Using Lytic Bacteriophages as a Biocontrol Intervention to Eliminate *Salmonella* in Romaine Lettuce, Mung Bean Sprouts, Mung bean Seeds and Mung Bean Germinated Sprouts and to Prevent Shiga-toxigenic *Escherichia coli* Biofilm Formation on Romaine Lettuce

BY

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A Thesis Submitted to the Faculty of Graduate Studies of

The University of Manitoba

In Partial Fulfilment of the Requirements of the Degree of

MASTER OF SCIENCE

Department of Food Science

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Winnipeg

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ACKNOWLEDGEMENTS

This dissertation is dedicated to my mother and father for their love, support, understanding, and help.

I would like to give my sincere gratitude to my supervisor Dr. Claudia Narvaez and my co-advisor Dr. Tim McAllister, for the great opportunity provided when they accepted me to Food Science program to pursue Master at the University of Manitoba. This experience would never have been possible without their guidance.

Thanks to Dr. Narvaez, for being more than an advisor, encouraging me to be a brave, patient, kind and generous person in both my career and my personal life. Thanks to Dr. Argenis Rodas-González, for all his patient assistance with the statistical analysis.

Thanks to my committee member Dr. Rick Holley and Dr. Celine Nadon, for their kind assistance and helpful suggestions throughout my research.

To Yiran Ding, my friend, colleague, and roommate, for the most fabulous collaboration experience we did together for the project.

To Matthew Wells for his time and patience of reviewing and suggestions to my thesis.

To Danyang Hu, Rui Ma, Hao Cheng Du and all my friends for their companion, support and encouragement throughout my study.

To all the technical and administrative staff in Food Science Department. Thanks for their help and support.

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ABSTRACT

Introduction: Produce commodities have been linked to Shiga-toxigenic *Escherichia coli* (STEC) and *Salmonella* outbreaks worldwide. *Salmonella* bacteriophage have been successfully applied alone or in combination with other bacteriophages to control *Salmonella* in food. *E. coli* O157:H7 as a critical pathogen related to foodborne illnesses can persist in many foods and produce biofilms. *E. coli* O157:H7 biofilm can be destroyed by the enzymes produced by lytic bacteriophages, making the phages a potential intervention to prevent the biofilm formation on lettuce surfaces.

Purpose: 1) To assess the effectiveness of a commercial phage SalmoFresh™ in combination with a novel STEC phage cocktail to reduce/eliminate *Salmonella* on the surface of Romaine lettuce, mung bean sprouts, and mung bean seeds; 2) To determine the efficiency of a novel STEC phage cocktail at preventing *E. coli* O157 biofilm formation on Romaine lettuce pieces; 3) To determine the effects of cofactors on the effectiveness of STEC phages.

Methods: 1) The effectiveness of SalmoFresh™ was tested against 5 *Salmonella* strains (Newport, Braenderup, Typhimurium, Kentucky, and Heidelberg) at exponential growth phase individually, at 2, 10 and 25 °C using duplicate microplate virulence assays. Lettuce and mung bean sprout samples were tested with both spot inoculation and immersion. For spot inoculation, lettuce pieces and mung bean sprouts were washed with tap water before inoculation with *Salmonella* (10 µL/lettuce piece; 6 µL/sprout piece). SalmoFresh™ (10^8 PFU/ml) was sprayed on lettuce & sprouts and held for 1, 24, 48 and 72 h (2, 10 and 25 °C). For immersion, scale-up lettuce, sprouts, and mung bean seeds were washed with tap and chlorinated water (150 ppm for lettuce and sprouts; 1000 ppm for seeds) and rinsed twice with non-chlorinated water before immersed in

Salmonella cocktail culture (10^5 CFU/ml). Lettuce and sprouts were exposed to SalmoFresh™ (10^8 PFU/ml) only or in combination with STEC phage cocktail by immersion for 1, 24, 48 and 72 h (2, 10 and 25 ºC). Seeds were immersed in bacteriophage cocktails for 1 h at 25 ºC. *Salmonella* reduction/survival was determined by total plate count [Xylose Lysine Tergitol-4 (XLT4) agar] and immunomagnetic separation (IMS). Mung bean seeds from control and treatment groups were germinated with sterile water, and *Salmonella* survival in sprouted seeds was examined.

Lettuce pieces were used to assess the ability of STEC phage applied with cofactors (Mg^{2+}, Ca^{2+}, L-tryptophan) to prevent the formation of biofilms on intact and damaged lettuce surfaces. Lettuce pieces were pretreated with STEC bacteriophage cocktail for 2 h and inoculated with 4 biofilm-forming *E. coli* O157:H7 strains (EO122, R508, 1931, and 161-84) and incubated for 24 h at 2, 10 and 25 ºC. Then the pieces were washed twice with sterile water, and the reduction/survival was determined by total plate count on MacConkey Agar with Sorbitol (SMAC).

Results: 1) Microplate assay results indicated that SalmoFresh™ (10^8 PFU/ml) reduced (P=0.007) *Salmonella* by an average of 5 log CFU/ml after 5 h of exposure. However, the spot plate technique showed *Salmonella* survival after overnight incubation 37°C. For spot inoculation, *Salmonella* was reduced by 1.5 log CFU/g and 1.65 log CFU/g on lettuce and sprouts, respectively, when SalmoFresh™ was delivered using a sprayer. For the scale-up test, application of SalmoFresh™ in combination with STEC bacteriophage (lettuce: 3.48 log reduction; sprouts: 2.36 log reduction) did not further improve phage efficacy (P=0.9975) compared to applying SalmoFresh™ alone (lettuce: 2.41 log reduction; sprouts: 2.16 log reduction), but both were more effective (P<0.0001) than using chlorinated water alone (lettuce: 2.41 log reduction; sprouts: 1.83 log reduction). However, the effect of SalmoFresh™ alone or in combination with STEC

bacteriophage cocktail of reducing *Salmonella* was not significant when applied to mung bean seeds (P=0.2395) and germinated sprouts (P=0.1219).

Pre-treatment with an STEC bacteriophage cocktail and cofactors reduced (P<0.0001) *E. coli* O157:H7 attachment from 0 to 3.5 log CFU/cm^2 at 2, 10 and 25 °C with an overall reduction of 1.31 log_{10} CFU/cm^2. The highest reduction (3.42 log) was achieved at 25°C using bacteriophages plus cofactors for *E. coli* O157:H7 strain EO122. However, when no cofactors were applied, the overall reduction of *E. coli* O157:H7 attachment (on average 0.75 log_{10} CFU/cm^2) was lower (P<0.0001) than the bacteriophage treatments with cofactors.

In summary, SalmoFresh™ alone or in combination with an STEC bacteriophage cocktail applied through immersion reduced *Salmonella* on fresh produce, but the effect was not significant when applied to mung bean seeds (P=0.2395) and germinated sprouts (P=0.1219). The STEC bacteriophage cocktail partially prevented *E. coli* O157:H7 attachment and therefore biofilm formation on fresh produce. When bacteriophages were applied with cofactors, the cocktail was even more effective at reducing biofilm formation.

1. CHAPTER 1. INTRODUCTION

Fresh produce related food safety problem caused by pathogens is a serious health threat in developed and developing countries. In North America, 9.4 million cases of foodborne illnesses caused by 31 common pathogens lead to 55,961 hospitalizations and 1,351 deaths each year, 11% of the hospitalizations and 28% of deaths are caused by non-typhoidal *Salmonella* (Scallan et al., 2011). STEC have been reported to cause 265,000 cases of illnesses each year, resulting in more than 3600 hospitalizations and 61 deaths in the United States (Bryan et al., 2015). The Food and Drug Administration (FDA) reported that $10-$83 billion is spent every year for medications to relieve pain and suffering from foodborne illnesses (FDA, 2013). Due to these reasons, efforts towards developing effective antimicrobial strategies aimed at controlling foodborne pathogens have been increasing (Viazis et al., 2011). From the year 2007 to 2008, most produce-generated foodborne illness outbreaks were linked to *Salmonella* and STEC in North America (Bryan et al., 2015; Lynch et al., 2009). According to Beutin and Martin (2012) and Brandl and Amundson (2008), fresh produce-related outbreaks caused by *Salmonella and* STEC have been reported in North America, Australia, and Europe.

Until 2009, leafy greens had been ranked as the No.1 outbreak-related food category, being linked to 363 outbreaks and 13,568 reported foodborne illnesses (Davidson et al., 2013a). The outbreaks are due to cross-contamination during growing, irrigation, harvesting, processing, transportation, packaging and storage. Many produce-related outbreaks are caused by norovirus-infected food handlers in restaurants, but the large multi-state outbreaks are usually caused by bacterial contamination early in production on the farm. Lack of effective antimicrobial treatments from planting to harvest can raise the risk of pathogen contamination of final product (Buck et al.,

2003). Therefore, the correct bacterial control intervention, an appropriate treatment time, and a proper concentration of the antimicrobial solution in washing and disinfection steps are indispensable factors for reducing outbreaks caused by pathogens like *Salmonella* and STEC (Parish et al., 2003).

E. coli O157:H7 can form biofilms on the surface of fresh produce (Silagyi et al., 2009), a factor that is responsible for its frequent linkage to produce-generated foodborne outbreaks. Biofilms can be described as sessile microbial communities that grow on surfaces and are embedded in an exopolysaccharide matrix (EPS) with a heterogeneous gel-like architecture (Gómez-López, 2012). The architecture is a highly hydrated three-dimensional structure consisting of dense areas interspersed with pores and channels (Allison, 2000; Garrett et al., 2008; Gómez-López, 2012). The resistance of biofilms to antimicrobial biocides, environmental and oxidative stressors, disinfectants and sanitizers used in the food industry make them a severe food safety problem (Almasoud et al., 2015; Silagyi et al., 2009). The formation of biofilms by pathogens on the surface of fresh produce can cause post-processing cross-contamination, and have a direct negative impact on produce safety and public health (Gómez-López, 2012; Kumar & Anand, 1998).

Among the conventional chemical sanitizers, chlorine is most commonly used by industry to disinfect fresh fruits and vegetables (Davidson et al., 2013b). Since the mechanisms of pathogen biofilm formation are not the same for various produce surfaces, specific exposure times and free chlorine concentrations are critical for effective control of bacteria (Suslow, 2014). Lettuce requires an exposure of 100-150 parts per million (ppm) available chlorine on continuous belts, whereas spinach needs to be sprayed on continuous belts with 70-150 ppm of free chlorine (Suslow, 2014). However, chlorine fumes released from treated water can cause discomfort to workers (Suslow, 2014). Discolouration and undesirable odors can also occur if levels of chlorine exceed

1% (Baskaran et al., 2013). Additionally, the surplus water with chemical sanitizers need to be specially purified or treated, which can add a significant cost to the final processed product (Neal et al., 2012). To satisfy consumers' increasing demand for organic fruits and vegetables, bacteriophages, have recently been used to control pathogens on fresh produce and have also been shown to be active against pathogens in biofilms (Sharma, 2013). Bacteriophages are viruses that can invade bacterial cells by attaching to the surface of target bacteria (Sharma, 2013). They are not only ubiquitous in nature, but exist in cheese, sausages and a wide variety of ready-to-eat foods as well (Sharma et al., 2009). Since bacteria may become resistant to a single bacteriophage, bacteriophage cocktails are recommended as means of more effectively killing bacteria on fresh produce (Fischer et al., 2013). SalmoFresh™ is a mixture of 6 bacteriophages, specifically for killing *Salmonella* (Magnone et al., 2013), and is FDA approved generally recognized as safe (GARS) (Magnone et al., 2013). BacteriophageGuard Listex., EcoShield™ and ListShield™ are other commercial bacteriophages reported in food safety research (Anany et al., 2014). Intracellular bacteriophage proteins induce normal bacterial lysis in a process called 'lysis from within' (LI) (Abedon, 2011). The limitation for LI is that most bacteria cease metabolic processes at storage temperatures below 4°C, which can cause the failure of bacteriophages to complete their life cycle and lyse bacterial cells (Ferguson et al., 2013). In contrast, 'lysis from without' (LO) can kill bacteria by an extracellular agent without the bacteriophages' internally infecting the bacterial cell (Abedon, 2011). When the number of bacteriophages attaching to the bacterial cell wall exceeds a threshold level (MOI > 50), the bacterial cell wall ruptures and cell contents are released (Abedon, 2011). In this way, bacteriophages can kill pathogens at lower temperatures through the production of phage endolysins (Abedon, 2011) as compared to the lytic pathway.

In summary, this research assesses the ability of SalmoFresh™ to reduce *Salmonella* on fresh lettuce, sprouts, and mung bean seed samples and determine if a STEC bacteriophage cocktail applied with cofactors prevents biofilm formation on intact and cut edges of Romaine lettuce.

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2. CHAPTER 2. LITERATURE REVIEW

2.1. *Salmonella*

2.1.1. *Salmonella* general information

Salmonella spp. are Gram-negative facultative anaerobic motile bacilli belonging to the family of *Enterobacteriaceae* (Doyle & Buchanan, 2007). *Salmonella* serotypes vary in their ability to cause infection and only a small portion of *Salmonella* (>2500 serotypes) cause human infections. Most of the diseases for warm-blooded animals are caused by one species (*S. enterica* subsp. *enterica*) and the most commonly reported disease-related serotypes are Enteritidis (~38%) and Typhimurium (~22%) (Agasan et al., 2002). The genus *Salmonella* consists of two species, *Salmonella enterica* and *Salmonella bongori* (Popoff et al., 2000). *S. enterica* can be subdivided into 6 subspecies: *S. enterica* subsp. *enterica* (I), *S.* enterica subsp. *salamae* (II), *S. enterica* subsp.*arizonae* (IIIa), *S. enterica* subsp. *diarizonae* (IIIb), *S. enterica* subsp. *houtenae* (IV), and *S. enterica* subsp. *indica* (VI) (Popoff et al., 2000). Both *Salmonella* Typhimurium and *Salmonella* Typhi belong to *S. enterica* subsp. *enterica.*

In the past 10 years, fresh produce has become a prominent vehicle of human salmonellosis. Many factors can result in this situation, including the growing demand for ready-to-eat cut and prepackaged fresh produce. During processing, produce is frequently chopped and the cut edges can release cellular nutrients that encourage the growth of associated bacteria. Outbreaks of human salmonellosis related to fresh produce such as lettuce [caused by *S. enterica* subsp. *enterica* serotype Typhimurium (Brandl & Amundson, 2008; Crook et al., 2003), *S. enterica* subsp. *enterica* serotype Braenderup (Gajraj et al., 2012), *S. enterica* subsp. *enterica* serotype Newport (Gillespie,

2004)], sprouts (Gordenker, 1999; Honish & Nguyen, 2001; Pönkä et al., 1995; Winthrop et al., 2003), tomatoes (Greene et al., 2008; Gupta et al., 2007), and mangoes (Sivapalasingam et al., 2003) have been repeatedly reported in the last decade. These foodborne outbreaks emphasize the importance of implementing pathogen control measures in the produce industry along with government regulations. Cross-contamination of fresh fruits and vegetables by *Salmonella* may occur not only through cellular damage during processing, but also through the root system and from surface soil or harvest equipment (Doyle & Buchanan, 2007). In recent years, foodborne illnesses related to sprouted seeds have been frequently reported (Rimhanen-Finne et al., 2011). Contamination can result from the presence of pathogens either within or on the surface of seeds, as the warm temperature and high moisture conditions during germination encourage bacterial growth. It has been reported that *Salmonella* readily attaches to the seed coat, as suggested by successful isolation of *Salmonella* from germinated sprouts (Doyle & Buchanan, 2007).

2.1.2. *Salmonella* epidemiology

There are various pathways whereby humans maybe exposed to *Salmonella*, including foodstuffs of both animal and plant origin (Hald, 2013). Since *Salmonella* is prevalent in the feces of both wild and domestic animals, fecal-oral transmission occurs as a result of the fecal contamination of food at both pre-harvest and post-harvest (Hald, 2013). For non-typhoid *Salmonella*, possible vehicles resulting in infection include unpasteurized milk, raw/uncooked rice, meat products and increasingly, the contamination of fresh fruits and vegetables (Cliver et al., 2011). In North America, the prevention of enteric fever focuses on improving sanitation to ensure food and water safety, and a typhoid vaccine was applied to the susceptive public to reduce the risk of infection (Crump & Mintz, 2010). The high fatality rate of typhoid fever (10%-20%) that

occurs with delayed treatment and inadequate health care can be reduced to 1% with antibiotic therapy (Bhan et al., 2005; Cliver et al., 2011). Since antimicrobial resistance in *S.* Typhi and *S.* Paratyphi has been reported (Crump & Mintz, 2010), outbreaks caused by antimicrobial resistant strains in the 1980s became a major concern (Bhan et al., 2005). Though direct person-to-person transmission rarely happens, fecal-oral transmission (Dritz & Braff, 1977) and human excreta contaminated food or water has frequently been linked to typhoid fever (Cliver et al., 2011). Typhoid and paratyphoid fever was popular among infants, children and adolescents in Southeastern and Northeastern Asia, while enteric fever caused by *S.* Enteritidis was endemic in Western Europe and North America (Crump & Mintz, 2010).

2.1.3. Outbreaks related to produce commodities

In the US, 9.4 million cases of foodborne illnesses caused by 31 common pathogens resulted in 55,961 hospitalizations and 1,351 deaths, among which non-typhoidal *Salmonella* generated 11% of the hospitalized outbreaks and 28% of the deaths (Scallan et al., 2011). Foodborne illnesses related to fresh vegetables have been increasing over the last 10 years. The Food and Drug Administration (FDA) reported that $10-$83 billion is spent in the field on foodborne illnesses each year for medications to relieve pain and suffering (FDA, 2013a). Due to the reasons mentioned above, efforts to develop effective antimicrobial strategies to control foodborne pathogens have increased (Viazis et al., 2011). From 2007 to 2008, most produce-generated foodborne illness outbreaks were related to *Salmonella* in North America. *S. enterica* in fresh produce has caused foodborne illnesses in thousands of people and resulted in mortalities (Lynch et al., 2009). The annual overall salmonellosis mortality rate is about 1% in the US

(Cummings et al., 2010). According to Brandl and Amundson (2008), lettuce has been linked to outbreaks caused by *Salmonella,* not only in the US, but also in Australia, Finland, and England.

