 3.7). Isolates from all serogroups remained viable in the scaled-up experiment. Spray delivery was the more effective phage application method against isolates of serogroups O26, O103, and O157 compared to immersion.

3.3.5.2. Sprouts

A similar trend of phage effectiveness was demonstrated in sprouts. The phage cocktail was more effective at controlling STEC in scaled-up than spot-inoculation experiments, and the reductions caused by immersion in the STEC phage cocktail were greater than by spraying the sprouts (Fig. 3.14). The phage cocktail remained effective at the lower temperatures (2 °C > 10 °C > 25 °C) on sprouts treated by either spraying or immersion in the STEC phage cocktail (Fig. 3.15). However, only 4 of 7 serogroups (O45, O111, O121, and O145) were detected from samples in the spot-inoculation experiment, and these were isolates from the same serogroups as with lettuce. STEC serogroups O26, O103, and O157 were not detected or recovered by PCR or IMS from any of the sprouts sprayed with the phage cocktail (Table 3.8). Thus, the STEC phage cocktail ultimately killed isolates from 3 of the 7 serogroups when sprayed onto sprouts. As with lettuce, all serogroups were detected on Rainbow agar in the scaled-up experiment with sprouts. The phage cocktail eliminated STEC O26, O103, and O157 when applied by spraying but it was less effective than immersion with both lettuce and sprouts.

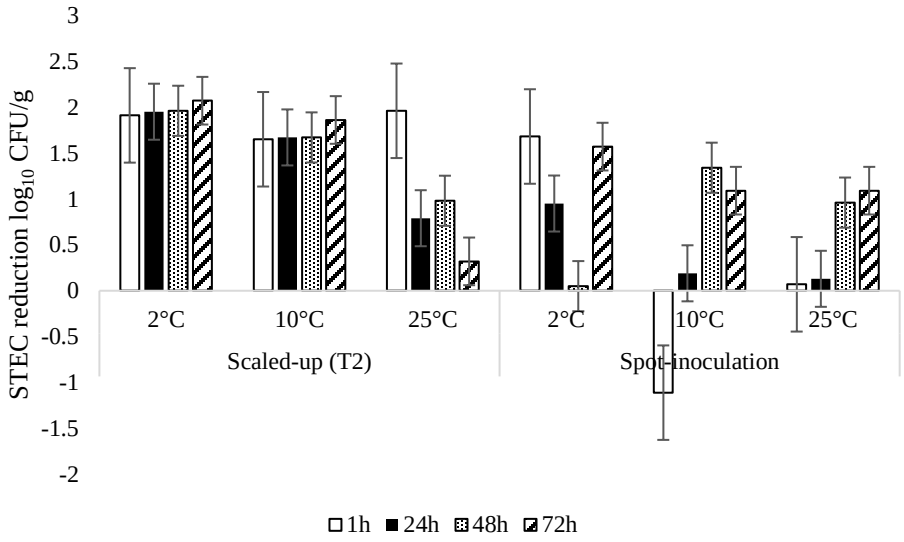

Figure 3.13. Reduction of total numbers of STEC on lettuce immersed in 10^5 $\log_{10}$ CFU/ml STEC and treated with a phage cocktail delivered by immersion (scaled-up) and spraying (spot-inoculation) at 2, 10, 25 °C for 72 h