According to the Center for Disease Control and Prevention (CDC), during the past decade, 16 out of 48 *Salmonella* outbreaks in the US were related to ready-to-eat fruits and vegetables (CDC, 2016a). As the consumption of leafy greens rose by 9% from 1995 to 2006, related foodborne illness outbreaks have increased by 38.6% (CDC, 2016a). Until 2009, leafy greens were ranked as the No.1 outbreak-related food category, linked to 363 outbreaks and 13,568 reported foodborne illnesses (Davidson et al., 2013b). Outbreaks arose as a result of cross-contamination during harvesting, processing, transportation, packaging and storage; likely as a result of improper antimicrobial treatment during pre-harvest and post-harvest processes. Therefore, it is important to select the right type of bacterial-control method, and employ the correct contact time and concentration of the disinfectant solution to achieve disinfection. These factors are indispensable for reducing outbreaks caused by pathogens like *Salmonella* (Parish et al., 2003).

Salmonella outbreaks related to fresh produce in recent years in the US and Canada are listed in Table 2-1. and Table 2-2, respectively.

Table 2-1. Foodborne outbreaks related to fresh produce caused by *Salmonella* from 2010 to 2016 in the US

Year	Pathogens	Produce	Cases (deaths)	References
2010	*Salmonella* Newport	Alfalfa sprouts	44	(CDC, 2010b)
2011	*Salmonella*	Alfalfa and mixed sprouts	140	(CDC, 2011b)
2011	*Salmonella* Newport	Alfalfa sprouts and spicy sprouts	25	(CDC, 2011c)
2011	*Salmonella* Panama	Cantaloupe	20	(CDC, 2011d)
2011	*Salmonella* Agona	Papaya	106	(CDC, 2011e)
2012	*Salmonella* Typhimurium *Salmonella* Newport	Cantaloupe	261 (3)	(CDC, 2012c)
	Salmonella Braenderup	Mango	103	
2013	*Salmonella* Saintpaul	Imported cucumbers	84	(CDC, 2013b)
2014	*Salmonella* Enteritidis	Bean sprouts	115	(CDC, 2014b)
2016	*Salmonella* Poona	Imported cucumbers	907 (6)	(CDC, 2016b)
2016	*Salmonella* Reading *Salmonella* Abony	Alfalfa sprouts	36	(CDC, 2016c)
2016	*Salmonella*	Alfalfa sprouts from one contaminated seed lot	26	(CDC, 2016d)
2017	*Salmonella* Kiambu *Salmonella* Thompson	Imported Maradol papayas	109 (1)	(CDC, 2017)

Table 2-2. Foodborne outbreaks related to fresh produce caused by *Salmonella* in Canada.

Time frame	Organism	Vehicle	Province	Venue	No. of outbreak cases (deaths)	References
2001 Feb. to Mar.	*Salmonella* Enteritidis	Mung bean sprouts (suspect)	AB, BC, SK	Various	84	(Kozak et al., 2013)
2002 Oct.	*Salmonella* Newport	Fruit trays	ON	Private Parties	34	
2002 Mar. to May	*Salmonella* Poona	Cantaloupe	ON	Various	2	
2004	*Salmonella* Brandenburg	Cucumber	BC	Community	12	
2004 July	*Salmonella* Javiana	Roma tomatoes (suspect)	ON	Restaurant	7	
2005	*Salmonella* Enteritidis	Mung bean sprouts	AB	Restaurant	8	
2005	*Salmonella* Enteritidis	Mung bean sprouts	ON	N/A	592	(Rohekar et al., 2008)
2006 June-Dec.	*Salmonella* Oranienburg	Fruit salad	ON	Health care facility	2	(Kozak et al., 2013)
2008 Jan.-April	*Salmonella* Litchfield	Cantaloupe	BC, AB, MB, ON, NB	Grocery store	9	
2008 May	*Salmonella* Saintpaul	Raw peppers	Traveled to the US	N/A	5	(CDC, 2008)
2009 July	*Salmonella* Cubana	Onion sprouts	AB, BC, NS, ON	N/A	20	(Kozak et al., 2013)
2012	*Salmonella* Braenderup	Mango	AB, BC, NS	N/A	21	(CDC, 2012c)

2.1.4. *Salmonella* foodborne illnesses symptoms

Salmonella infections can cause severe clinical disease in humans including typhoid fever, uncomplicated enterocolitis/diarrhea, bacteremia and chronic asymptomatic carriage (Coburn et al., 2007). Non-typhoid *Salmonella* has been reported to be one of the most frequent causes of gastroenteritis and bacteremia worldwide (Scallan et al., 2011). Onset to symptoms takes 12 to 72 h, with the illness lasting 4 to 5 d followed by a fatigue period (Hald, 2013). Human foodborne diseases such as typhoid fever and intestinal/diarrheal disease are the most common syndromes of *S. enterica* infection (Coburn et al., 2007). Although salmonellosis typically has a low mortality rate (<1%), some invasive infections can cause fatality rates as high as 20-30% (Hald, 2013). *Salmonella* infective dose varies among different age groups; the eldly and young children are especially more susceptible than the rest groups of people (Kothary & Babu, 2001). Kothary & Babu (2001) indicated that the general infective dose for *Salmonella* was 10^5 to 10^{10} in the previous volunteer study; while Fontaine et. al. (1980) reported less than 100 organisms infective dose for different *Salmonella* serotypes prevalent in high fat and protein food vehicle, which may protect them from gastric acidity.

2.1.5. *Salmonella* virulence factors.

Host immunopathology, morbidity, and mortality involve *Salmonella* virulence factors interacting with the host defense system in various tissues as the infection develops (Coburn et al., 2007). Bacteria must be exposed to the correct selective pressures within a suitable environment for virulence genes to be retained within the host genome (Bowe et al., 1998). Gene products coding for virulence factors can be hosted on plasmids or the chromosome in either a singular form

or in the form of gene clusters known as pathogenicity islands (Marcus et al., 2000). Virulence genes for a number of different factors including toxins, adhesion or invasion maybe contained within these pathogenicity islands (Hacker et al., 1997). Pathogenicity islands are found in both Gram-positive and Gram-negative bacteria and are present in the genome of pathogenic strains and often absent from non-pathogenic strains of bacteria (Hacker et al., 1997). For many enteric bacteria, the presence of one pathogenic island usually results in the bacterium exhibiting a pathogenic phenotype (Marcus et al., 2000). *Salmonella* pathogenicity islands (SPIs) are large gene cassettes in the chromosome that encode for the virulence determinants required for bacterial virulence in the host (Marcus et al., 2000). Since SPIs have a lower G+C content (37% and 47%) as compared to the rest of the bacterial chromosome (about 58%), SPIs are easier to transfer horizontally within bacteriophage or plasmids and are likely to be conserved across *Salmonella* serotypes (Marcus et al., 2000).

2.2. Shiga-toxigenic Escherichia coli (STEC)

2.2.1. STEC General Information

E. coli is a Gram-negative facultative, rod-shaped bacterium belonging to the family *Enterobacteriaceae*. It is commonly found in the gastrointestinal tract of humans and warm-blooded animals. Commensal *E. coli* strains seldom cause illnesses, however, many of the pathogenic strains with virulence factors can cause intestinal gastroenteritis, urinary tract infections and neonatal meningitis in immunocompromised individuals who are vulnerable to infections (Meng & Schroeder, 2007). One of the pathogenic groups of *E. coli*, known as shiga-toxin producing *E. coli* (STEC) possess specific virulence attributes that enable them to cause illnesses including urinary tract infections, diarrhea, sepsis, and meningitis (Meng & Schroeder,

2007; Pennington, 2010). STEC, also known as 'verocytotoxin-producing *E. coli*' are capable of producing Shiga toxin type 1 (*Stx1*) and type 2 (*Stx2*) either singularly or in combination (Bryan et al., 2015; Padola & Etcheverría, 2014). There are more than 100 serogroups of *E. coli* that can produce Shiga toxins, among which *E. coli* O157:H7 and the 'big six' group (O26, O45, O103, O111, O121, O145) are most well-known (Elder et al., 2016; Law, 2000). STEC are important foodborne pathogens and are the causative agents of both outbreaks and sporadic cases of human illness ranging in severity from mild intestinal discomfort to hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) (Majowicz et al., 2014; Padola & Etcheverría, 2014). HUS is the most common cause of acute renal failure in children younger than three years old (Bryan et al., 2015). Shiga toxins produced by STEC result in bloody diarrhea, and in severe cases can cause HUS (Amirlak & Amirlak, 2006).

2.2.2. STEC epidemiology

In 1982, two outbreaks of hemorrhagic colitis in the US resulted in O157:H7 STEC being recognized as a major human pathogen (Karch et al., 1999). In 1983, as the sporadic cases of uncomplicated watery diarrhea and HUS continued to be identified, STEC was shown to be linked to these symptoms on all 6 populated continents (Bryan et al., 2015; Karch et al., 1999). STEC have been reported to cause at least 265,000 human illnesses each year, and were responsible for 3600 hospitalizations and 61 deaths in the US. The non-O157 STEC are estimated to cause 112,752 infections each year (Bryan et al., 2015; Scallan et al., 2011). From 2006 to 2013, the incidence of laboratory confirmed *E. coli* O157:H7 infection in the US was 1.15 cases per 100,000, which is almost the same as non-O157 STEC (1.17 cases per 100,000) (Crim et al., 2014). The Economic Research Service (ERS) of the US Department of Agriculture estimated that STEC O157 outbreaks

cost $405 million yearly, including premature deaths ($370 million), medical care ($30 million), and lost productivity ($5 million) (Frenzen et al., 2005).

E. coli O157:H7 is responsible for most of the severe foodborne diseases that are linked to STEC (Bosilevac & Koohmaraie, 2011; Luna-Gierke et al., 2014). Human infections by non-O157 STEC, with the most common serogroups of O26 (190; 21%), O103 (178; 20%) and O111 (106; 12%), are steadily increasing in the year of 2017 comparing to the year from 2013 to 2016 (Marder et al., 2017). The consumption of undercooked ground beef was the most frequent source of STEC outbreaks in Canada (Bosilevac & Koohmaraie, 2011). Unpasteurized dairy products (Guh et al., 2010), unpasteurized juice (Cody et al., 1999), and fresh produce (Breuer et al., 2001; Hilborn et al., 1999; Sharapov et al., 2016) are also reported as primary sources of STEC in foodborne outbreaks.

Although the primary reservoir of STEC is healthy cattle and other ruminants such as sheep, goats, and deer, they can also infect humans through direct contact or through the consumption of contaminated food or water (EFSA, 2011). Three transmission routes have been identified: 1) contaminated food, drinking or swimming water, 2) person to person transmission, and 3) direct contact with animals (Karch et al., 1999). Food contamination can occur as a result of contamination of meat or milk during harvest and processing, and contamination of water used for irrigation or cleaning and processing of cultivated vegetables and fruits (Neil et al., 2011). Besides the consumption of contaminated food, swimming in polluted lakes or rivers can also be a point source of infection with STEC (Paunio et al., 1999; Samadpour et al., 2002). It has been reported that 6% of *E. coli* O157 infections and 8% of non-O157 STEC infections result from animal contact (Hale et al., 2012). STEC infections from direct contact with animals in open zoos (Goode et al., 2009; Stirling et al., 2008), petting/dairy farms (Crump et al., 2002; Møller-Stray et al., 2012;

Stirling et al., 2008) and household pets (Lambertini et al., 2016) has been reported. Person-to-person transmission of STEC has been recognized as the cause of outbreaks in child-care settings, a particularly tragic outcome given the susceptibility of young children to developing HUS (Wikswo & Hall, 2012). The easy person-to-person transmission of STEC is a reflection of its low infective dose (~10-100 CFU) (Rahal et al., 2012)..

2.2.3. STEC outbreaks related to produce commodities

An updated report by Public Health Agency of Canada (PHAC) estimated that 4 million Canadians (1 in 8) are affected by foodborne illnesses each year, while the CDC estimated this number at 48 million, resulting in 128,000 hospitalization and 3000 deaths in the US (PHAC, 2016; Scallan et al., 2011). Produce has been reported to be a prominent food vehicle of STEC outbreaks in both Canada and the United States (Tables 2-3. and 2-4.). From 1982 to 2002 in the US, produce was linked to 21% (38 out of 183) of foodborne outbreaks, in which 34% of the total of 5269 cases were caused by *E. coli* O157:H7 (Matthews, 2006). Restaurants and other food service organizations/activities are the primary sources of *E. coli* O157:H7 produce-related foodborne illnesses (40%), with cross-contamination during food preparation being responsible for 47% of outbreaks (Matthews, 2006). The processing steps used to prepare ready-to-eat fresh produce such as cutting, slicing, peeling and shredding can expose interior cellular surfaces, which can increase microbial levels 6 to 7 fold (Kozak et al., 2013). PHAC (2016) reported that among the 11,600 hospitalizations caused by foodborne illnesses in Canada, *E. coli* O157 was responsible for 245 (6%) cases and 8 deaths each year. Since the continuously increasing trend of consuming fresh fruits and vegetables is expected to continue to at least 2020 (Lin, 2004), outbreaks linked to

produce will almost certainly continue to occur. Canada's greenhouse vegetable production was reported to have a value of $1.2 billion dollars in 2006 (Kozak et al., 2013).

Table 2-3. Outbreaks linked to fresh produce caused by STEC from 2010 to 2016 in the United States and Europe.

Location	Year	Pathogens	Produce	Cases (deaths)	References
USA	2010	*E. coli* O145	Shredded Romaine Lettuce	26	(CDC, 2010a)
USA	2011	*E. coli* O157:H7	Romaine Lettuce	58	(CDC, 2011a)
Europe	2011	*E. coli* O104:H4	Sprouts	3911 (47)	(EFSA, 2011)
USA	2011	*E. coli* O157:H7	Strawberries	15 (1)	(FDA, 2011)
USA	2012	*E. coli* O157:H7	Spinach	33	(CDC, 2012a)
USA	2012	*E. coli* O26	Raw Clover Sprouts	29	(CDC, 2012b)
USA	2014	*E. coli* O121	Raw Clover Sprouts	19	(CDC, 2014a)
USA	2016	*E. coli* O157:H7	Alfalfa Sprouts	11	(CDC, 2016a)

Table 2-4. Outbreaks linked to fresh produce caused by *E. coli* O157:H7 in Canada.

Time frame	Vehicle	Province	Venue	No. of outbreak cases (deaths)	References
2002 Oct. to Nov.	Salad/sandwiches	PEI	Long-term care facility	17 (2)	(Kozak et al., 2013)
2006 Aug. to Sept.	Spinach	ON	N/A	1	
2006 Sept.	Lettuce (suspect)	ON	N/A	7	
2008 Oct. to Nov.	Romaine Lettuce	ON	Restaurant	29	
2008 Oct. to Nov.	Spanish Onions (suspect)	ON	Restaurant	235	
2008 Oct.	Iceberg Lettuce	ON	Restaurant	3	
2008	Lettuce	N/A	N/A	134	(Warriner & Namvar, 2010)
2010	Vegetable soup	MB	N/A	26	(Holley, 2017)
2013	Lettuce	NS, NB, ON	N/A	20	

2.2.4. STEC main virulence factors

The primary virulence factors in STEC are Stx1, Stx2, and their subtypes or combinations, which are more often associated with more severe disease such as HUS (Bolton, 2011; Shen et al., 2015). Stx1 has the subtypes Stx1a, Stx1c, and Stx1d, while Stx2 consists of subtypes Stx2a, Stx2b, Stx2c, Stx2dact, Stx2e, Stx2f and Stx2g (Shen et al., 2015). Tesh et al. (1993) suggested that the difference in the structure/function between Stx1 and Stx2 resulted in variation in holotoxin stability and receptor affinity which were responsible for the difference in virulence between the two toxins. As a result Stx2, especially Stx2a, Stx2c, and Stx2dact, have been reported to be more frequently associated with hemorrhagic colitis (HC) and HUS than Stx1 (Bielaszewska et al., 2006).

The locus of enterocyte effacement (LEE), contains the genes *eae, tir, espA, espB, espC, and espD*, and is a 35–45 kb pathogenicity island found on the chromosome (Bolton, 2011; Farrokh et al., 2013). LEE is essential for intimate attachment to intestinal epithelial cells, the initiation of host signal transduction pathway and the formation of attaching and effacing (A/E) lesions (Bolton, 2011; Shen et al., 2015). Since most of the common STEC serotypes associated with human diseases (O157:H7, O26:H11, O111:NM and O145:NM) are LEE positive, LEE is considered to be associated with enhanced virulence (Bolton, 2011). The outer membrane protein intimin, encoded by the *eae* gene on LEE, is another well characterized STEC virulence factor (Bolton, 2011; Shen et al., 2015). The LEE-encoded type III secretion system (TTSS), which includes TTSS structural and translocator-proteins (*espA, espB, espD*) allows the bacterium to inject proteins into the host cell (Bolton, 2011; Farrokh et al., 2013).

Enterohaemolysin, encoded by *e-hlyA* on a 92kb plasmid (pO157), is an important virulence factor for O157:H7 and other STEC serotypes (Bolton, 2011; Shen et al., 2015). It is capable of causing erythrocyte haemolysis, providing iron to support the infection process while at the same time it can act as a pore-forming cytolysin that damages host cells (Bolton, 2011; Shen et al., 2015). Some other putative virulence factors, such as a cytolethal distending toxin, Subtilase cytotoxin, adhesions (*EfaI, Iha, Saa, and ToxB*), lipopolysaccharides (LPS), iron acquisition system, flagella, and protease are also contributors to the infection process (Bolton, 2011; Shen et al., 2015).

2.3. Produce processing practices

2.3.1. Background information

The consumption of fresh fruits and vegetables are widely acknowledged as crucial for a healthy diet because they can provide nutrients, vitamins, and fiber (Olaimat & Holley, 2012). Epidemiological data indicated that plant-based foods such as fresh produce can lower the risk of coronary artery disease and stroke (Hu, 2003). The World Health Organization promotes the consumption of fresh fruits and vegetables and recommends a minimum daily intake of 400 g of fresh produce to prevent chronic diseases (World Health Organization, 2012). The consumption of fresh fruits and vegetables in Canada increased by 56% and 26% respectively, from 1963 to 2010 (Statistics Canada, 2001). Between 2001 to 2009, there were 27 produce-related outbreaks resulting in approximately 1549 illnesses in Canada, among which 50% were caused by *Salmonella* followed by *E. coli* O157:H7 (33%) (Kozak et al., 2013).

2.3.2. Lettuce, sprouts and seeds processing

In North America, *E. coli* O157:H7 and *Salmonella* outbreaks in raw produce resulted in 700 illnesses and 4 deaths in 2006 (Forsythe, 2011). The most predominant types of fresh produce associated with foodborne outbreaks are tomatoes, sprouted seeds and leafy greens (Table 2-5.) (Doyle & Erickson, 2008; Warriner et al., 2009). It has been widely acknowledged that sprouted seeds are a primary concern as a source of pathogens as the warm temperatures (~30°C) and high humidity during germination promote bacterial growth (Ye et al., 2010). The rapid growth of pathogens under these conditions can pose a serious threat even if the initial level of contamination on seeds is low (Fu et al., 2008; Rimhanen-Finne et al., 2011). Disinfection after sprouting is largely ineffective as pathogens in the sprouts are often harbored in inner tissues that are inaccessible to the disinfectant (Ferguson et al., 2005). Moreover, the fact that mung bean sprouts and alfalfa sprouts are often unwashed prior to consumption makes consumers more vulnerable to acquiring pathogens from these sources. Therefore, it is important for both the industry and the growers to take measures to counteract these food safety risks.

2.3.3. Current pre-harvest and post-harvest risk and interventions

Microbial contamination can happen at any of the pre-harvest and post-harvest steps in the farm-to-consumer chain including growing, irrigation, harvest, processing, storage, transportation, wholesale and household consumption (Olaimat & Holley, 2012).

Pre-harvest risk and interventions. During the pre-harvest phase, Good Agriculture Practices can effectively reduce pathogen prevalence in produce (CanadaGAP, 2015), but if the pathogen survives on the plant surface, cross contamination can occur. Pre-harvest STEC and

Salmonella contamination sources (soil, water, manure) and methods of transmission (irrigation and contact with feces) to leafy green vegetables have been identified (Liu et al., 2013). It has been reported that *E. coli* O157:H7 can become internalized in spinach leaves and an outbreak of *Salmonella* Newport was linked to mangoes contaminated by drip irrigation water (Berger et al., 2010). Therefore, a reduction of human and animal activity in crop-growing areas, decontamination of irrigation water and effective pest-control are necessary for pre-harvest interventions to decrease the prevalence of pathogens on produce. High intensity rainfall events can increase the chances of manure and soil particles being transferred to plant surfaces, resulting in an increase in the contamination of produce by both *Salmonella* and STEC (Cevallos-Cevallos et al., 2012; Liu et al., 2013).

Post-harvest risk and interventions.

During post-harvest, poor personal hygiene of workers and improper food-handling procedures can increase the risk of pathogen contamination of fresh produce (Matthews, 2006). The different steps in post-harvest processing including reception, shredding, washing, rinsing, and storage are processes in which under favorable conditions, pathogens can rapidly multiply (Allende et al., 2004).

Physical intervention. Conventional physical treatment such as heat treatment is used as an alternative to chemical decontamination for harvested fresh fruits and vegetables. Heat treatments include hot water dips, saturated water vapor heat, hot dry air and hot water rinses (Mahajan et al., 2014). Besides killing insects and pathogens, heat treatment has additional benefits such as reducing chilling injury and delaying ripening as a result of the inactivation of enzymes (Lurie, 1998). Heat treatment has been applied to firm potatoes, strawberries, tomatoes, carrots, lettuce, beans, and bean sprouts and preserves color, prevents over ripening and increases shelf-

life (Mahajan et al., 2014). However, high energy and labor costs limit the application of heat treatment to large scale production (Mahajan et al., 2014). Detrimental effects such as: i) reducing the initial content of ascorbic acid; ii) promoting microbial growth rates during storage due to the liberation of nutrients as a result of plant cell destruction also need to be considered (Moreira et al., 2006).

Chemical intervention. Chlorine-based solutions, organic acids and hydrogen peroxide (H_2O_2) are the most common antimicrobial chemical agents used due to their low cost and oxidative properties (Mahajan et al., 2014). Washing with tap water, chlorine water or antimicrobial agents has been reported to reduce *E. coli* O157:H7 and *Salmonella* by 1.8-2.8 and 2.6-3.8 log CFU/g, respectively (Leverentz et al., 2001; Park & Beuchat, 1999). Although chlorinated water is effective at reducing pathogen levels to some extent, its efficiency as an antimicrobial agent depends on the level of chlorine and organic matter in solution. Although treatment with 1% chlorine can reduce pathogens on produce to an undetectable level in a short time, high levels of chlorine (>1%) may result in undesirable odors and taste of the produce (Baskaran et al., 2013; Mahajan et al., 2014). Moreover, standard chlorine concentrations (50-200 ppm), do not sufficiently reduce pathogens attached to the surface of freshly-cut produce (Mahajan et al., 2014). Limitations such as internalized pathogen contamination and biofilm formation on fresh produce can reduce the effectiveness of post-harvest decontamination methods (Olaimat & Holley, 2012; Oliveira et al., 2014). Although the antimicrobial effects of 4-5% H_2O_2 are comparable to 100-200 ppm of chlorinated water, the application of H_2O_2 is undesirable in the food industry as it can cause severe browning of shredded lettuce (Garg et. al., 2016). Citric, acetic, and lactic acids are the common organic acids applied due to their ability to reduce environmental pH, disrupt bacterial membranes and result in the accumulation of anions (Parish et al., 2003).

However, the strong unacceptable flavor and impact of organic acids on water quality has limited their widespread use in the produce industry (Garg et al., 2016).

Since a high proportion of fresh produce is destined for the organic market (Vukasovič, 2016), where antimicrobial agents and chemical washes are often prohibited (Matthews, 2006), biocontrol methods such as bacteriophages are being increasingly considered to improve the preservation and safety of produce (Leverentz et al., 2003; Magnone et al., 2013b).

2.3.4. Limitations for pathogen decontamination of produce

Although appropriate decontamination methods can reduce the risk of foodborne illnesses, some laboratory experiments with plant tissues or intact whole produce suggests that pathogens irreversibly attach to the plant surface and cannot be readily removed by traditional washing or agitation processes (Solomon & Sharma, 2009). Both endophytic and epiphytic colonization of fresh produce by STEC and *Salmonella* has been reported to be the cause of foodborne illnesses (Ölmez, 2016; Steenackers et al., 2012). The extent of endophytic colonization depends on the types of pathogen as well as the pH and sugar content of plant tissue and the plant growth stage (Zheng et al., 2013). Epiphytic attachment depends on several factors including curli, fimbriae, flagella, and the formation of biofilms (Kroupitski et al., 2013; Lee et al., 2015; Ölmez, 2016). Damaged plant tissues are found to be more vulnerable to colonization as compared to intact whole fruits or vegetables (Solomon & Sharma, 2009). Studies showed little correlation between bacterial surface appendages, charge, or hydrophobicity and the ability to attach to the surface of lettuce (Boyer et al., 2007). Other studies using knock-out bacterial pathogens that lacked the ability to produce extracellular carbohydrates, colonic acid, *OmpA* and poly n-acetylglucosamine were less likely to firmly attach to alfalfa sprouts (Solomon & Sharma, 2009). Shaw et al. (2008) indicated

that *E. coli* O157:H7 (10^6 CFU/ml) could attach to lettuce and spinach with the filamentous type III secretion system at 37°C.

Table 2-5. Outbreaks of foodborne disease associated with fresh produce.

Date	Pathogen	Produce	Comments	Reference
December 2005	*Salmonella*	Mung bean sprouts	Canada, 618 confirmed cases	
February 2006	*Salmonella*	Alfalfa sprouts	Canada, recall for suspected contamination	
February 2006	*Salmonella*	Alfalfa sprouts	Australia, 100 confirmed cases	
June 2006	*E. coli* O121:H19	Lettuce	United States, 4 confirmed cases	
July 2006	*Salmonella*	Fruit salad	United States and Canada, 41 confirmed cases	
August 2006	*Salmonella*	Alfalfa sprouts	United States, recall for suspected contamination	
September 2006	*E. coli* O157:H7	Spinach	United States, 205 confirmed cases, 3 deaths	
September 2006	*Clostridium botulinum*	Pasteurized carrot juice	United States and Canada, 6 cases	
October 2006	*E. coli* O157:H7	Lettuce	Canada, 30 confirmed cases	(Warriner et al., 2009)
October 2006	*E. coli* O157:H7	Lettuce	Canada; recall due to suspected contamination	
October 2006	*Salmonella*	Tomatoes	United States; 183 cases	
November 2006	*E. coli* O157:H7	Lettuce	United States; 81 confirmed cases	
November 2006	*E. coli* O157:H7	Lettuce	United States; 71 confirmed cases	
November 2006	*Salmonella*	Peanut butter	United States; 481 confirmed cases	
April 2007	*Salmonella*	Lettuce	UK, recall for suspected contamination	
August 2007	*Shigella sonnei*	Baby carrots	Canada, 4 cases	
April 2008	*Salmonella*	Cantaloupe	Canada, United States and Mexico, 64 confirmed cases	

June 2008	*Salmonella*	Tomatoes/peppers	United States and Canada, 1442 confirmed cases	
September 2008	*E. coli* O157:H7	Lettuce	United States and Canada, 134 confirmed cases	
September 2008	*Salmonella*	Alfalfa sprouts	United States, 14 confirmed cases	
November 2008	*Salmonella*	Basil	UK, 32 confirmed cases	
December 2008	*Salmonella*	Alfalfa sprouts	United States, recall for suspected contamination	
2010	*Salmonella*	Domestic green onions	20 cases of foodborne illnesses	(Denis et al., 2016)
December 2011	*Salmonella* Newport	Ready-to-eat watermelon slice	Europe; 63 confirmed cases	(Byrne et al., 2014)
December 2012	*E. coli* O157:H7	Lettuce	Canada, imported from the US, affected 31 people	(PHAC, 2014)

2.4. Biofilms

2.4.1. General information

Microorganisms in food systems are likely to associate with solid surfaces that are rich in nutrients promote reproduction and growth (Kumar & Anand, 1998). During this process they form specific proteinaceous adhesins that enable them to be retained on surfaces and resist physical forces such as hydrodynamic shear (Klemm & Schembri, 2000). These adhesins support the formation of biofilms which are structured communities of bacterial cells enclosed in a self-produced polymeric matrix that adheres to inert or living surfaces (Steenackers et al., 2012). Biofilms are formed on food product contact surfaces, food processing equipment and within potable water distribution systems (Gómez-López, 2012). Attached biofilms on surfaces are not easily removed and cannot be dislodged by rinsing as the cells are embedded within an extracellular polymeric matrix (Blaschek et al., 2008). In food industries, the most effective way to remove biofilms is through mechanical action such as high pressure spray and mechanical scrubbing (Gibson et al., 1999). Biofilms can serve as long-term sources of contamination within the food production environment. Thus, it is important to prevent the attachment of bacteria and therefore avoid biofilm formation rather than to try and remove them after they have formed.

Various STEC serotypes have been shown to be able to form biofilms on meat, poultry and fresh produce (Silagyi et al., 2009). The resistance of biofilms to antimicrobial biocides, environmental and oxidative stress, disinfectants and sanitizers used in the food industry make them a serious food safety problem (Almasoud et al., 2015; Silagyi et al., 2009). Therefore, the formation of pathogen biofilms on fresh produce surfaces can result in post-processing and cross-

contamination, having a direct impact on produce safety and public health (Gómez-López, 2012; Kumar & Anand, 1998).

2.4.2. Biofilm formation

The formation of biofilms occurs in several distinct steps: 1) the initial process involves reversible attachment (by van der Walls attraction forces, electrostatic forces and hydrophobic interactions) followed by proliferation of bacterial cells and their accumulation in multi-layer cell clusters; 2) the production of surface polysaccharides and the formation of extracellular polymeric matrix results in irreversible attachment and acts as a bridge between the bacterial cell and substratum; 3) the establishment of a biofilm architecture and 4) the dispersion of single cells to enable the biofilm to spread over the surface and colonize downstream surfaces (Gómez-López, 2012).

Biofilms can be described as sessile microbial communities that grow on surfaces and are embedded in an exopolysaccharide (EPS) matrix with a heterogeneous gel-like architecture that forms a highly hydrated three-dimensional structure (the dense areas, the pores, and the channels) (Allison, 2000; Garrett et al., 2008; Gómez-López, 2012). The three-dimensional biofilm structure has been described as a water channel model (Gómez-López, 2012). In this model, substructures with void sectors protrude from substratum to the top of the biofilm, representing the channels through which waste and substrate products can move (Gómez-López, 2012). The mushroom shaped microcolonies in the discrete matrix community are composed of 10-25% cells and 75%-90% EPS (Costerton et al., 1987). EPS composition varies with environmental conditions in biofilms and has been reported to account for approximately 50%-90% of total organic carbon (Allison, 2000). For Gram-negative bacteria, exopolysaccharides such as glucose, alginate,

cellulose, colonic acid (*E. coli*) and β-1,6 linked N-acetylglucosamine (*Salmonella* and *E. coli*) play a significant role in biofilm formation (Gómez-López, 2012).

Biofilm adhesion is influenced by bacterial cell surface structures as well as environmental conditions. Cell surface structures including outer membrane proteins, capsular polysaccharides, LPS, curli, fibrillae and flagella can influence the adhesion of biofilms by changing the physicochemical properties (hydrophobicity and cell-charge) of bacteria cells (Gómez-López, 2012). Ryu et al. (2004) reported that curli-producing *E. coli* O157:H7 did not form biofilms on the surface of stainless steel coupons in the presence of lettuce juice, but did form biofilms in minimal salt broth. This suggests that the availability of nutrient can influence biofilm formation. During food processing, the high shear forces generated as a result of the food flowing through the processing system will decrease biofilm attachment, especially when the biofilms are at the reversible attachment phase (Frank, 2001). Therefore, rough surfaces can help promote biofilm attachment as they prevent shear forces from readily disrupting the biofilm (Lin et al., 2013).

2.4.3. Biofilm inactivation/removal on produce surfaces

Biofilms have been reported to form on numerous contact surfaces including stainless steel (Shen et al., 2012; Woolston et al., 2013), glass (Woolston et al., 2013), polyvinyl chloride (Hammond et al., 2010) as well as food product surfaces (meat, poultry and produce) (Gómez-López, 2012; Jahid et al., 2015). Cross-contamination happens when pathogens are transferred from surface-attached biofilms to ready-to-eat food products, placing consumers at risk. The high levels of carbohydrate and optimal water activity associated with produce commodities encourages pathogenic and spoilage bacteria to grow and form biofilms as observed by Field Emission Scanning Electron Microscopy (FESEM) (Jahid et al., 2015).

Early research using scanning electron microscopy (SEM) showed that pathogens tended to attach to stomata, broken trichomes, or mainly at points where the waxy cuticle of plants was breached (Frank, 2001). Although some pathogens may colonize produce by dissolving the waxy layer, this approach to colonization is rarely observed (Frank, 2001). The attachment of biofilms depends on plant nutritional conditions, hydrophobicity, cellulose surface charge, damage to the waxy outer layer and the interaction of contaminating bacteria with natural epiphytic microflora (Jahid & Ha, 2012). In natural environments, biofilms have been reported to account for 80% of the total microbial population (Jahid & Ha, 2012). In the laboratory, biofilm formation has been reported to occur on lettuce (Jahid et al., 2015; Niemira & Cooke, 2010; Patel & Sharma, 2010), spinach (Niemira & Cooke, 2010), cabbage (Patel & Sharma, 2010) and sprouts (Fransisca et al., 2011). Seo and Frank (1999) found that entrapped *E. coli* O157:H7 were located 20 to 100 µm below the cuticle surface in stomata and cut edges. Khalil and Frank (2010) reported that damaged parts of leafy greens were a distinct growth niche, with various bacterial responses in different types of leafy greens. For example, *E. coli* O157:H7 did not grow at damaged sites in baby Romaine lettuce at 8 ℃, but did establish substantial populations on the surface of damaged spinach at the same temperature (Khalil & Frank, 2010). This differential response likely reflects the higher level of tissue oxidation associated with Romaine lettuce as compared to spinach.