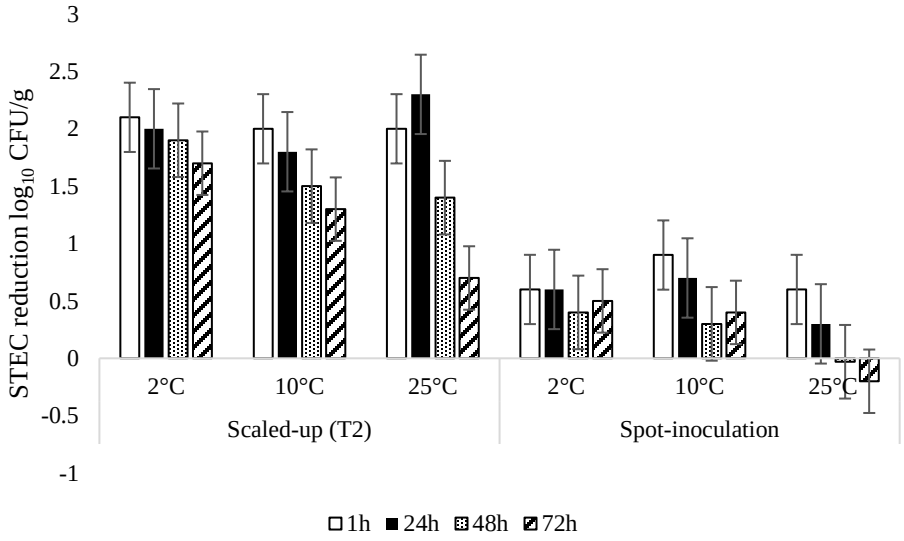

Figure 3.14. Reduction of total numbers of STEC on sprouts immersed in 10^5 $\log_{10}$ CFU/ml STEC and treated with a phage cocktail delivered by immersion (scaled-up) and spraying (spot-inoculation) at 2, 10, 25 °C for 72 h

3.3.6. Phage-resistant mutant

No phage resistant STEC strains were found from either spot-inoculation or scaled-up trials for fresh produce and seeds. The phage cocktail remained effective against those STEC isolates from targeted serogroups after being re-exposed to the phage cocktail.

3.4 Discussion

3.4.1. Phage effectiveness *in vitro* influenced by temperatures

Phages used in this study exhibited potential to control all of the targeted STEC strains when used at high concentrations at 2, 10, and 25 °C. Refrigeration is the most common method for food preservation as low temperatures slow or stop microbial growth. However, these storage conditions can also reduce the activity of phage as they depend on the metabolic activity of the host to complete their lytic cycle. As the temperature decreased during washing and storage, the lytic activity of phages would also be expected to decrease since optimal phage activity has been reported to be in the range of 30 to 45 °C (Obeso et al., 2008). However, in the present study it was found that phages were effective at refrigeration temperatures, which suggested that some of the phages might be active through the LO mechanism. A higher MOI of phages was required to eliminate STEC at 2 and 10 °C than 25 °C. Refrigeration slows bacterial growth, which limits the ability of phages to enter the lytic cycle, as the bacterial cell machinery needed for phage replication is less active at lower temperatures. *E. coli* growth and metabolism are generally limited at low temperature as membrane fluidity is decreased because membranes become rigid (Parola et al., 1990; Graumann and Marahiel, 1996). *E. coli* can exhibit the homeoviscous adaptation which allows their membrane composition to alter and adapt to changes in temperature (Sinensky, 1974). At low temperature, the increasing incorporation of unsaturated fatty acids will partially

compensate for the loss in membrane flexibility (Parola et al., 1990). As temperature is decreased, the affinity of the bacterial enzymes and their thermal energy is also reduced, which slow down bacterial metabolism (Amato and Christner, 2009). In contrast, for LO, bacterial growth is irrelevant, as the high MOI results in a substantial number of phages being adsorbed to the host and cell wall destruction occurs as a result of the absorption of a mass of gp5 proteins produced by the phage (Asami et al., 1997). The observation that the MOI was higher at 2 and 10 °C than 25 °C suggests that these phages killed STEC via a similar mechanism. Some phages (AYO145A) eliminated targeted hosts at 2 and 10 °C but not at 25 °C, suggesting that LO activity or a mechanism other than LO could have been responsible for the effectiveness of this particular phage. Another possibility could be the production of lytic enzymes by these phages that may remain active during storage under low temperatures, which of course needs to be tested in a future study.

For the purpose of reducing multiple STEC serogroups during processing, storage, and transportation, applying phages as a cocktail on food products is encouraging as all phages showed activities reducing the STEC population at 2, 10, and 25 °C, especially for STEC O26, O103, O145, and O157.

3.4.2. The effectiveness of phage cocktail decreased on fresh produce as compared to *in vitro*

The first possible explanation for the decreased effectiveness of phages O45, AXO111A, AXO121A, and AYO145A in fresh produce is that conditions were more conducive for bacterial growth in the liquid media of the microplate assay than on vegetables. Researchers have previously shown that bacteria are more easily grown in planktonic cultures as compared to solid food due to their greater mobility in liquid (Jeanson et al., 2015). The reduced growth of bacteria on produce

is a possible reason for why the phages were not as effective since the bacteria were not as metabolically active. The larger number of target bacteria in liquid media results in a greater rate of phage amplification, generating more phage to infect and kill bacteria. The cofactors added to the liquid media also promoted phage attachment to bacteria, which was absent in produce. Furthermore, phages may also diffuse more freely in liquid media of the microplate assay, increasing the likelihood of them contacting their targeted host. Previous research has applied similar principles using phages to reduce *L. monocytogenes* on ready-to-eat food (both liquid and solid products) that was stored up to 6 days at 6 °C (Kazi and Annapure, 2016). In their study, phages were shown to be more effective in liquid foods such as milk and cheese brine, where bacteria were eliminated and remained undetectable after storage. In contrast, with solid foods such as hot dogs and salad leaves, phages reduced bacterial counts by up to 5 logs, but bacteria were still detectable by plating. More research is needed to determine the extent that differences in the absorption efficiency of the phage is associated with differences in the chemical composition of growth media and foods (Chan et al., 2012).

Another possible explanation for more bacteria surviving on plant material after phage treatment is that the bacteria invaded into the inner structure of the plant tissue which makes it more difficult for phage to attach (Beuchat, 2002; Sofos et al., 1998; Zeng et al., 2014). STEC outbreaks in vegetables were often related to shredded produce or salad where cutting or mechanical forces damage produce surfaces (Beuchat, 1999). Bacteria that invade the cut edges and stomatal openings of produce leaves may be less accessible to phages, reducing their ability to decontaminate vegetables (Solomon et al., 2003; Stine et al., 2005). A previous study assessing STEC invasion of lettuce found that *E. coli* O157:H7 was associated with 7% of the lettuce leaves that had been surface sterilized after inoculation, suggesting that the bacterium within the leaves

evaded sanitation (Mootian et al., 2009). STEC bacteria used in the current research could also have penetrated to the interior of leaves, reducing the efficacy of phage. However, results from this and previous research are not sufficient to explain the difference in effectiveness of phage during *in vitro* and *in vivo* tests.

It is important to mention that the concentration of STEC in naturally contaminated produce is likely at least 2-3 log CFU/g lower than the 5 log CFU/g used in the present study. This may also have caused differences in the effectiveness of phage. A previous study by Althaus et al., (2012) investigated the number of microorganisms present on fresh produce by screening 142 salads, 64 fresh-cut fruits, and 27 sprout samples. Generic *E. coli* was only found in 5 lettuce samples at a concentration of 2-3 log CFU/g. STEC were also detected in one lettuce sample using multiplex real-time PCR at < 1 log.

A pre-trial was done following the scaled-up experiment with phage cocktail against STEC on lettuce at 10^3 log CFU/ml, and stored for 24, 48, and 72 h at 2 ^{o}C. The phage cocktail caused > 2 log CFU/g reductions in total STEC numbers over 72 h. Phages may have more effectiveness when applied during commercial fresh produce production as numbers of STEC would be far lower than 5 log CFU/ml. Further, an additional situation that needs to be considered is that if the STEC concentration is too low in the produce, the phage may not come in contact with target hosts due to poor phage motility on solid food. More studies are needed for analysis of the actual effectiveness of phage cocktails used on real industrial production lines where the STEC numbers on contaminated produce are likely to be lower.