2.4.4. Effect of disinfectants on biofilm removal/prevention on vegetables

The efficacy of disinfectants to inactivate biofilm-associated bacteria is influenced by several factors including the duration between contamination and sanitizer application, contact time, internalization of bacteria within produce, temperature, pH, bacterial load, and the maturity of formed biofilms (Jahid & Ha, 2012). Standard sanitizers aim to achieve a 5 log reduction within

a contact time of 30 sec on food contact surfaces, while disinfectants must irreversibly inactivate or destroy all specified microorganisms within 10 min (Pfuntner, 2011). Many chemicals, such as chlorine, can function as both a sanitizer and disinfectant (Gaulin et al., 2011). Recent studies on using disinfectants to reduce *E. coli* O157:H7 biofilms are presented in Table 2-6.

The effectiveness of disinfection depends on the type and load of microorganisms associated with the produce (Jahid & Ha, 2012). Since most fresh fruits and vegetables are high in soluble carbohydrates that promote bacterial growth, disinfectants may be less effective on these foods. Jahid and Ha (2012) reported that the US FDA recommended the application of 20,000 ppm of free chlorine in the form of $Ca(OCl)_2$ for 15 min to sanitize *E. coli* O157:H7 and *Salmonella* on seeds. However, few studies have explored the ability of disinfectants to prevent the formation of biofilm on fresh produce.

Table 2-6. A summary of research on the efficacy of disinfectants for inactivation of mature biofilm of *E. coli* O157:H7 on produce.

Type (concentration)	Stage of biofilm formation	Contact time	Pathogen applied	Produce name	Procedure	Efficiency	References
Bacteriophage BPECO 19 (10^8 PFU/ml)	Mature biofilm	Submerge in bacteriophage for 2 h	*E. coli* O157:H7	Lettuce	24 h at 10, 20, and 25 °C	≥2 log CFU/cm^2	(Sadekuzzaman et al., 2017)
Tannin extracts from *Acacia mearnsii* De Wild. (tannin AQ (2 %, w/v), tannin SG (1 %, v/v) and tannin SM (1 %, v/v)	Mature biofilm	10 min	*E. coli*	Lettuce	2-24 h at room temp.	2 log CFU/cm^2	(Klug et al., 2017)
Electrostatically sprayed with malic and lactic acid solutions alone (1.0/2.0/3.0/4.0% w/v) or in combination	Mature biofilm	N/A	*E. coli* O157:H7 and *Salmonella* Typhimurium	Spinach and cantaloupe	8 °C for 72 h	Spinach: 4.14 log CFU/disk; Cantaloupe: 3.6 CFU/disk	(Almasoud et al., 2015)
100 and 200ppm chlorine, short chain fatty acid (0.4%)	Mature biofilm	22 °C for 2 min	*E. coli* O157:H7	Romaine lettuce	4 °C for 18 to 24 h	< 2 log CFU/g	(Keskinen & Annous, 2011)
200 ppm NaOCl soaking followed by 0.04% calcinated calcium spray	Mature biofilm	20 min at room temp.	*E. coli* O157:H7	Radish sprouts	Germination in dark at room temp.	<1.65 log CFU/ml	(Fransisca et al., 2011)
Sodium hypochlorite solution (300, or 600 ppm), irradiation (0, 0.25, 0.5, 0.75, or 1 kGy)	Mature biofilm	3 min	*E. coli* O157:H7	Romaine lettuce, spinach leaves	Submerged for 2 min and incubate 4 °C 24, 48, or 72 h	Chlorine: 1.3 log CFU/g for baby spinach, 1.8 log CFU/g for Romaine; Irradiation: <3.0 log	(Niemira & Cooke, 2010)

						CFU/g for 1 kGy after 72 h	
Chlorine dioxide (3 mg/L), sodium hypochlorite (100 mg/L)	Mature biofilm	1 min	*E. coli*	Lettuce	3 days at 4 °C plus 4 days at 7°C	Hypochlorite: 1.3 to 0.31 log CFU/g; chlorine dioxide: 0.8 to 0.01 log CFU/g	(López-Gálvez et al., 2010)

2.5. Bacteriophages

2.5.1. General background of bacteriophages

Bacteriophages, are natural bacterial predators, that as viruses can infect bacteria (Douglas, 2013; Kelley et al., 2015). The International Committee on Taxonomy classifies bacteriophages into one order, 13 families and 31 genera (van Regenmortel et al., 2000). There are two orders include *Caudovirales* and *Ligamenvirales* while other phage families are Unassigned (Douglas, 2013). Approximately 96% of bacteriophages are tailed bacteriophages with double-stranded DNA (dsDNA) molecules (binary symmetry) (Abedon, 2005). They are classified into the order *Caudovirales* which includes three distinct phylogenetically related families (*Myoviridae, Siphoviridae, Podoviridae*) based on tail mophology (Abedon, 2005; Kutter, 2004). Bacteriophages belonging to the *Myoviridae* (represent 25% of *Caudovirales*) possess a long contractile tail. *Siphoviridae* (61%) possess flexible non-contractile tails and *Podoviridae* (14%) have short non-contractile tails (Abedon, 2005; Fokine & Rossmann, 2014; Wurtz, 1992). All *Caudovirales* have heads with dsDNA, central tubes and sheaths separated from the heads by necks (Abedon, 2005).

2.5.2. Bacteriophage infection cycle

The bacteriophage life cycle includes adsorption, infection, release, and decay (Abedon, 2005). Bacteriophages can be classified as *virulent* or *temperate* (Kutter & Sulakvelidze, 2004). Virulent bacteriophages replicate through a lytic cycle by injecting their genome into the host cell after absorption, followed by cell lysis and liberation of new bacteriophage progeny (Kutter &

Sulakvelidze, 2004). In contrast, temperate bacteriophages [including bacteriophage lambda (λ), P1, Mu and dairy bacteriophages] may sometimes enter a lysogenic cycle, in which the bacteriophage genome integrates into the host genome and remains in a quiescent state (Kutter & Sulakvelidze, 2004). Most of the bacteriophages used to control food pathogens are lytic. In addition to the lytic cycle, Sharma (2013) and Abedon (2011) indicated that some bacteriophages (T-even and certain other bacteriophages with a large genome) can employ 'lysis from without' (LO) and can efficiently lyse bacteria at low temperature after adsorbing to the cell surface and without completing the infection cycle.

Lysis from within. Lysis from within (LI) is the standard intracellular bacterial cell lysis process brought about by the adsorption of one or few bacteriophage particle(s) to the bacterial surface (Abedon, 2011; Delbrück, 1940). During initial infection, multiple tail fibers enable bacteriophages to reversibly adsorb to the bacteria cell surface through specific receptors (Molineux, 2006). Bacteriophage tails orientate perpendicularly to the cell surface and the tail protein binds irreversibly to the receptor (Molineux, 2006). Bacteriophage adsorption is host specific, depending on the typical receptor sites present in the cell wall of the bacterium (Douglas, 2013). A particular bacteriophage strain can only infect a narrow host range of bacteria due to the natural and structural characteristics of receptors on bacteria cell surfaces (Rakhuba et al., 2010). The receptors located on the bacterial surface include pili (M13, D3112, F116), flagella (χ, SP3, PBP1), lipopolysaccharide (LPS) (T7, P22), surface proteins, (T1, T5, λ, AR1), teichoic acids (SP50, ϕ25) and capsules (K29, K1F, H4489A) (Clokie & Kropinski, 2008b). Lindberg (1973) and Rakhuba et al. (2010) reported that receptors for Gram-negative bacteria (*E. coli* and *Salmonella*) consist of proteins, LPS, and phospholipid complexes. Bacteriophages require cations such as Ca^{2+} and Mg^{2+} as cofactors to complete their lytic cycle (Clokie & Kropinski, 2008b).

Although the binding of bacteriophages with LPS-protein complex is reversible, precipitation occurs when the cofactor Mg^{2+} interacts with the complex, resulting in irreversible attachment (Rakhuba et al., 2010). The receptor of T5 bacteriophages has been isolated and found to be a lipoprotein molecule with high molecular weight (Douglas, 2013). The adsorption to a particular host of the T-even group bacteriophages (T2, T4, and T6) has been found to be influenced by cofactors (Brenner et al., 1962). T4 and T6 bacteriophages require L-tryptophan as a cofactor to unwind the tail fibers from the sheath surface, before interaction with the bacteria-host surface (Anderson, 1945; Brenner et al., 1962; Douglas, 2013). In contrast, indole can inhibit the adsorption of T2 bacteriophage due to increased contraction of the tail fiber into the sheath (Brenner et al., 1962).

For most of the dsDNA bacteriophages, after virion proteins penetrate the outer membrane/cell wall, bacteriophage can generate a murein-degrading enzyme (endolysin) and a second membrane-embedded protein (holin), known as the 'holin-endolysin' strategy to create and enlarge a hole within the peptidoglycan layer of the bacterial cell (Abedon, 2005; Moak & Molineux, 2004; Molineux, 2006). This conformational change allows bacteriophage DNA to be released from the capsid and enter the host cell (Storms et al., 2012). Endolysin, is responsible for the enzymatic cleavage of peptidoglycan, and is produced during the lytic cycle in the late phase of gene expression (Garcia et al., 2010). Holin is a small hydrophobic protein that can enable endolysin to cross the cytoplasmic membrane and reach the cell wall (Wang et al., 2000). At this point, the bacteriophage enters either a lytic or lysogenic phase. For virulent lytic bacteriophages, nucleic acid replication followed by structural protein synthesis of bacteriophage particles occurs before new bacteriophage particles are assembled and released from the host cell (Campbell, 2003). The whole cycle takes up to 40 min and can produce approximately 100 new bacteriophage

particles (Campbell, 2003). As for temperate bacteriophages in the lysogenic phase, they are harbored in host cells in an inactive form (Campbell, 2003). Since temperate phage encoded functions can enhance the fitness of a lysogen, the lysogenic cell will be maintained within the bacterial population due to the selective advantage conferred by the phage genes (Kutter & Sulakvelidze, 2004). In this way, the prophages can protect the lysogenic host against extra phage infection and temporarily protect the host from attack by other bacteriophages (Campbell, 2003; Kutter & Sulakvelidze, 2004).

Lysis from without. Although bacteriophage have been used to decontaminate fresh fruits and vegetables, they may have low efficacy at the low temperatures (4°C and 10°C) encountered during produce storage and processing (Sharma, 2013). Most bacteriophages cannot complete their lytic cycle at temperatures below 4°C as most host bacteria are not metabolically active at low temperatures (Sharma, 2013). However, bacteriophages may still cause 'lysis from without (LO)', even at low temperatures. LO is a phenomenon exhibited by T-even and other bacteriophages with large genomes, especially T2 and T4 (Abedon, 2011; Molineux, 2006). It happens when a sufficient number of bacteriophage particles (more than 50) simultaneously adsorb to the bacterial cell wall. However, bacteriophage must be present in the environment at high concentrations to achieve LO (Arisaka et al., 2003; Kutter & Sulakvelidze, 2004; Lone et al., 2016). Unlike lysis from within (LI), no bacteriophage are liberated from the host cell as a result of LO (Abedon, 2011). LO can cause a decrease in culture turbidity as bacterial cell lysis occurs (Anany et al., 2014; Clokie & Kropinski, 2008a; Delbrück, 1940; Kutter & Sulakvelidze, 2004). Lysis of the bacterial cell occurs as a consequence of penetration of bacteriophages into the cell envelope during adsorption (Abedon, 2011). The penetration activity in T4 bacteriophage is reported to be encoded by gene *5*, whose gene product (*gp5*) is a tail-associated protein that acts as a lysozyme

(Abedon, 2011; Arisaka et al., 2003; Kao & McClain, 1980). Once a substantial number of bacteriophages absorb, the damage caused by *gp5* can result in cell lysis (Abedon, 2011). If the number of bacteriophage capable of absorption is low, insufficient levels of *gp5* may fail to cause bacterial lysis (Abedon, 2011).

2.5.3. External factors influencing bacteriophages

Temperature. Although many bacteriophages can survive low and high temperatures, bacteriophage infection can be affected by temperature (Jończyk et al., 2011). At low temperatures, reduced Brownian motion can influence the efficiency by which bacteriophage contact and penetrate host cells, resulting in less bacteriophage entering the multiplication stage (Tey et al., 2009). In contrast, high temperature can prolong the latency period of bacteriophage (Burns, 1982), increasing the duration of transition from the infective stage to multiplicative stage (Tey et al., 2009). Moreover, when the temperature is out the of the optimum range of enzyme activity (20-40°C), lower bacteriophage production will occur as a result of the slow host-growth rate (Tey et al., 2009).

pH. Gallusser and Kuhn (1990) reported that there is an electrostatic interaction between bacteriophage proteins and cytoplasmic membrane surfaces during the binding of bacteriophage to the bacterial surface, and as a result pH can influence bacteriophage propagation (Tey et al., 2009). Most bacteriophages are reported to be stable over a pH range from 3.5 to 6.8 (Abedon, 2008; Oliveira et al., 2014), while some research suggests that bacteriophage become unstable at a pH lower than 4.4 (Leverentz et al., 2001; Leverentz et al., 2003). Jończyk et al. (2011) reported that an immediate coagulation occurs when bacteriophages are exposed to a $pH < 2$. When pH is between 3 and 4, reversible precipitation occurs as bacteriophage can be redispersed by shaking

(Jończyk et al., 2011). Moreover, the enzymatic activity of host cells can be significantly influenced by pH and thus it has an impact on the efficiency of bacteriophage propagation, infection and host cell growth rate (Tey et al., 2009).

Salinity and ions. It has been reported that a rapid change of osmotic pressure (from high salt concentration to low salt concentration solutions) can cause the extrusion of DNA from broken heads or tails of bacteriophage (Jończyk et al., 2011; Lark & Adams, 1953). The inactivation rate of bacteriophage caused by NaCl or trivalent metal ions (i.e., Al^{3+}) can be substantially decreased if divalent ions such as Ca^{2+} and Mg^{2+} are present at millimolar concentrations (5-10 mmol/L) (Jończyk et al., 2011; Lark & Adams, 1953; Mylon et al., 2009). This is due to the divalent cations neutralizing the negative charge on the surface of bacteriophage (Jończyk et al., 2011). However, Jepson and March (2004) reported that, even though tap water contained Ca^{2+} and Mg^{2+}, bacteriophage were more stable in distilled water (Jepson & March, 2004). The titer of bacteriophage was decreased by 2-3.5 logs after storage for two weeks at ambient temperature in tap water. It was suggested that halogenating agents in the tap water were responsible for the bacteriophage instability (Jepson & March, 2004). Moreover, water containing organic matter has been reported to decrease bacteriophage stability as a result of the formation of complexes between Ca^{2+} and organic matter which lower the overall surface charge and destabilize bacteriophage particles. (Mylon et al., 2009).

2.5.4. Previous work using bacteriophages in food

Nowadays consumers consider food that contains minimal additives and is only heat treated as being more healthy (Juneja et al., 2012). Thus, natural antimicrobials, as an alternative to 'synthetic' chemical preservatives, have been applied to food to improve food safety, enhance food

hygiene quality and extend shelf-life (Anany et al., 2014; Juneja et al., 2012). Bacteriophages, with the ability to adapt to various environments, have the advantage of exerting their natural antimicrobial properties in complex matrices such as food (Anany et al., 2014). Bacteriophages have shown an impressive ability to reduce pathogens at both the pre-harvest and post-harvest stages of food production (Loneragan & Brashears, 2005; Soni & Nannapaneni, 2010; Woolston et al., 2013). They have been shown to reduce pathogens in beef and poultry (Carter et al., 2012; Seo et al., 2016; Sukumaran et al., 2015b) as well as fresh fruits and vegetables (Leverentz et al., 2003; Magnone et al., 2013b; Sharma, 2013). In 2006, the US Food and Drug Administration (FDA) approved the application of the bacteriophage LMP-02 from Intralytix Inc. as an additive to control *Listeria monocytogenes* on ready-to-eat poultry and meat (Coffey et al., 2010). Commercial bacteriophages that are recognized by the FDA as Generally Recognised As Safe (GRAS) have been employed in food safety by a number of companies (Table 2-7.).

Previous researchers have shown that bacteriophages can effectively reduce *E. coli* and *Salmonella* to some extent (Table 2-8.).

Table 2-7. Commercially available bacteriophage-based products to enhance food safety (Taylor et al., 2015)**.**

Company	**Product**	**Target bacteria**	**Website**
Applied Bio Research Inc.	Smart bacteriophage	Not specific	http://www.appliedbioresearch.co/
Intralytix Inc.	ListShield™	*Listeria monocytogenes*	http://www.intralytix.com/
	EcoShield™	*Escherichia coli* O157:H7	
	SalmoFresh™[a]	*Salmonella*	
Micreos Food Safety	BacteriophageGuard Listex.[a]	*L. monocytogenes*	http://www.micreos.com/
	BacteriophageGuard S.	*Salmonella*	
Omnilytix	AgriBacteriophage™	*Xanthomonas campestris pv. vesicatoria*, or *Pseudomonas syringae pv. tomato.*	http://www.omnilytics.com/

[a] FDA approved as GRAS

Table 2-8. Studies that have used bacteriophage as a biocontrol method to reduce pathogens on fresh produce.