3.4.4. The effectiveness of phage cocktail varies on different produce types

Beside the difference in phage efficacy evident between the microplate assay and fresh produce, the effectiveness of phages varied among produce types. For the proposes of safety, the properties of produce that influence the effectiveness of phage should also be considered in designing phage cocktails that will control pathogens across a range of produce types. In the present research, the phage cocktail was more effective at reducing STEC on lettuce than on sprouts, particularly at 2 °C. Phages may be less effective on sprouts compared to lettuce because conditions are more conducive for the growth of STEC. Pathogens found in sprouts mostly originate from seeds that are contaminated during growth in the fields, during harvest, handling, processing, or distribution (Taormina et al., 1999). Warm temperatures (24-35°C) and high moisture levels are required for seeds to germinate, conditions that are ideal for microbial growth (Montville and Schaffner, 2005). Itoh et al. (1998) found that sprouts were highly contaminated (> 7 logs CFU/g) in both the cotyledons and hypocotyl after soaking in a suspension of *E. coli* O157:H7, and suggested that these plant structures could also shield this pathogen from phages. Also, the complex waxy structure of the surface lettuce leaves can interfere with microorganisms entering the plant tissue, possibly making them more accessible for phages to attach (Agrios, 1997).

3.4.5. The effectiveness of the phage cocktail varied with isolates from different STEC serogroups

When looking at treatment effectiveness against individual serogroups, isolates from serogroups O26, O103, and O157 were not detected from either lettuce or sprouts. The phage cocktail was more effective against some serogroups than others, and especially for *E. coli* O157:H7. Phages intended for use in food should have strong lytic activities and broad host ranges

that cover the target microorganism. Wang et al. (2015) reported that phages used in the present study were selected with bias towards their effectiveness against O157, with the exception of phage AKFV33, phages AYO26A, and AXO121A could lyse the 4 common STEC O157:H7 strains. Multiple phages used in the current study were effective against serogroup O26 (AYO26A, AXO103A, and AXO111A) and O103 (AYO26A, AXO103A, and AXO121A) (Wang et al., 2015), which may explain why the phage cocktail had the highest effectiveness against these serogroups. In addition, differences in the sensitivities of STEC serogroups to phages maybe due to pharmacokinetic aspects. For example, with fresh produce, phage attack of some serogroups including *E. coli* O157:H7 may be less difficult than for other non-O157 serogroups (Chan et al., 2012).

The findings of the present research on phage could be a critical step towards solving the food safety issue associated with STEC in fresh produce such as lettuce and sprouts. According to a CDC report, the STEC serogroups associated with outbreaks caused by lettuce during 2006 to 2016 were O157: H7 and O145. The phage cocktail in the current study caused a > 2 log CFU/g in isolates from both of these serogroups. Outbreaks caused by sprouts were associated with serogroups O157:H7, O26, and O121 and the STEC phage cocktail used in the present study was proven to be effective at reducing two of them (CDC, 2017).

3.4.6. Storage times and temperatures impacted the effectiveness of the phage cocktail under scaled-up conditions

Treatment of fresh produce with a STEC phage cocktail reduced but did not eliminate 7 STEC serogroups at the storage temperatures of 2, 10, and 25 °C. Phages were more effective at 2 and 10 °C than 25 °C, at storage times over 1 h with both lettuce and sprouts. The best conditions

to use the phage cocktail as a biocontrol method for STEC was to apply it at 2 and 10°C over 72 h of storage, or at 25 °C for 1 h. The observation of stable phage cocktail effectiveness as the storage time increasing in 2 and 10 °C is encouraging since the recommended shelf-life for fresh lettuce is 1 week (168 h), and 3-5 days (72 h to 120 h) for sprouts at refrigeration temperature (4 °C) (Renee and McKinney, 2009). However, the STEC population cannot be controlled after longer periods of storage if they were not eliminated early at 25 °C. Data obtained in this research suggests that the use of phage to control STEC on lettuce and sprouts would not be effective at ≥ 25 °C. However, a previous study reported that phages could eliminate *E. coli* O157:H7 in broth at 30 or 37 °C, but was less effective at 12 °C, likely due to reduced metabolic activity of the host cells at this lower temperature (Kazi and Annapure, 2016). These differences could be explained by differences in the phage's efficacy and mechanisms employed by the phage used to reduce STEC. The higher growth rate and more metabolically active cells at 25 °C should enhance the effectiveness of phage. However, results obtained from the present scaled-up and spot-inoculation experiments indicated that STEC phages were more effective at controlling STEC on lettuce and sprouts stored at ≤ 10 °C for periods longer than 1 h. In support of the current findings, Sharma et al. (2009) reported a similar result when using phage ECP-100 to reduce *E. coli* O157:H7 on fresh-cut lettuce stored at different temperatures. In this study, ECP-100 was more efficient at reducing *E. coli* O157:H7 at 4°C than at 20°C (Sharma et al., 2009). In addition, other researchers have reported that phage more effectively control other pathogens at temperatures lower than 10 °C. For example, applying phages on fresh-cut honeydew melons reduced *S.* Enteritidis populations by 3.5 log CFU/wound when stored at 5 °C and *Salmonella* spp. populations by 2 to 4.6 $\log_{10}$ CFU/sample at 10 °C (Leverentz et al., 2001; Leverentz et al., 2003). In these cases, LO was suggested as the most probable mechanism whereby phages were killing bacteria at 2/10 °C

(Ferguson et al., 2013). Phages in the present study may also have used the LO mechanism at low temperatures, but more research is required to understand the mechanisms that phage use to control bacterial populations at refrigeration temperatures. The investigation of phage activity at lower temperatures is essential since most fresh produce is stored at ≤ 4 °C prior to sale.

Other possible explanations for the superior efficacy of phage cocktail at 2 and 10 °C on fresh produce may be due to unexamined factors, such as that the phages were more active at lower temperatures, or the lack of growth of the bacterial population at low temperatures on produce (Sharma et al., 2009). Other researchers examined the stability of *Stx*2 phages stored at 4, 24, and 37°C in LB broth over 60 days (Rode, 2011). Phages stored at 4 °C initially exhibited a 3 log reduction in titre after one day, but their activity remained relatively constant after 60 days of storage. In contrast, phages stored at 24 °C exhibited a continuous decline in activity with a 5 log reduction in titre at the end of storage. If the phages used in the present study behave similarly, the loss in phage activity at 25 °C as compared to at 2 and 10 °C could reflect a reduction in titre at higher temperatures.

Storage time is also a critical variable, especially for the STEC phage cocktail at 25 °C, where the effectiveness of the phage declined after just 1 h of storage of lettuce and sprouts in both spot-inoculation and scaled-up experiments. At 25 °C, the cocktail may have been effective in the first hour as a result of completion of a lytic cycle which typically requires 20-40 min (Mathews, 1994; Shao and Wang, 2008). In the current study, lytic activity declined after the first lytic cycle, possibly due to the lack of diffusion on solid food, where phages did not come in contract with host receptors. However, with longer periods of exposure to the higher temperatures, the activity of the phage may have been reduced with longer storage times.

3.4.7. Survival of isolates after phage cocktail treatment under scaled-up conditions

Based on the results obtained for produce, the STEC phage cocktail could not completely eliminate any of the serogroups in either lettuce or sprouts in scaled-up trials. Surviving bacteria were detected for all serogroups even when the cocktail achieved a high level of reduction (> 5 $\log_{10}$ CFU/g) in STEC populations. A possible explanation for the survival of bacterial cells may be related to phage lysogeny. Researchers have shown that phages have a mechanism to communicate with one another through the production of a small peptide molecule called phi3T (Erez et al., 2017), which allows phages to "control" the way they attack bacterial cells. Phage can synthesize and release phi3T which can control the number of host cells infected by either the lysogenic or lytic pathways. Phage attacks the bacterial host via lysogenic pathway and produces prophages in hosts, where the host becomes phage-resistant to lytic infection from the same phage type (Skurnik and Strauch, 2006). Directing some phage to towards the lysogenic pathway can ensure that some host cells remain viable for future replication of phage as environmental conditions change.