Pathogens	Bacteriophage	Bacteriophage control	References
Salmonella	Bacteriophage-A: target was *S.* Typhimurium and Enteritidis; Bacteriophage-B target was *S.* Montevideo	Bacteriophage-A achieved a 1.37 log suppression of *Salmonella* growth on mustard seeds. The mixture of Bacteriophage-A and Bacteriophage-B reduced 1.50 log of *Salmonella* growth in the soaking water of broccoli seeds.	(Pao et al., 2004)
	Cocktail composed of UAB_Phi 20, UAB_Phi78, and UAB_Phi87	0.9 log CFU/g of reduction was achieved when dipped the lettuce into bacteriophage cocktail solution for 60 min at 4°C.	(Spricigo et al., 2013)
	The combination of *E. asburiae* JX1 (an antagonistic bacteria) and bacteriophage cocktail	The reduction level of *Salmonella* was 5.7 to 6.4 log CFU/ml.	(Ye et al., 2010)
E. coli O157:H7	EcoShield™	On spinach, *E. coli* O157:H7 RM4407 (EHEC) declined by 2.38 and 2.49 log CFU/cm^2 at 4 and 10°C after 30 min application. On lettuce, EHEC declined at 4°C by 2.49 and 3.28 log in 30 min and 2 h, respectively.	(Boyacioglu et al., 2013)
	EcoShield™	A significant ($p < 0.05$) reduction of experimentally contaminated lettuce by 87% after 5 min contact time. The reduced levels of bacteria were maintained for at least one week at refrigerated temperatures.	(Carter et al., 2012)
	EcoShield™	Spraying bacteriophages onto inoculated fresh cut lettuce can significantly reduce *E. coli* O157:H7 by 0.82 log CFU/cm^2 and 1.32 log CFU/cm^2 on day 0 and day 1, respectively.	(Ferguson et al., 2013)

	ECP-100	The reduction of *E. coli* O157:H7 on lettuce treated with ECP-100 on 0, 1, and 2 d were 1.92, 1.57, 1.64 CFU/cm^2 (P<0.05) when stored at 4°C.	(Sharma et al., 2009)
	BEC8	There was 1-2 log reduction within 24 h at 8°C for spinach and lettuce.	(Viazis et al., 2011)
E. coli, Shigella Salmonella combination	EcoShield™, SalmoFresh™, ShigActive	Bacteriophage cocktail combined with levulinic acid produce wash with high organic content reduced a5 strain cocktail culture > 4 logs.	(Magnone et al., 2013a)

2.5.5. SalmoFresh™ as a pathogen reduction intervention

SalmoFresh™ is a commercial, lytic bacteriophage cocktail produced by Intralytix Inc. that can selectively kill most common pathogenic *Salmonella* serotypes including Typhimurium, Enteritidis, Heidelberg, Newport, Hadar, Kentucky, Thompson, Georgia, Agona, Grampian, Senftenberg, Alachua, Infantis, Reading, and Schwarzengrund (Intralytix, 2015; Pérez Pulido et al., 2016). SalmoFresh™, contains 6 individual lytic bacteriophages, and has been approved by Health Canada to be applied directly on fresh and processed fruits and vegetables before slicing or ready-to-eat poultry products prior to or after grinding (Intralytix, 2015).

It has been reported that SalmoFresh™ can effectively reduce pathogenic *Salmonella* prevalent on glass coverslips and stainless steel coupons (Woolston et al., 2013), in turkey breast cutlets and ground turkey (Sharma et al., 2015), on chicken breast fillets (Sukumaran et al., 2015a), in fresh fruits and vegetables (Magnone et al., 2013b), individually or in combination with other bacteriophages.

However, when applied on the surface of raw turkey breast cutlets, SalmoFresh™ only achieved 0.8, 0.6 and 1.3 log CFU/g reductions in *Salmonella* Heidelberg on day 0, 1 and 7, respectively, and resulted in no reduction with ground turkey (Sharma et al., 2015). A similar result has been reported by Sukumaran et al. (2015a) with only a 0.8 log CFU/g reduction when chicken breast fillets were immersed in a 10^9 PFU/ml solution of SalmoFresh™. SalmoFresh™ has been reported to cause a 2.37 log CFU decline in *Salmonella* Newport on cucumbers at 22 ºC. However, this decline was insufficient to prevent the transfer of this pathogen to fresh-cut slices (Sharma et al., 2017). When SalmoFresh™ was combined with EcoShield™ and ShigActive™ in the washing solution, a > 4 log reduction of the pathogen was achieved for cantaloupe, broccoli and

strawberries (Magnone et al., 2013b). Therefore, SalmoFresh™ has potential as a pathogen reduction intervention for fresh produce, especially when combined with other bacteriophages.

2.5.6. Bacteriophage cocktail for biofilm mitigation

As the second step during biofilm formation, in addition to producing polysaccharides, *E. coli* can produce a long, thin and wiry fibre called curli (Pawar et al., 2005). Curli can only be produced at ambient temperatures and low osmotic pressure during the stationary growth phase (Pawar et al., 2005). It is a bi-subunit (*CsgA* and *CsgB*) protein, that was shown to make *E. coli* O157:H7 more virulent than the non-curli expressing variant in a mice model (Uhlich et al., 2002). It has been shown that curli expression plays a key role in increasing the resistance of STEC to sanitizers (Wang et al., 2012). The formation of curli fimbriae structural proteins is controlled by *CsgD*, which is a central regulator of biofilm production that is regulated by RpoS and RpoD (Uhlich et al., 2013). The transcription factor *mrlA* can bind to the *RpoS* promoter to enhance the *RpoS*-dependent transcription of *CsgD* (Uhlich et al., 2013). It has been reported that bacteriophages carrying stx_1 can interrupt *mrlA* function by using an insertion site in the proximal *mlrA* coding region of *E. coli* O157:H7 strains (Uhlich et al., 2013). The loss of *mlrA* can lead to poor curli expression and biofilm formation by *E. coli* O157:H7 (Uhlich et al., 2013), raising the possibility that bacteriophage can interrupt the formation of biofilms by *E. coli* O157:H7.

Bacteriophages have the potential of controlling biofilms through several mechanisms. Firstly, bacteriophages can rapidly multiply during the lytic cycle and reach numbers that are sufficient to disrupt biofilms. This self-replicating phenomenon has the potential for a single bacteriophage to degrade the biofilm matrix as bacteriophage progeny infect adjacent bacterial

cells within the biofilm (Fu et al., 2010). Secondly, some bacteriophages can produce polysaccharide degrading enzymes to degrade the EPS matrix of the biofilm (Fu et al., 2010).

Previous research has successfully used bacteriophage as a pre-treatment to reduce the formation of biofilms on abiotic solid surfaces (Curtin & Donlan, 2006; Fu et al., 2010). Curtin and Donlan (2006) reported that bacteriophage cocktails with divalent cations achieved a 4.47 $\log_{10}$ CFU/cm^2 reduction of catheter-associated *Staphylococcus epidermidis* within biofilms, while Fu et al. (2010) reported 4.37 $\log_{10}$ CFU/cm^2 reduction in *Pseudomonas aeruginosa* in biofilms in catheters.

According to all the information above, my research had the following specific objectives:

1. To determine the effectiveness of SalmoFresh™ for reducing *Salmonella* on Romaine lettuce, mung bean sprouts and mung bean seeds.
   - 1.1. To determine the effectiveness of using SalmoFresh™ to eliminate 5 *Salmonella* strains using an *in vitro* micro plate assay
   - 1.2. To test the effectiveness of SalmoFresh™ at reducing a *Salmonella* cocktail inoculated onto fresh produce and mung bean seeds.
     - 1.2.1. To test the effectiveness of SalmoFresh™ at reducing *Salmonella* on Romaine lettuce and sprout samples with spot inoculation at 2, 10 and 25 ºC over a period of 1, 24, 48 or 72 h.
     - 1.2.2. To test the effectiveness of SalmoFresh™ at reducing *Salmonella* on scaled-up lettuce, sprouts and mung bean seed samples inoculated through immersion at 2, 10 and 25 ºC after 1, 24, 48 or 72 h.
2. To determine the effectiveness of an STEC bacteriophage cocktail applied with or without cofactors at preventing biofilm formation by *E. coli* O157:H7 on the intact and cut surfaces

of Romaine lettuce.

2.1. Use a micro plate biofilm formation assay to determine the ability of 4 *E. coli* O157:H7 strains to form biofilms *in vitro*.

2.2. To determine the effectiveness of a STEC bacteriophage cocktail applied with or without cofactors at preventing biofilm formation by the 4 *E. coli* O157:H7 strains on intact and cut surfaces of Romaine lettuce.

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3. CHAPTER 3. OBJECTIVE I

Efficacy of a Lytic Bacteriophage Preparation to Reduce *Salmonella* on Romaine Lettuce, Mung Bean Sprouts and Mung Bean Seeds.

3.1. Abstract

Introduction: Produce commodities are linked to *Salmonella* outbreaks worldwide. *Salmonella* bacteriophage have been successfully applied alone or in combination with other bacteriophages to control *Salmonella* in food.

Purpose: To evaluate the effectiveness of a *Salmonella* lytic bacteriophage cocktail to reduce/eliminate *Salmonella* on the surface of Romaine lettuce, mung bean sprouts, and mung bean seeds; to assess if the activity of *Salmonella* bacteriophages is affected when combined with an STEC phage cocktail and disinfectant (chlorinated water).

Methods: A commercial lytic bacteriophage preparation SalmoFresh™ (Intralytix, Inc.) was obtained and tested against 5 *Salmonella* strains (Newport, Braenderup, Typhimurium, Kentucky, and Heidelberg) individually, at 2, 10 and 25 ºC for 5 h using an *in vitro* microplate virulence assay. SalmoFresh™ alone or in combination with STEC bacteriophage cocktails were applied to lettuce, sprouts and mung bean seeds by either; 1) spray-application of SalmoFresh™ (10^8 PFU/ml) to lettuce (3×3cm^2 pieces) (n = 98) and sprouts (n = 98) after spot-inoculation with *Salmonella* (10^5 CFU/ml); 2) immersion of 600 g lettuce, 300 g sprouts or 30 g mung bean seeds in SalmoFresh™ alone (10^8 PFU/ml) or in combination with an STEC bacteriophage cocktail (10^8 PFU/ml) for 15 min (lettuce and sprouts) or 1 h (seeds) after immersion-inoculation with *Salmonella* (10^5 CFU/ml) followed by exposure to chlorine for 30 sec. Bacteriophage was not

applied to positive controls, while the negative controls were neither inoculated with bacteria nor subject to any treatment. Lettuce and sprouts in treatment and control groups were stored at 2, 10 and 25 ºC and analyzed for viable *Salmonella* after 1 h and every 24 h for up to three days. Seeds were drained, dried for up to 1 h, packaged and stored dry at room temperature before the *Salmonella* population was determined after 72 h. Mung bean seeds were then germinated and *Salmonella* reduction/survival was determined by total viable *Salmonella*cells on xylose lysine tergitol-4 (XLT4) agar plates and immunomagnetic separation (IMS).

Results: Microplate virulence assays indicated that SalmoFresh™ (10^8 PFU/mL) reduced (P=0.007) *Salmonella* by an average of 5.34 logs CFU/mL after 5 h at 25° C. However, the spot plate technique showed that some *Salmonella* remained viable. Spraying SalmoFresh™ onto lettuce and sprouts reduced *Salmonella* by 0.7 log_{10} CFU/g and 0.8 log_{10} CFU/g, respectively. Although SalmoFresh™ did not significantly reduce *Salmonella* on mung bean seeds (P=0.2395) and germinated sprouts (P=0.1219), delivery of bacteriophage through immersion did control (P<0.0001) this pathogen on both lettuce and sprouts. Samples treated with a combination of chlorinated water, SalmoFresh™ and STEC phages resulted in the highest reduction for lettuce (3.02 log) and sprouts (2.26 log).

Conclusion Application of SalmoFresh™ in combination with STEC bacteriophage (lettuce: 3.48 log reduction; sprouts: 2.36 log reduction) did not further improve phage efficacy (P=0.9975) compared to applying SalmoFresh™ alone (lettuce: 2.41 log reduction; sprouts: 2.16 log reduction), but both were more effective (P<0.0001) than using chlorinated water alone (lettuce: 2.41 log reduction; sprouts: 1.83 log reduction).

3.2. Introduction

Fresh produce has been linked with several foodborne illness outbreaks caused by *Salmonella* and in the last decade has become a serious health threat in developed and developing countries. In the US, 11% of the outbreaks that resulted in hospitalization and 28% of the deaths were caused by non-typhoidal *Salmonella* over this period (Scallan et al., 2011). Foodborne illness outbreaks associated with fresh vegetables have been increasing. Consequently, efforts towards developing effective antimicrobial strategies aimed at controlling foodborne pathogens has also been an area of growing interest (Viazis et al., 2011).

The consumption of fresh fruits and vegetables is widely acknowledged as crucial for a healthy diet because produce can provide nutrients, vitamins, and fiber for humans (Olaimat & Holley, 2012). Epidemiological data indicated that plant-based foods such as fresh produce can lower the risk of coronary artery disease and stroke (Hu, 2003). As the consumption of leafy greens rose by 9% from 1995 to 2006, related foodborne illness outbreaks have increased by 38.6% (CDC, 2016b). Lettuce linked to outbreaks caused by *Salmonella* has been reported in North America, Australia, Finland, and England (Brandl & Amundson, 2008). Until 2009, leafy greens ranked as the No.1 outbreak-related food category, causing 363 outbreaks and 13,568 reported foodborne illnesses (Davidson et al., 2013b).

Outbreaks can occur due to adulteration of the produce at any stage of the production process from the growing stage through to packaging and storage. Lack of effective antimicrobial treatments from planting to harvesting to processing can raise the risk of contamination of the final product (Buck et al., 2003). Therefore, it is crucial to identify a biocontrol hurdle approach that is

compatible with produce processing procedures to reduce outbreaks caused by pathogens such as *Salmonella* (Parish et al., 2003).

Antimicrobial treatments have been applied in many food processing industries to decrease the risk of pathogen contamination (Jahid & Ha, 2012). Among the conventional chemical sanitizers, chlorine is extensively used by industry to disinfect fresh fruits and vegetables (Davidson et al., 2013c). Lettuce requires exposure to 100-150 parts per million (ppm) of free chlorine on continuous belts whereas spinach needs to be sprayed with 70-150 ppm of free chlorine (Suslow, 2014). However, chlorine gas released from treated water can cause discomfort to workers (Suslow, 2014). Discolouration and undesirable odor are other drawbacks of chemical disinfectants when chlorine levels in water exceed 1% (Baskaran et al., 2013). Chlorine can react with organic material and form carcinogenic organochlorine compounds such as chloramines (Jahid & Ha, 2012; Stopforth et al., 2008). Moreover, wastewater that contains chemical sanitizers also requires additional purification and treatment, significantly increasing produce production costs (Neal et al., 2012).

To satisfy the growing consumer demand for organic fruits and vegetables, bacteriophages, which have been recently used to control foodborne pathogens in fresh produce offer a feasible alternative method to kill pathogens (Sharma, 2013). Bacteriophages are abundant in nature and are already present in cheese, sausages and a variety of ready-to-eat foods (Sharma et al., 2009). Lytic bacteriophages replicate through a lytic cycle, which includes adsorption, infection, and release. After adsorption, injection of nucleic acids from the phage to the host takes place. During DNA translocation across the host cell, nucleic acid replication followed by structural protein synthesis of bacteriophage particles occur before new bacteriophage particles are assembled and

released from the host cell (Campbell, 2003). The whole cycle takes up to 40 min and can produce approximately 100 new bacteriophage particles (Campbell, 2003).

Many commercial bacteriophage cocktails such as Guard Listex, EcoShield™, and ListShield™ have been reported to be effective at controlling their targeted host in food (Taylor et al., 2015). SalmoFresh™, a *Salmonella* lytic bacteriophage cocktail, has been granted GRAS (Generally Recognized As Safe) status by the FDA (FDA, 2013b; Intralytix, 2015). One of the reported limitations of lytic bacteriophages is their low effectiveness when used at refrigeration temperatures (< 4°C). At 4°C, most bacteria stop metabolism which can prevent bacteriophages from completing their lytic cycle (Ferguson et al., 2013). Fortunately, the lytic cycle is not the only mechanism whereby phages can kill bacteria. Bacteriophages can also cause "lysis-from-without" (LO) whereby a substantial number of phages are adsorbed on the host and cause lysis through cell wall destruction (Abedon, 2011; Ferguson et al., 2013). When the number of attached bacteriophages is above a threshold level (MOI > 50), the bacterial cell wall is destroyed, cell contents are released, but no progeny are generated (Abedon, 2011). Through this mechanism, bacteriophage can potentially kill pathogens at lower temperatures (Abedon, 2011). Another primary concern in phage studies is selection for bacteriophage-resistant bacteria (Demerec & Fano, 1945; León & Bastías, 2015). The alteration or deletion of the receptors on the outer membrane of the cell surfaces can help protect the bacteria from bacteriophage attack (Kudva et al., 1999; Tanji et al., 2004). Because bacteria are likely to develop resistance to a single bacteriophage, simultaneous application of multiple bacteriophage within a cocktail is recommended as a method of thwarting resistance (Fischer et al., 2013).

This study determined the effectiveness of SalmoFresh™ against 5 *Salmonella enterica* serovars (Typhimurium, Enteritidis, Heidelberg, Kentucky, and Newport) on lettuce, sprouts and

mung bean seeds when applied alone or in combination with an STEC bacteriophage cocktail and chlorine at 2, 10 and 25 °C for 1, 24, 48 and 72 h.

3.3. Method and Material

3.3.1. Preparation of bacterial cultures and bacterial cocktail preparation

Five *Salmonella* strains were tested in this study (Table 3-1.). All strains were obtained from the culture collection of the Food Science Department, University of Manitoba. The bacterial cultures were stored in Trypticase Soy broth, TSB (Difco, Becton Dickson Co., Franklin Lakes, NJ, US) containing 15% glycerol (Sigma Chemical Co., St. Louis, MO, US) at -80 °C. Each culture was thawed and streaked on Trypticase Soy agar, TSA (Difco) and revived after incubation at 37 °C for 24 h.