3.4.8. The effectiveness of phage cocktail was enhanced on lettuce and sprouts when combined with chlorinated water

Wash water containing sodium or calcium hypochlorite is the primary method of preventing pathogen contamination during produce production. Chlorinated water has been proven effective at killing pathogenic *E. coli* attached to surfaces, or cut edges and stomata in produce (Sharma et al., 2009). Chlorine cause an irreversible oxidation on sulphydryl group of the bacterial enzymatic sites that located in the cytoplasmic membrane, which can damage the structure of the cell membrane and make the phage penetration easier (Estrela et al., 2001). Neo et al. (2003)

reported that 170 ppm chlorinated water could reduce *E. coli* O157:H7 by 1.5-2.0 logs on mung bean sprouts, which was similar to the reduction of STEC with chlorinated water in the current study (1.99 $\log_{10}$ CFU/g).

Based on the results obtained from the present study, it was found that the effectiveness of a phage cocktail at controlling STEC on lettuce, sprouts, and MB was enhanced when combined with chlorinated water. Ferguson et al. (2003) reported similar findings for lettuce leaves contaminated with *E. coli* O157:H7 and treated for 30 sec with 50 μg/ml sodium hypochlorite (NaOCl) or with NaOCl and a commercial phage cocktail called Ecoshield or with EcoShield alone. The samples were stored at 4 °C for 24 h. The combination treatment resulted in a 1.54 $\log_{10}$ CFU/cm^2 reduction in *E. coli* O157:H7, which was more effective than either NaOCl (1.38 $\log_{10}$ CFU/cm^2) or EcoShield (1.32 $\log_{10}$ CFU/cm^2) alone. However, differences among treatments were not statistically significant. This previous research confirmed our finding that using chemical sanitizers can enhance the effectiveness of lytic phages at reducing STEC on lettuce. The current study provides evidence that using a combination of chlorinated water and STEC phage cocktail on fresh produce has the potential to reduce STEC populations greater than either treatment alone.

In support of the current findings, other researchers reported that a combination of chemical treatment and phage achieved higher reductions of bacteria compared to using either of them alone, and these studies were not only associated with *E. coli* serogroups but also with other pathogens. For example, Sukumaran et al. (2015) showed that the application of Salmofresh^{TM} combined with 30 ppm chlorine achieved greater reductions of *Salmonella* than either phage or chlorine alone on raw chicken breast at 4 °C for 24 h. Additionally, the combined treatment of a phage with activity against *Pseudomonas aeruginosa* and chlorine reduced the biofilm growth of this bacterium by

94 ± 2% and removed 88 ± 6% of existing biofilms (Zhang et al. 2013). The combination treatment was more effective than either the phage or chlorine treatment alone.

3.4.9. The effectiveness of phage cocktail and chlorinated water in Mung beans (MB) and germinated MB sprouts

In MB, phage cocktail treatment alone was more effective than chlorinated water. A possible explanation is the potency of active chlorine was diminished rapidly when came in contact with seeds which contain high levels of organic matter. The phage cocktail effectiveness is likely not influenced by organic matter (Jaquette et al., 1996). In addition, chlorinated water has different effects on STEC serogroups. According to a previous study, some non-O157 STEC strains appear to be more susceptible to chlorine treatment than *E. coli* O157:H7 in the presence of organic matter (Shen et al., 2013). This may explain why some of the non-O157 serogroups were more affected than others by treatments with chlorinated water.

Studies of using phages as a biocontrol method are meaningful for seeds and sprout production since sprouts pose a higher risk of containing pathogens as compared to other fresh produce because the bacterial population can grow exponentially during the germination process. During sprout germination, any pathogens that survive in the seeds can grow rapidly (FSAI, 2011), and this cannot be prevented by phage cocktail or chlorinated water. It is essential to find an effective disinfection method to control the number of STEC in seeds since a previous study demonstrated that *E. coli* O157:H7 numbers in alfalfa seeds can increase to 10^6 - 10^7 CFU/g within 48 h during germination, a level that remained unchanged at 9 ± 2 °C for 6 days (Taormina and Beuchat, 1999). Currently, soaking seeds in chlorinated water is the most common method of reducing food-borne pathogens commonly associated with seeds and sprouts. Chlorinated water