Salmonella cocktail suspensions were prepared by transferring bacterial colonies (fresh culture 16-18 h) from plates into fresh TSA (Criterion™, Hardy Diagnostics, Santa Maria, CA, US) and incubated at 37 °C to obtain a bacterial suspension that was adjusted to 10^8 CFU/mL (OD_{600} = ~ 0.13; Thermo Scientific GENESYS 20, Thermo Fisher Scientific, Inc., Rochester, NY, US). *Salmonella* isolates were streaked on XLT4 agar plates (Criterion™ Dehydrated Media, Hardy Diagnostics) containing XLT4 supplement (BD Difco™, Sparks, MD, US). A single colony was selected from the plates and transferred to 10 mL TSB and incubated overnight at 37 ºC. Each culture (10^8 CFU/mL) was transferred into bottles containing 20 mL of TSB and incubated overnight at 37 ºC. After incubation, the bottles were centrifuged (Beckman-Coulter Allegra X-22R Centrifuge, Kansas City, MO, US) for 10 min at 4000 × g and the supernatant was discarded.

The bacterial pellet was suspended in 2 mL of mTSB [TSB+10% glycerol] and the total volume was brought to 30 mL with mTSB. The concentration of *Salmonella* cocktail stock culture was confirmed as 10^8 CFU/mL through plating on XLT4 agar plates.

Table 3-1. *Salmonella* strains used in the current experiment.

Genus	Serotype	Strain No.	Origin
Salmonella enterica	Newport	1194	Ground beef
Salmonella enterica	Braenderup	H9812 (ATCC BAA6644)	N/A
Salmonella enterica	Typhimurium	ATCC 19585	N/A
Salmonella enterica	Kentucky	F41-2	Chicken carcasses
Salmonella enterica	Heidelberg	32SusP	Chicken nuggets

3.3.2. Shiga-toxigenic *E. coli* (STEC) bacteriophage cocktail and SalmoFresh™

Commercial SalmoFresh™ was purchased from Intralytix Inc. (Baltimore, MD, US). The components of the SalmoFresh™ phage cocktail are listed in Table 3-2. and Table 3-3. with additional information available on the company's website (Intralytix, 2015). The study tested 5 lots (250 mL/lot) of SalmoFresh™.

The STEC bacteriophage cocktail used for this study contained bacteriophages isolated from beef feces from feedlots in Alberta, Canada (Table 3-4.). Fecal samples (47 cattle per trailer) were collected every two weeks from May 2013 to August 2013 as described by Stanford et al. (2010). This previous study isolated 37 lytic bacteriophages against non-O157 STEC (O26, O45, O103, O111, O121, O145) and O157:H7. The lytic capability and host range of the bacteriophages was assessed (Niu et al., 2012; Wang et al., 2015) and the STEC bacteriophage cocktail was propagated prior to its use in the present study. All the bacteriophage cocktail preparation steps have been done by the researchers in the previous studies, and the present study used the ready-to-use STEC bacteriophage preparation.

Both SalmoFresh™ and STEC bacteriophage cocktail preparations are mixtures of virulent lytic bacteriophages and would not enter the lysogenic phases in prophage status.

Table 3-2. Genome size and composition of phage contained in SalmoFresh™ provided by Intralytix Inc. (FDA, 2013b)

Phage	#ATCC	GenBank Accession	GC%	Size (bp)	No. of open reading frames	Undesirable genes*
SBA-1781	PTA-5282	JX181814 - JX181821	39	88 124	741	None
SKML-39	PTA-12380	JX181829	50	159 624	1547	None
SPT-1	PTA-5281	JX181822 - JX181823	39	87 248	725	None
SSE-121	PTA-5283	JX181824	45	147 745	1455	None
STML-13-1	PTA-8365	JX181826 - JX181828	51	161 646	1650	None
STML-198	PTA-12381	JX181825	37	158 160	1350	None

*Bacterial toxin-encoding genes.

Table 3-3. Summary of *Salmonella enterica* host strain for SalmoFresh™ provided by Intralytix Inc. (FDA, 2013b)

Phage	Host strain	Serotype	Biochemistry	PFGE	Endogeneous phage	Antibiotic Susceptibility
SBA-1781	S.H178	Hadar	*Salmonella* spp.	+	-	Susceptible to all tested
SKML-39	S.K39	Kentucky	*Salmonella* spp.	+	-	Susceptible to all tested
SPT-1	S.E378	Enteritidis	*Salmonella* spp.	+	-	Susceptible to all tested
SSE-121	S.A121	Agona	*Salmonella* spp.	+	-	Susceptible to all tested
STML-13-1	S.E236	Enteritidis	*Salmonella* spp.	+	-	Susceptible to all tested
STML-198	S.T198	Typhimurium	*Salmonella* spp.	+	-	Susceptible to all tested

Table 3-4. Taxonomy, genome size and morphology of 7 STEC phage stocks.

Taxonomy		Serotypes	Strain	Estimated genome size (kb)	Head dimensions (nm)	Tail dimensions (nm)		Undesirable genes	References
Genus	Family					Length	Width		
T4	*Myoviridae*	O26	EC19960464	177	115-117	109-118	21-24	None	(Wang et al., 2015)
To be determined	To be determined	O45	EC19940040	To be determined	To be determined	To be determined	To be determined	None	
T5	*Siphoviridae*	O103	EC20010670	126	82-85	178-196	10	None	
ViI	*Myoviridae*	O111	EC20030053	151	92-95	100-102	16-20	None	
rV5	*Myoviridae*	O121	EC20040083	140	93	122	17	None	
O1	*Myoviridae*	O145	EC20020231	68-89	78-79	113-114	17-18	None	
T5	*Siphoviridae*	O157	R508	108	78	185	8.5	None	(Niu et al., 2012)

3.3.3. *In vitro* microplate virulence assay

Susceptibility of *Salmonella* isolates to SalmoFresh™ was assessed using a microplate phage virulence assay similar to that described by Niu et al. (2009). *Salmonella* bacteriophage were serially diluted 10-fold by adding 20 µL volumes of phage in 180 µL volumes of mTSB (TSB with 10 mM $MgSO_4$) in 96 well microplates (Nunc™, Roskilde, Denmark) in triplicate. Then, 20 µL of the overnight culture (10^8 CFU/mL) of each *Salmonella* isolate was added to each well, and the plates were incubated at 2, 10 and 25 ˚C for 5 h. The negative controls contained mTSB only, while positive controls consisted of mTSB plus the *Salmonella* isolate cultures (10^8 CFU/mL). After incubation, the optical density (OD) of all wells was measured with a microplate reader (BioTek ELx800; BioTek Instruments Inc., Winooski, VT, US) at 630 nm. Reduction of the *Salmonella* population in microplate wells was determined after 5 h. Treatment and control groups in microplates were serially diluted from 10^{-1} to 10^{-7} by adding 20 µL of each sample into 180 µL buffered peptone water (BPW) (Criterion™, Hardy Diagnostics). Diluted samples were spread-plated onto XLT4 agar and incubated at 37 ºC overnight. The multiplicity of infection (MOI) for each bacteriophage host was calculated as the initial number of phages in the greatest-dilution wells (PFU/ml) divided by the initial number of bacteria added (CFU/mL). Three repetitions and 12 observations for each strain at the three different temperatures were conducted for the experiment.

3.3.4. Spot plate technique for determining *Salmonella* survival

After microplates were prepared and incubated as described in section 3.3.3, 2 µL of sample from control and treatment groups was taken from each well and plated on XLT4 agar.

Agar plates were incubated at 37 ºC overnight and examined for the presence of *Salmonella* colonies.

3.3.5. Commercial fresh romaine lettuce, mung bean sprouts, and mung bean seeds

Fresh lettuce (Dole™ prepacked romaine hearts, Winnipeg, MB) and sprouts (Bulk, Winnipeg, MB) and mung bean seeds (Sprout Master, Elmvale, ON) were purchased from a local supermarket or online. Romaine lettuce was prepared by removing the outer leaves and parts of stems (3 cm from the base of the heart) before washing. Whole sprouts with root, stem, and cotyledon were selected for the study. Mung bean seeds were examined under a stereoscopic microscope (Richter Optica U2B Binocular Lab Microscope, China) and only those seeds that exhibited no visible damage were selected for the study.

3.3.6. Effect of SalmoFresh™ against *Salmonella* cocktail on spot-inoculated lettuce and sprout pieces.

To evaluate the effectiveness of SalmoFresh™ on *Salmonella* spot-inoculated produce, lettuce and sprouts were washed three times with tap water at 10°C. This washing temperature (~10 °C) is recommended by FDA (2008) to the produce industry to suppress the growth of background flora and avoid cross-contamination. Lettuce pieces were cut with a sterile blade to obtain 3x3 cm^2 (12 pieces/treatment), and whole sprouts (12 pieces/treatment) were selected and placed on square Petri dishes (10x10 cm^2 plastic, Fisherbrand®, Pittsburg, PA, US) using sterile tweezers. Lettuce and sprouts were sprayed with 70% ethanol to reduce the interference of background flora.

The effectiveness of spraying SalmoFresh™ (10^8 PFU/mL) at reducing or eliminating *Salmonella* on lettuce (0.84 mL/piece) and sprouts (0.63 mL/piece) was evaluated. Samples were spot-inoculated (2 µL/spot, 5 spots/lettuce piece, 3 spots/sprout) with *Salmonella* cocktail culture (10^5 CFU/mL). The 5 spots for lettuce pieces were evenly inoculated with the pipette onto the leaf surface, and the three spots for sprouts were inoculated onto the cotyledon, stem, and root. After spot inoculation, all samples were air-dried for up to 3 h. After air-drying at room temperature, samples from treatment groups were treated with SalmoFresh™ with commercial plastic sprayer (HX50; Yuyao Dongxia Sprayer Plastic Industrial Co. Ltd, Ningbo, China) by pressing the trigger of the sprayer for each time of spray (0.21ml/ spray, 1 spray/ lettuce piece or sprout). Then the samples were packed in sterile bags (Whirl-Pak®, Nasco, Fort Atkinson, WI, US) and stored at 2, 10, and 25 ℃ for 1, 24, 48, and 72 h. The negative control groups were without any treatment or inoculation. Buffered peptone water (90 mL) was added to the sample storage bags and homogenized (Stomacher blender, Interscience, France) for 1 min. Serial dilutions from 10^{-1} to 10^{-7} were conducted for all samples by using dilution blanks with 9 ml of sterile BPW and 1 ml of the sample. Dilutions were plated onto XLT4 agar plates with supplement.

3.3.7. Small scale trial to assess the effectiveness of STEC bacteriophage cocktail and SalmoFresh™ mixture to reduce *Salmonella* on lettuce, mung bean sprouts and mung bean seeds

To assess phage efficacy under industry conditions, a small-scale trial was conducted. SalmoFresh™ was applied alone or in combination with the STEC bacteriophage cocktail and chlorinated water in 4 treatments (described in Table 3-5). Lettuce leaves (600 g), 300 g sprouts, and 30 g mung bean seeds were washed three times with tap water at 10 °C. Excess water was

removed using a sterile salad spinner. Samples were adulterated by immersion in a *Salmonella* cocktail culture (10^5 CFU/mL) for 30 min to allow bacterial attachment and the inoculated samples were assigned to 4 different treatments. For treatment 1 (Tr1), the inoculated samples (lettuce leaves/sprouts/seeds) were washed with chlorinated water (150 ppm for lettuce and sprouts, 1000 ppm for seeds) with agitation for 3 min, followed by three rinses with deionized water (10 ºC); Treatment 2 (Tr2), the inoculated lettuce leaves/sprouts/seeds samples were treated with SalmoFresh™ and the STEC bacteriophage cocktail (10^8 PFU/mL); Treatment 3 (Tr3), the inoculated lettuce leaves/sprout/seed samples were washed with chlorinated water (150 ppm for lettuce and sprouts, 1000 ppm for seeds) using the same method as Tr1 before treatment with the SalmoFresh™ and STEC bacteriophage cocktail combination; Treatment 4 (Tr4), the inoculated samples were treated only with SalmoFresh™. For Tr2, Tr3, and Tr4 samples were immersed in solutions containing bacteriophages for 15 min (lettuce and sprouts) or 1 h (mung bean seeds). Positive and negative controls were included; positive controls consisted of a set of samples inoculated without any treatment, while negative controls consisted of samples without treatment or inoculation. The chlorinated water used for Tr1 and Tr3 was prepared by mixing a commercial bleach (sodium hypochlorite as the main ingredient) (Old Dutch®, Montreal, QC) in cold (10 °C) deionized water (150 ppm: 9.375 mL bleach + 990.625 mL water, 1000 ppm: 62.5 mL bleach + 937.5 mL water). Concentrations of the chlorinated water were tested by Quantab test strips (Hach Co., Loveland, CO, US) before application. Lettuce and sprouts (10 g of each) were randomly collected from treatment and controls groups, packaged in bags and stored at 2, 10, 25 ºC for 1, 24, 48, and 72 h. Adulterated seeds (10 g) were taken from treatment and control groups, dried for up to 1 h and stored in sterile bags in the dark at 25 ºC for 72 h.

Salmonella enumeration: Lettuce and sprouts were homogenized in 90 mL of BPW in a stomacher (Interscience, France) for 1 min; while seed samples were sonicated for 30 sec (ultrasonic bath, Fisherbrand®, UK). Serial dilutions were prepared by using dilution blanks with 9 ml of sterile BPW and a 1 mL aliquot of each sample. Samples were serially diluted from 10^{-1} to 10^{-7} and samples were spread-plated on XLT4 agar plates. For Tr1 and Tr3, 9 mL D/E Neutralizing Broth (Difco, BD Diagnostic Systems, Palo Alto, CA, US) was used for the first dilution to neutralize chlorine and prevent inhibition of *Salmonella*. Immunomagnetic separation (IMS) was used in attempts to detect *Salmonella* if no growth on XLT4 agar plates was observed.

Table 3-5. Treatments for the scale-up study.

Treatment group No.	Treatment
1	The inoculated lettuce leaves/sprouts/seeds samples washed with chlorinated water (150 ppm for lettuce and sprouts, 1000 ppm for seeds)
2	The inoculated lettuce leaves/sprouts/seeds samples treated with SalmoFresh™ and STEC bacteriophage cocktail combination (10^8 PFU/mL)
3	The inoculated lettuce leaves/sprouts/seeds samples washed with chlorinated water (150 ppm for lettuce and sprouts, 1000 ppm for seeds) before treatment with the SalmoFresh™ and STEC bacteriophage cocktail combination
4	The inoculated lettuce leaves/sprout/seed samples treated by SalmoFresh™ only

3.3.8. Immunomagnetic separation (IMS)

Samples were enriched in a modified Tryptic soy broth [TSB (Oxoid, Nepean, ON) with 10 mg/L Novobiocin (Alfa Aesar Co. Inc., Ward Hill, MA, US). The enrichment was carried out by adding 1 mL of sample into tubes containing 9 mL of mTSB. Samples were thoroughly vortex mixed and incubated for 24 h at 37 °C. After incubation, IMS was conducted using Dynabeads® (Thermo Fisher Scientific, Waltham, MA, US) using the BeadRetriever Tube Rack (Dynal, Lake

Success, NY, US) following manufacturer recommendations. Briefly, 1 mL of the enriched sample from mTSB was mixed with 10 µL of anti-*Salmonella* beads (Dynal) in a 1.5 mL microcentrifuge tube for 15 min. Beads were then washed three times with 1 mL phosphate buffered saline (PBS)-Tween 20 (TEKnova, Hollister, CA, US) before 100 µL of the bead-bacteria mixture was spread-plated on XLT4 agar plates using a sterilized cotton swab. Plates were incubated overnight at 37 ºC. Recovered *Salmonella* colonies were confirmed using a *Salmonella* latex kit (Oxoid, Nepean, ON).

3.3.9. Bacteriophage resistance test for surviving colonies from each trial.

A representative number of surviving *Salmonella* colonies from each bacteriophage treatment group were isolated by streaking one loop of sample on XLT4 agar plates. Up to 10 isolated colonies from each treatment group were used to make up a pure stock culture (10^8 CFU/ml) with 200 µL TSB+15% glycerol at 37 °C for 24 h. Cultures were then stored at -80 °C. The susceptibility of the culture for survival to SalmoFresh™ was tested by the *in vitro* microplate virulence assay described in 3.3.3.

3.3.10. Statistical analysis

Data were analyzed by analysis of variance using the ANOVA procedure of SAS (SAS version 9.2, SAS Institute Inc., Cary, NC, US). A complete random design (CRD) was used with the MIXED procedure of SAS. The fixed effects of treatments, time and temperature were included in the model. Effects were considered significant at $P<0.05$.

Bar charts of total *Salmonella* numbers for treatment and control groups together with a bar chart of the reduction in *Salmonella* population are provided as the initial exploration of the

data. Tables for the two-way interaction of treatment and temperature effect have been constructed based on the SAS output to show the significance of different treatments.

3.4. Result and Discussion

3.4.1. *In vitro* microplate virulence effect of SalmoFresh™

Lytic effects against individual *Salmonella* strains varied with temperature. A two-way interaction ($P<0.0001$) of the lytic activity against bacteria strains and temperature was found, which means the lytic activity increased with temperature for all 5 strains. After 5 h, the OD of treatments at 2, 10, and 25 ℃ were similar (OD = 0.054, 0.054, 0.053, respectively); while the positive control groups at 25 ℃ exhibited a higher OD (OD = 0.123) than controls at 2 and 10 ℃ (OD = 0.52 and 0.54, respectively, Figure 3-1.). Bacteriophage lytic activity was the highest at 25 ℃ for all 5 strains, while no difference ($P>0.05$) in lytic activity towards strains at 2 and 10 ℃ was observed. Obeso et al. (2008) confirmed that phage lytic activity was temperature-dependent as the activity of endolysin was higher at 25 ℃ than at 4 ℃. Endolysin is an enzyme produced by phage that is responsible for lysis of the bacterial cell wall (Groman & Suzuki, 1963). Consequently, temperature can influence the lytic activity of phage through its impact on endolysin. However, Leverentz et al. (2001) reported a temperature-independent phage effect when applying phage mixtures to reduce *Salmonella* on honey dew slices. These results suggest that the degree to which temperature influences the activity of endolysin may differ among phage strains.

Salmonella population/reduction obtained in the *in vitro* microplate virulence assay at 25 °C for treatment and control groups can be observed in Figure 3-2. The lytic phage (10^8 PFU/mL)

caused an overall 5.34 log_{10} CFU/mL reduction in the 5 *Salmonella* strains. Previous research reported a 4 log reduction of *Salmonella* on fresh fruits and vegetables surfaces with application of SalmoFresh™ in combination with EcoShield™, an STEC lytic bacteriophage cocktail (Magnone et al., 2013a).

The MOI values at 2, 10 and 25 ºC, calculated as the number of plaque forming units (pfu) of phage used for infection divided by the number of cells (cfu) of *Salmonella* being infected (Table 3-6). The overall MOI values were higher at 2 and 10 ºC (MOI = 90.82) than at 25 ºC (MOI = 0.047), indicating that more bacteriophage particles were required to lyse *Salmonella* at lower temperatures. At 25 ºC, bacteriophage can continuously cause cell lysis through particle amplification as part of the normal lytic cycle. While at 2 and 10 ºC, the mechanism of 'lysis from without (LO)' requires a greater number of bacteriophage particles to attack the host cell and be effective. Since the optimum temperature for bacteriophage lytic activities is 30-45 ºC (Obeso et al., 2008), lower temperatures can suppress the normal infection cycle. Therefore, LO is responsible for the effectiveness of phage at refrigeration temperatures as these temperatures impede the normal lytic cycle (Ferguson et al., 2013). LO can be induced by high-multiplicity virion adsorption (MOI > 50) to the bacterial cell, causing swelling of the host cell within 5 to 10 min, but the process produces no phage progeny (Abedon, 2011; Kazi & Annapure, 2016). During the adsorption of substantial numbers of bacteriophages, the bacterial cell envelop can develop a 'weak point' which can lead to the lysis of the host cell (Abedon, 2011). These mechanisms are supported by the fact that destruction of the host cell by LO resulted in a higher MOI at 2 and 10 ºC than 25 ºC.

Since the control cultures did not grow well in microplates at 2 and 10 °C, the spot plate technique was used to assess the survival and lysis of all strains at the three temperatures. Using

the spot plate method, it was clear that *Salmonella* survived in microplates, 5 h after bacteriophage treatment (10^8 PFU/mL) at 25 ºC (Table 3-7.). At 2 and 10 ºC, SalmoFresh™ killed all 5 strains at 10^8 PFU/mL, but *Salmonella* did survive if phage were applied at 10^7 PFU/mL. A previous microplate virulence assay using STEC phage (Niu et al., 2009) killed all host bacteria in microplate wells, however, the presence of viable *Salmonella* 5 h after phage treatment indicates the present treatment method was not as effective. Application of SalmoFresh™ at 10^8 PFU/mL was the only level that lysed all *Salmonella* isolates after 5 h at all temperatures.

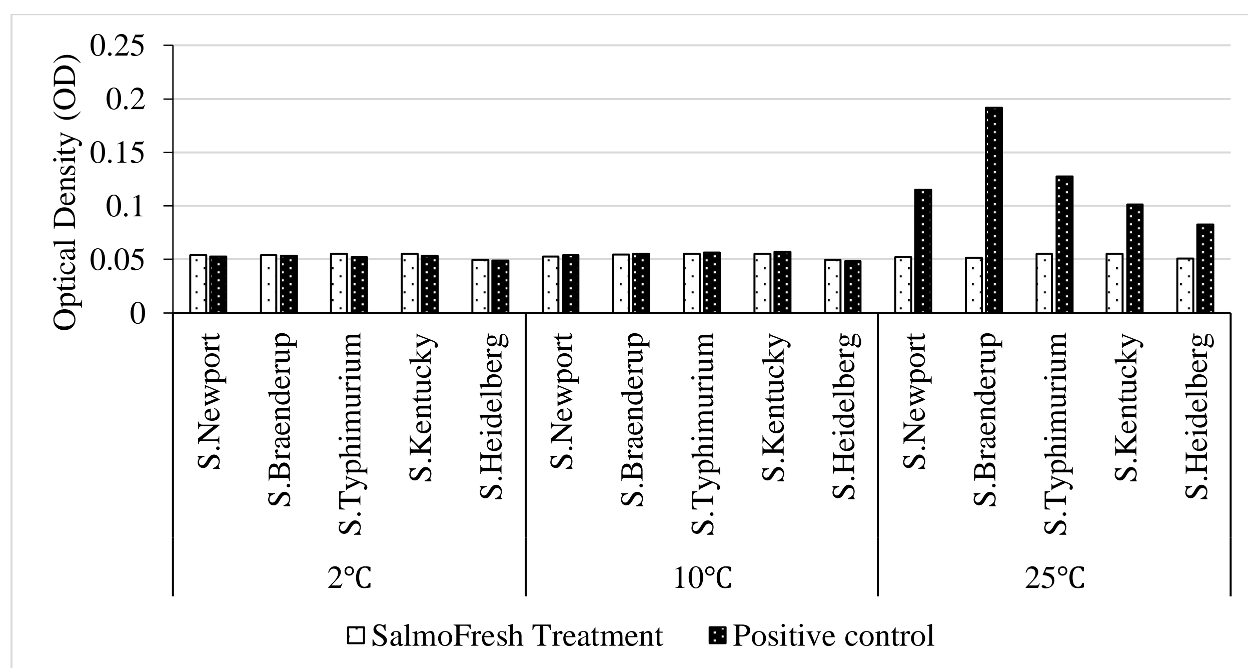

Figure 3-1. The effectiveness of SalmoFresh™ against the 5 individual *Salmonella* strains shown in the form of optical density (OD) values tested by *in vitro* microplate virulence assay.

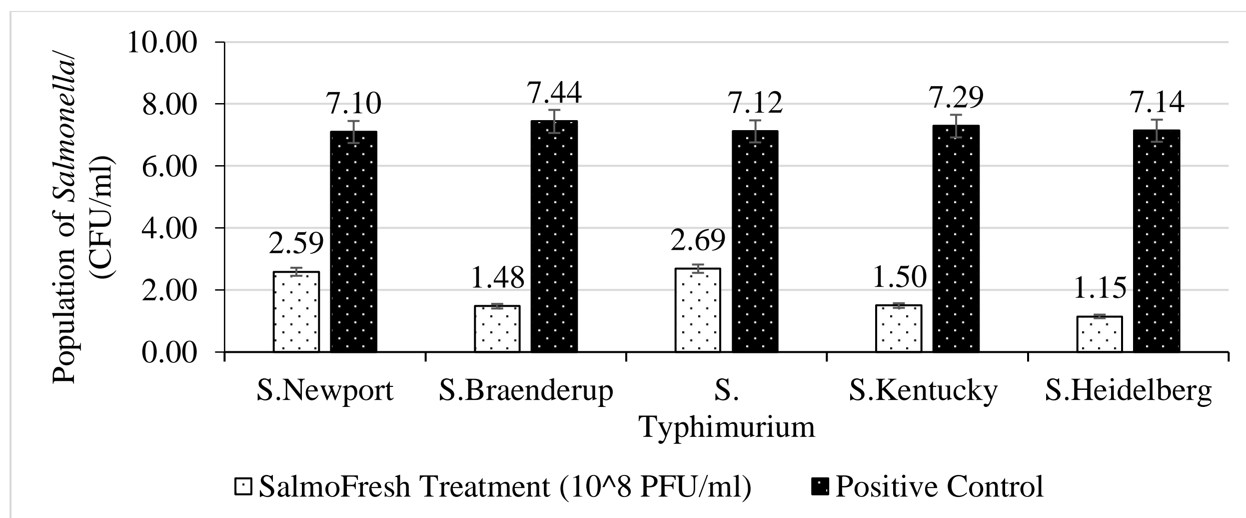

Figure 3-2. *Salmonella* population of *in vitro* microplate virulence assay for control and treatment groups at 25°C.

Table. 3-6. Temperature effect on the multiplicity of infection of (MOI) of SalmoFresh™ against the 5 *Salmonella* strains at three different temperatures.

Salmonella strains	Temperature		
	2℃	10℃	25℃
S. Newport	145.2^{A}	145.2^{A}	0.1^{B}
S. Braenderup	95.5^{A}	95.5^{A}	0.006^{B}
S. Typhimurium	64.9^{A}	64.9^{A}	0.06^{B}
S. Kentucky	49.4^{A}	49.4^{A}	0.03^{B}
S. Heidelberg	100^{A}	100^{A}	0.1^{B}

$^{A, B}$: distinct letters for the effect of different temperatures on the same *Salmonella* strain for SalmoFresh™ treatment groups, indicate differences (P<0.05).

Table 3-7. Result for survival (spot plate assay) and lysis (microplate assay) of *Salmonella* strains at 2 ℃, 10 ℃ and 25 ℃ after being treated with SalmoFresh™ at various concentrations.

Temp.	SalmoFresh™ Conc. (PFU/mL)	*S.* Newport		*S.* Braenderup		*S.* Typhimurium		*S.* Kentucky		*S.* Heidelberg	
		lysis	survival	lysis	survival	lysis	survival	lysis	survival	lysis	survival
2℃*	10^8	+	-	+	-	+	-	+	-	+	-
	10^7	+	+	+	+	+	+	+	+	+	+
10℃*	10^8	+	-	+	-	+	-	+	-	+	-
	10^7	+	+	+	+	+	+	+	+	+	+
25℃	10^8	+	+	+	+	+	+	+	+	+	+
	10^7	+	+	+	+	+	+	+	+	+	+

*Salmonella numbers were too few to be detected after 5 h of treatment with SalmoFresh™ at 2 and 10 ℃, but all strains grew at ambient temperature after removal from cold storage.

3.4.2. Effectiveness of a SalmoFresh™ and STEC bacteriophage cocktail against *Salmonella*

3.4.2.1. Spot-inoculation with SalmoFresh™ spray treatment.

For the *in vivo* spot-inoculated lettuce and sprout pieces with SalmoFresh™, the overall reduction (regardless of temperature or storage time) was 0.76 $\log_{10}$ CFU/g for lettuce and 0.83 $\log_{10}$ CFU/g for sprouts (Table 3-8. and Table 3-9.). The maximum reduction observed on lettuce was about 1 $\log_{10}$ at 2 & 10 °C after 72 h and after 24 h at 25 °C ($P<0.0001$) (Figure 3-3.). Viazis et al. (2011) reported a 1 $\log_{10}$ CFU/leaf reduction in *Salmonella* at 4 °C after treatment of spot-inoculated lettuce with lytic bacteriophages for 10 min. Similarly, other researchers showed a 1-2 log CFU/cm^2 reduction in *E. coli* O157:H7 on fresh-cut spot-inoculated lettuce surfaces stored at 4 °C when lytic bacteriophages were sprayed on the surface (Sharma et al., 2009). For sprouts, the highest reduction was achieved at 2 °C after 48 h (0.9 $\log_{10}$ CFU/g), and at 10 and 25 °C after 24 h storage (~1 $\log_{10}$ CFU/g) ($P = 0.001$) (Fig. 3-5.). This indicates that the reduction of *Salmonella* on lettuce and sprouts was virtually negligible when phages were applied using sprayers. The ability of phage to reduce *Salmonella* (1 $\log_{10}$ CFU/g) was low when they were sprayed onto produce. The effect of spray application of bacteriophages has been reported to be more immediately effective than immersion in other research (Ferguson et al., 2013). Ferguson et al. (2013) indicated that spraying could restrict bacteriophage particles to the surface of lettuce tissues rather than penetrating deeply into the tissue. However, these previous findings do not support my data. One possible reason for this discrepancy could be that when samples were immersed in bacteriophage rather than delivered by sprayer, the bacteriophage particles can move in the solution and have a better chance to find the specific receptor to attach. Although the effect of

Salmonella reduction was not immediate, bacteria reduction could be greater than that achieved when phages were applied using a sprayer.

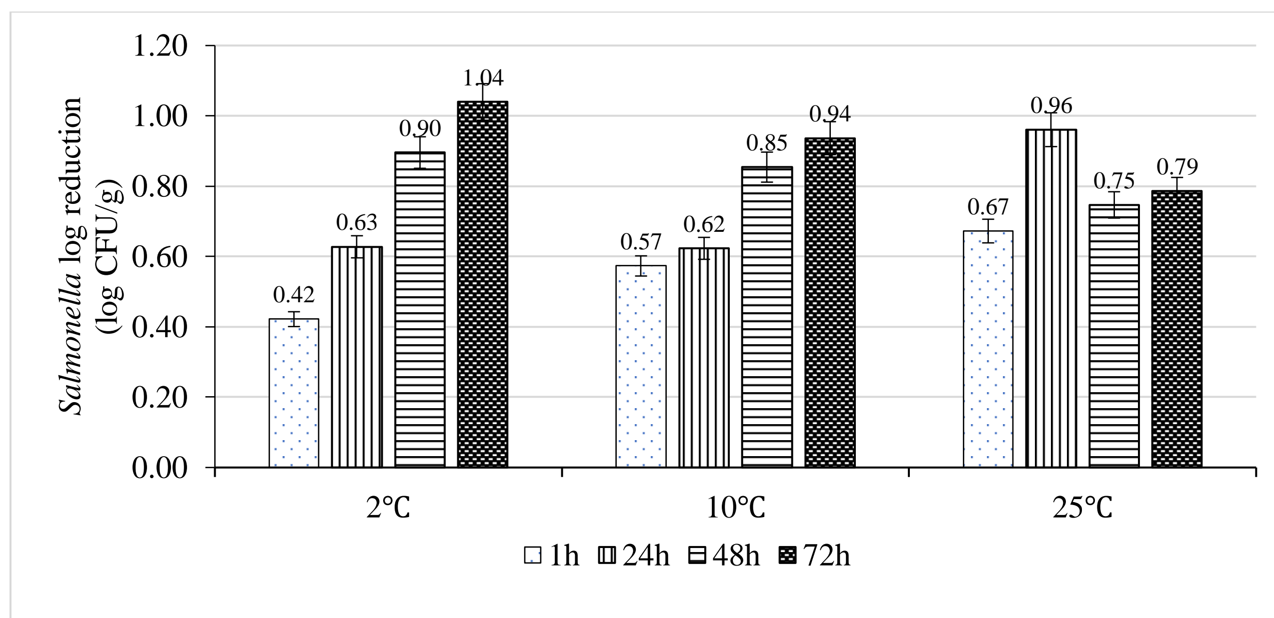

Figure 3-3. *Salmonella* reduction on lettuce pieces using spot inoculation of bacterial mixture followed by spraying with SalmoFresh™ before stored at 2, 10 and 25°C for 1, 24, 48 and 72 h.

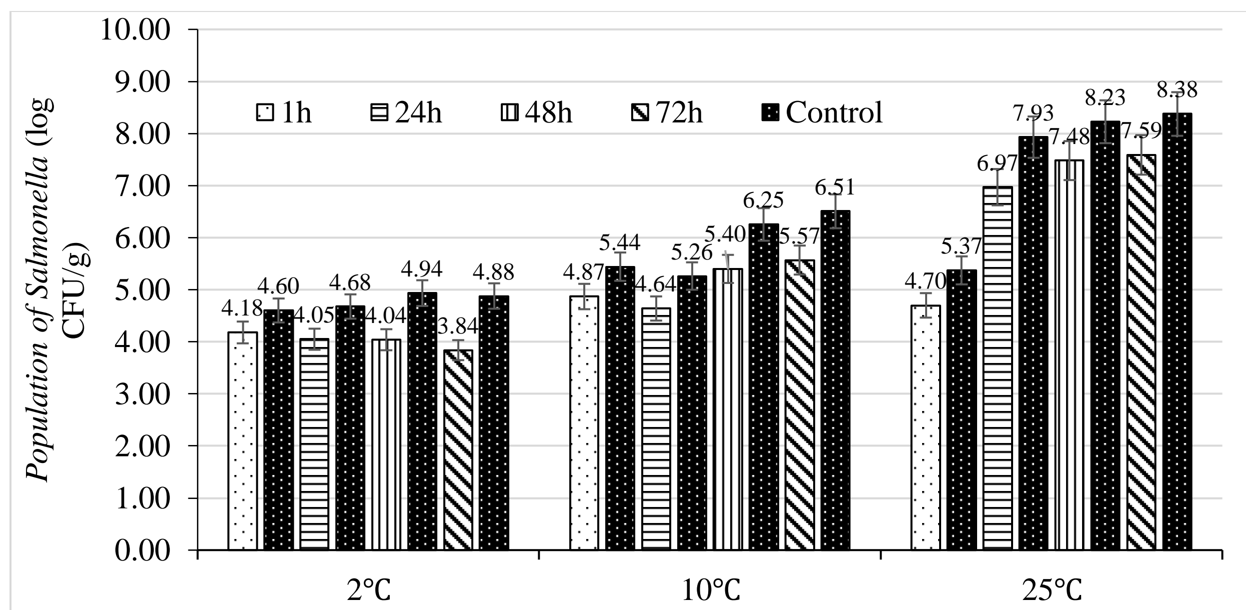

Figure 3-4. *Salmonella* population for the treatment and control groups of spot-inoculated lettuce. Each treatment group at 2, 10 and 25 ℃ was treated by spraying SalmoFresh™ and assessing survival after 1, 24, 48 and 72 h relative to a positive control group.

Table 3-8. *Salmonella* reduction (log CFU/g) for spot-inoculated lettuce as a result of treatment with SalmonFresh™ delivered by sprayer (10^8 PFU/ml) at different temperatures for various times.