applied to seeds before germination has proven effective at reducing pathogens. Presoaking seeds in $Ca(OCl)_2$ or NaOCl at concentrations of 2,000 ppm chlorine for 10 min can reduce *E.coli* O157:H7 on seeds and sprouts by up to 2.8 $\log_{10}$ CFU/g (Taormina and Beuchat, 1999). There has been a lack of studies examining the ability of the direct application of phages to reduce STEC on sprouts, especially for non-O157 serogroups. As a potential alternative for commonly used disinfection methods in the food industry, STEC phage cocktail has potential since it reduced *E. coli* O157:H7 3.58 $\log_{10}$ CFU/g, indicating that it was more effective than conventional chemical disinfectant on mung bean seeds. In addition, the concentration of chlorine in the water in the present study was below the CFIA recommended concentration for washing seeds of 2000 ppm calcium hypochlorite for 15 to 20 min (CFIA, 2007). In the present study, seeds were immersed in 1000 ppm chlorinated water for 15 min, a higher concentration may even further reduce the STEC population in seeds but may decrease the germination rate.

3.4.10. Phage delivery method influences effectiveness of the phage cocktail on lettuce and sprouts

The phage delivery method used is essential as it may influence the ability to control STEC on produce. My hypothesis that spraying would be the most effective phage delivery method was based on previous studies which demonstrated that spraying chlorinated water was more effective at reducing pathogens on fresh produce than dipping or submerging the produce in the chlorinated water (Beuchat et al., 1998; Sharma et al., 2009). It is important to mention that the produce processing conditions, and pathogens used in previous studies were different than in the current study. In addition, phages were not applied in the previous studies. Additionally, as a result of the present work, it became apparent that in the context of the earlier hypothesis, spraying was not the ideal delivery method for STEC phage at temperatures ≤ 10 °C.

The present research suggested that phage effectiveness was influenced by delivery method in both lettuce and sprouts. A potential explanation for the greater reductions by immersion of the fresh produce in the STEC bacterial cocktail is that this practice increased the area of produce contact surface with pathogens. The lettuce pieces/sprouts were fully immersed in the STEC bacterial cocktail in the scaled-up experiment as compared to only a small region of the leaf or sprouts in the spot-inoculation tests (10 µl per lettuce piece, 6 µl per sprouts). With immersion, STEC may also gain access to the inner tissues of the plant through the cut edges and therefore may be more unlikely to come in contact with phages. In support of the findings in the current study, a previous study found differences between immersion and spraying phage on green leafy vegetables (Ferguson et al., 2013). Similar to the current results, bacterial populations increased 24 h after spraying phage at 4 °C, while immersion in the phage cocktail resulted in a greater reduction of the *E. coli* O157:H7 population after 24 and 48 h. This supported the finding in the present study of the STEC phage cocktail having increased effectiveness at low temperatures when substrates were immersed as compared to being sprayed. Phage have greater mobility with immersion compared to spraying. Phage particle dispersion was increased as phage cocktail was sprayed onto lettuce leaves, which allowed the phage cocktail to be more active on produce surfaces, but they did not penetrate deeply into the tissue. Although immersion may not be as rapidly effective as spraying on produce surfaces in 1 h, it is more effective afterward (Ferguson et al., 2013).

3.4.11. Phage-resistant mutants are less likely developed using cocktails containing multiple phages

The phage cocktail remained effective against those STEC strains that survived, suggesting that none of those isolates developed resistance to phage. A possible explanation is that using

multiple phages could increase phage efficiency by attaching to receptors of multiple STEC serogroups. The combination of phages can target multiple STEC strains or cover multiple species that are associated with enteric diseases; a bacterium which is resistant to one phage type can be killed by another type (Chan et al., 2012).

Mutation is a way of bacterial evolution which is modification of the bacterial genomes to develop a new function to replace the old function through nucleotide exchange, insertion, and deletion. Bacterial mutation rates are generally between the range of 10^{-5} to 10^{-9} per nucleotide in one generation (Brüssow et al., 2004). The likelihood of developing a mutant is influenced by the cell metabolic rate and thus related to storage temperature. At higher temperature (25°C), bacteria have a faster metabolism than at 2 and 10 °C. As temperature increases, bacteria with higher metabolic rate will have more frequent DNA substitution caused by the generation of oxygen radicals, which are highly reactive molecules that may lead to DNA damage (Andrew et al., 1992). The oxidative DNA damage will be repaired continuously by bacteria (Shigenaga et al., 1989), but incorrect repair may cause mutation. Therefore, the mutation rates will increase as the bacteria with higher metabolic rate have more turnover of DNA.

Chapter 4. Appendix studies

4.1 STEC phage cocktail combined with Salmofresh™

Previous studies have shown that the different types of phage may compete with each other for resources when applied together. Phages differ in their pharmacokinetic and pharmacodynamic properties, which have the potential to influence the ability of different phages to eliminate targeted bacterial populations (Chan et al., 2012). Competition between phages may lead to lower reduction of pathogens on food. This led to the hypothesis that the STEC phage cocktail would be less effective at reducing STEC if it was combined with another phage cocktail product. The scaled-up validation experiments were also conducted with the STEC bacterial cocktail combined with a commercial phage cocktail against multiple *Salmonella* strains called Salmofresh™ (contains 7 *Salmonella* phages), a product approved for use by the USDA. My hypothesis was not supported as Salmofresh™ did not hinder the STEC phage cocktail's effectiveness and actually slightly increased the reduction in STEC due to unknown reasons. A possible explanation could be that Salmofresh™ produced enzymes that killed both *Salmonella* and STEC. Researchers have reported the release of lysins from phages with activity against *S. pyogenes* and *B. anthracis*, can damage the bacterial cell wall and kill the host using a LO mechanism. In my case, the Salmofresh™ may also be able to produce lysins that have a broad spectrum of activity and negatively impact both *Salmonella* and STEC. However, more research is needed to characterize the factors responsible for the greater reduction in STEC by the combined phage cocktails.

Chapter 5. Conclusion

Overall, the present study demonstrates the possibility of using a phage cocktail to effectively reduce STEC in fresh produce. The reduction was affected by time and temperature interaction; phages were effective at 2, 10, and 25 °C *in vitro*. At 2 and 10 °C, phages were effective over 72 h in both lettuce and sprouts even when bacterial metabolism was limited by low temperatures, which suggested the mechanism phages used was LO. STEC phage cocktail was effective against isolates from 7 STEC serogroups in lettuce, sprouts, and mung bean seeds. This was particularly true for isolates of serogroups O26, O103, and O157, while the effectiveness on serogroups O45, O111, O121, and O145 was less noticeable. The STEC phage cocktail can be used as an individual treatment or combined with the chlorinated water and Salmofresh™. The STEC phage cocktail has the potential to be an effective antimicrobial treatment in the fresh produce industry.

Even though the phage cocktail used in this study proven effective against isolates of STEC O157:H7 and the "big 6" serogroups in fresh produce and mung beans, there are still some hurdles that need to be overcome for this phage cocktail to be accepted by the produce industry. Firstly, this phage cocktail will have to be approved by regulatory as GRAS to be used on food. Moreover, phages in this cocktail were propagated using pathogenic *E. coli* by trained personnel, but it unlikely that the strains would be approved for amplification of phage for industrial use. Thus, the cost of using the phage cocktail is another important factor that needs to be considered. Also, consumer acceptance of applying virus onto foods may be another barrier to the introducing of phage cocktails into industry (Goodridge and Bisha, 2011). The general negative impression of viruses may stop consumers from buying food treated with phage. More education on phage specificity will be needed to increase consumer acceptance of phage-treated foods.

For future studies, the phage cocktail can be further tested to better characterize its host range and effectiveness under different conditions. Such studies should be designed using the phage cocktail against other strains within the same STEC serogroups used in the present study. It can also be tested on other produce types since the phage cocktails had different responses among lettuce, sprouts, and MB. Storage times longer than 72 h may be tested in the future as produce may be stored longer before consumption. In addition, phage cocktail should be tested with lower bacteria concentration and larger produce quantity to ensure the phage cocktail will be effective in the food industry. Last but not least, future study should also develop methods that integrate phage cocktail approaches with other antimicrobial agents such as bacteriocins and essential oils.

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