	2℃	**10℃**	**25℃**	**SEM**
1h	0.42^{a}	$0.57^{a/d}$	$0.67^{a/d}$	
24h	0.63^{a}	$0.62^{ab/d}$	$0.96^{b/e}$	0.1619
48h	0.9^{a}	$0.85^{ab/e}$	$0.75^{b/f}$	
72h	1.04^{a}	$0.94^{ab/e}$	$0.79^{b/f}$	

[a,b]: distinct letters for the effect of different treatment times on the same temperature of SalmoFresh™ treatment groups, indicate differences (P<0.05).

[d,e,f]: distinct letters for the effect of different temperature on the same treatment times of SalmoFresh™ treatment groups, indicate differences (P<0.05).

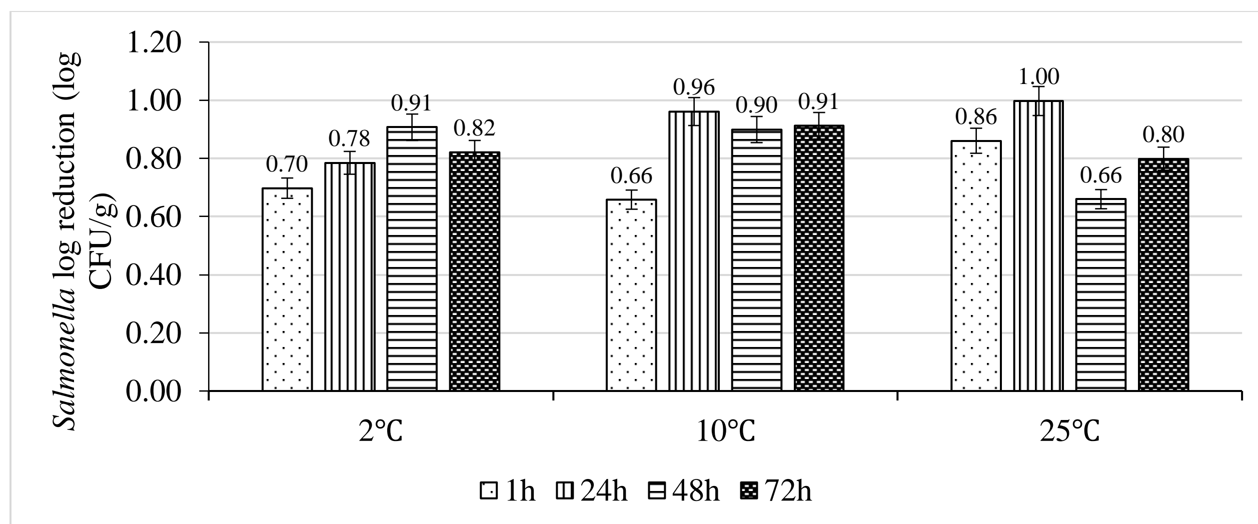

Figure 3-5. *Salmonella* reduction on sprouts using spot inoculation of a bacterial mixture followed by spraying with SalmoFresh™ before stored at 2, 10 and 25°C for 1, 24, 48 and 72 h.

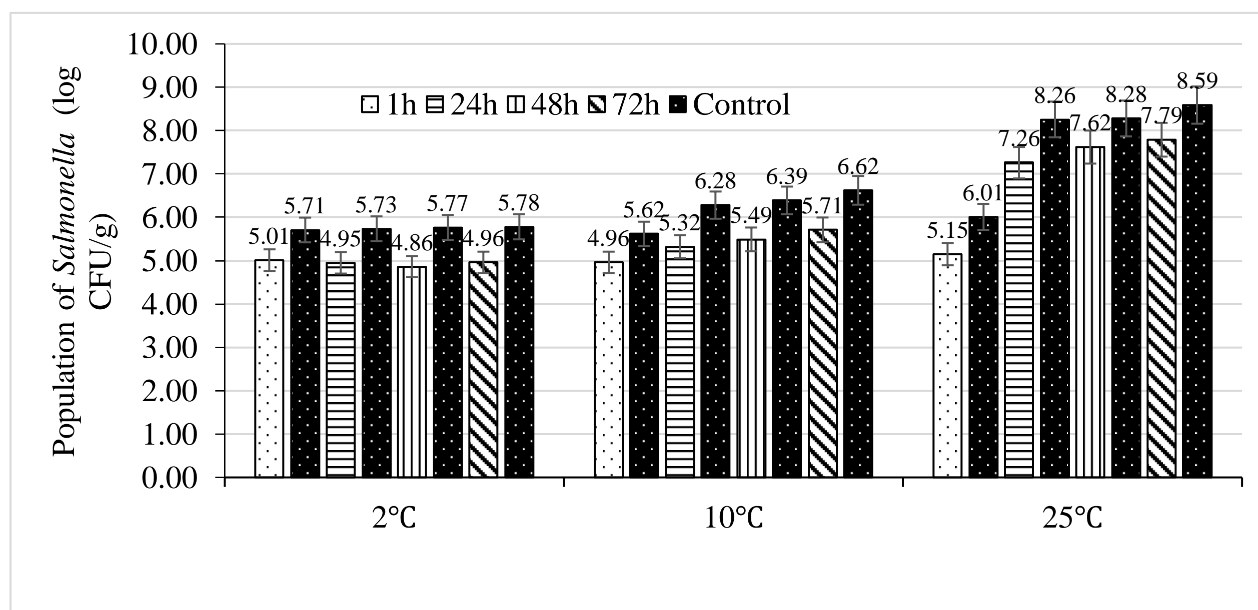

Figure 3-6. *Salmonella* population for the treatment and control groups of spot-inoculated sprouts. Each treatment group at 2, 10 and 25 ℃ was treated by spraying SalmoFresh™ before stored for 1, 24, 48 and 72 h relative to a positive control group.

Table 3-9. *Salmonella* reduction (log CFU/g) for spot-inoculated sprouts treated at different temperatures for various times.

Time	2℃	10℃	25℃	SEM
1h	0.70[a]	0.66[a/d]	0.86[a/d]	0.08

24h	0.78[a]	0.96[ab/d]	1.00[b/e]
48h	0.91[a]	0.90[b/e]	0.66[bc/f]
72h	0.82[a]	0.91[b/e]	0.80[c/f]

[a,b,c]: distinct letters for the effect of different treatment times on the same temperature of SalmoFresh™ treatment groups, indicate differences (P<0.05).
[d,e,f]: distinct letters for the effect of different temperature on the same treatment times of SalmoFresh™ treatment groups, indicate differences (P<0.05).

3.4.2.2. Scale-up test with immersion inoculation of SalmoFresh™ and STEC bacteriophage treatments

Scaled-up Lettuce Samples. The overall reduction obtained on scaled-up lettuce samples was 3.02 $\log_{10}$ CFU/mL of *Salmonella* (Table 3-11.), which was higher than previously observed with the spray treatment. Tr2 (STEC bacteriophage cocktail and SalmoFresh™) and Tr3 (STEC bacteriophage cocktail and SalmoFresh™ plus chlorinated water) were the most effective at reducing *Salmonella* (3.4-3.8 $\log_{10}$ CFU/g) on lettuce. Magnone et al. (2013a) reported a 3.05 log reduction when a broccoli sample inoculated with *Salmonella* was treated with a bacteriophage cocktail solution containing a mixture of SalmoFresh™ and EcoShield™. Tr1 (chlorinated water alone at 150 ppm) achieved a 2.4 $\log_{10}$ CFU/g reduction, which was higher than the 0.44-log reduction reported by Magnone et al. (2013a) when produce was washed with a 200 ppm chlorine solution for 1 min. Treatment efficacy was affected by temperature (P<0.001), with the efficacy of SalmoFresh™ being higher at 2 and 10 °C than at 25 °C (Table 3-12.). The application of SalmoFresh™ only (Tr4) resulted in a higher reduction (2.41 log) of *Salmonella* compared to fresh-cut lettuce treated only with EcoShield™ (1.32 log for *E. coli* O157:H7) (Ferguson et al., 2013).

Scaled-up Sprouts. The reduction in *Salmonella* on sprouts (2.26 $\log_{10}$ CFU/mL) as a result of bacteriophage treatment (Figure 3-8.) was slightly lower than with lettuce. Tr3 (STEC bacteriophage cocktail and SalmoFresh™ plus chlorinated water) was most effective as it reduced

Salmonella by 2.69 $\log_{10}$ CFU/g. The reductions for Tr2 (STEC bacteriophage cocktail and SalmoFresh™) (2.36 $\log_{10}$ CFU/g) and Tr4 (SalmoFresh™) (2.16 $\log_{10}$ CFU/g) (Table 3-13.) were almost the same as for SalmoFresh™ alone or in combination with the STEC bacteriophage cocktail. Treatment with 150 ppm chlorinated water (Tr1) caused the lowest reduction (1.83 $\log_{10}$ CFU/g) in *Salmonella*. The higher reduction of *Salmonella* resulted from immersion in phage as opposed to spraying phage, an outcome attributed to the phage achieving greater access to the targeted host with emersion. Pao et al. (2004) did not achieve as great a reduction (1.5 $\log_{10}$ CFU/g) in *Salmonella* when broccoli sprouts were dipped in *Salmonella* bacteriophage for 24 h at 25 ºC. In contrast, Ye et al. (2010) reported a slightly higher reduction in *Salmonella* (3.4 $\log_{10}$ CFU/g) when mung bean sprouts were dipped in *Salmonella*-infecting bacteriophages combined with antagonistic bacteria for 20 min at 25 ºC. Lee et al. (2002) reported that when mung bean sprouts were submerged in 200 ppm sodium hypochlorite alone for 10 min at room temperature (22 ºC) no *Salmonella* reduction was observed, although I found a 1.83 $\log_{10}$ CFU/g reduction by washing only with 150 ppm chlorinated water.

Scaled-up Mung Bean Seeds and Germinated Sprouts. The overall reduction in *Salmonella* on mung bean seeds was 1.25 log (Figure 3-9.). To be precise, reductions for mung bean seed samples were as follows: Tr1: 0.04 $\log_{10}$ CFU/g, Tr2: 1.4 $\log_{10}$ CFU/g, Tr3: 1.28 $\log_{10}$ CFU/g and Tr4: 2.28 $\log_{10}$ CFU/g. However, none of the treatments (P = 0.2395) effectively reduced *Salmonella* on mung bean seeds. The seeds spiked with the *Salmonella* cocktail (10^5 CFU/mL) and treated with 1000 ppm chlorinated water for 3 min in Tr1 exhibited the lowest reduction (0.04 log reduction). Tr2 and Tr3 had an intermediate reduction of 1.3-1.4 $\log_{10}$ CFU/ml *Salmonella*; while Tr4 (only SalmoFresh™) showed the highest reduction (~60%). However, after germination, *Salmonella* were recovered (10^4 CFU/g) due to the favorable humidity and

temperature conditions which promoted the growth of this bacterium (Figure 3-10.). SalmoFresh™ and chlorinated water alone, or in combination with a STEC bacteriophage cocktail were not adequate to reduce or prevent further multiplication of *Salmonella* cells on contaminating seeds, an observation that was blatantly obvious after germination (0.37 to 0.54 log reduction). Kocharunchitt et al. (2009) reported that, although the application of lytic bacteriophage resulted in a 1 log reduction of *Salmonella* on pre-inoculated alfalfa seeds after 3 h of treatment, the phages did not inhibit the growth of *Salmonella* during germination. Warriner et al. (2003) reported that once *Salmonella* contacted mung bean seeds, they were present on the surface and in inner tissues after germination. Since internalized *Salmonella* populations are hard to remove by postharvest biocidal washing, care should be taken to ensure that mung bean seeds are not contaminated during harvest and processing. Previous research reported two steps in the internalization of bacteria in plants: 1) bacteria enter the plant through physical/biological damage or natural openings on the tissue surface (i.e., stomata, lenticels, sites of lateral root emergence); 2) the existence of natural water (i.e., irrigation water, soaking water) that through capillary action promotes the flow of bacteria into the internal tissue (Deering et al., 2012). Besides the sprouted seeds, research has also documented the internalization of *E. coli* O157:H7 in lettuce tissues during washing (Erickson et al., 2010; Li et al., 2008).

Moreover, *Salmonella* has been reported to have the advantage of more effectively attaching to sprouted seeds as compared to *E. coli* O157:H7, which may partially explain why sprout-related outbreaks were more common for *Salmonella* (Barak et al., 2002). One possible reason for this difference is that *Salmonella* has a better ability to produce aggregative fimbriae (curli) in low temperature, low osmolarity environments (such as plant surfaces) than other bacteria (Barak et al., 2002).

When the surviving *Salmonella* colonies from the *in vivo* treatment groups for the scaled-up trials described in 3.3.8 were tested for phage resistance, they did not appear to exhibit permanent resistance to phage.

The fact that bacteriophage alone or combined with disinfectant could not permanently eliminate *Salmonella* complicates the use of phage as a biocontrol intervention. Therefore, it is important to consider the complex reaction between bacteriophage and host cells as well as the impact of environmental conditions. Factors like the minimum host threshold, host avoidance, resistance, and host specificity, are important to further understand the application of bacteriophage as a biocontrol intervention for food (Hudson et al., 2005). Environmental factors such as the irradiation by UV light and desiccation can all influence the activity of phage populations (Iriarte et al., 2007). These additional variables need to be considered in future studies.

Another factor that must be considered for future research is the bacterial inoculation level. Since the practical post-harvest pathogen cross-contamination level in food processing industries is usually low on fresh produce, conducting studies with low pathogen inoculum levels would present a situation that is more representative of industrial produce production conditions. Researchers have reported that when the pathogen contamination level is lower than 4 $\log_{10}$ CFU/mL, treatment of bacteriophage combined with other interventions (i.e., organic acid) can reduce *Salmonella* to a safe level (Hara-Kudo & Takatori, 2011; Viazis et al., 2011). Therefore, it would be worthwhile to conduct experiments using bacteriophage combined with chlorinated water and cofactors as a biocontrol intervention to reduce/eliminate low inoculum levels of *Salmonella* on produce surfaces.

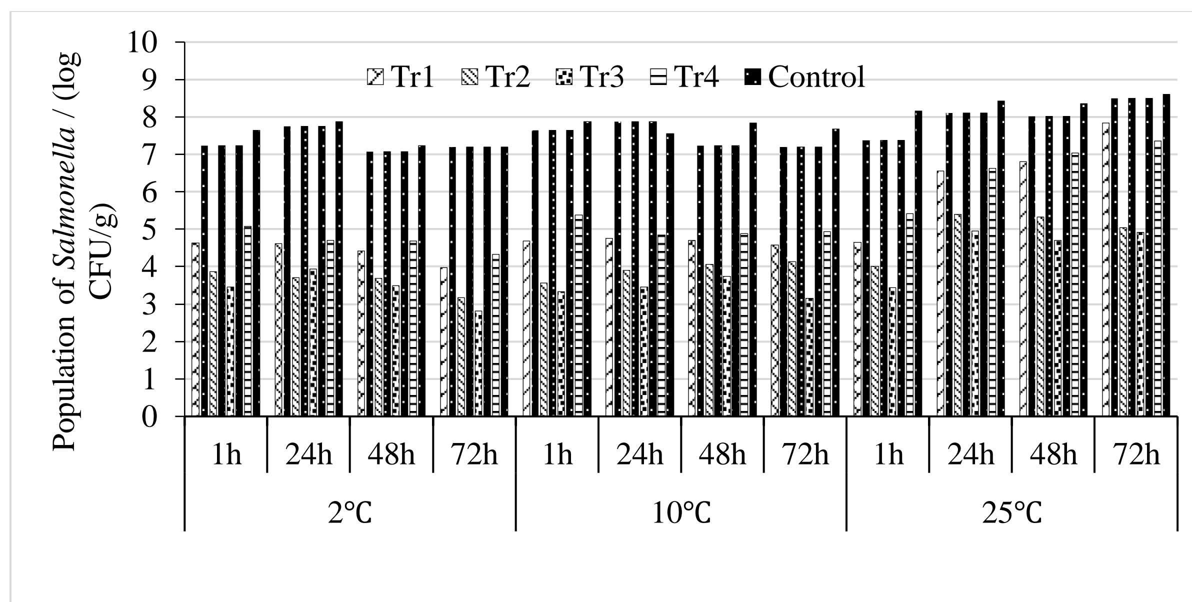

Figure 3-7. Population of *Salmonella* for scaled-up lettuce treated by SalmoFresh™ (10^8 PFU/ml) delivered through immersion. Each treatment group at 2, 10 and 25 ºC was treated for 1, 24, 48 and 72 h and compared relative to a positive control group.

Table 3-10. Treatment effects on *Salmonella* reduction on scaled-up lettuce samples.

Treatments	Tr1*	Tr2*	Tr3*	Tr4*	SEM	P value
Reduction (log_{10} CFU/g)	2.41^a	3.48^b	3.81^b	2.41^a	0.15	< 0.0001

$^{a, b}$. Least square means within a row lacking a common superscript letter differ ($P < 0.05$)

*: Tr1: The inoculated lettuce leaves/sprouts/seeds samples washed with chlorinated water (150 ppm for lettuce and sprouts, 1000 ppm for seeds); Tr2: The inoculated lettuce leaves/sprouts/seeds samples treated with SalmoFresh™ and STEC bacteriophage cocktail combination (10^8 PFU/mL); Tr3: The inoculated lettuce leaves/sprouts/seeds samples washed with chlorinated water (150 ppm for lettuce and sprouts, 1000 ppm for seeds) before treatment by SalmoFresh™ and STEC bacteriophage cocktail combination; Tr4: The inoculated lettuce leaves/sprouts/seeds samples treated by SalmoFresh™ only.

Table 3-11. Temperature effects on *Salmonella* reduction on spiked scaled-up lettuce (10^5 CFU/mL) after exposure by immersion at 25°C to SalmoFresh™ (10^8 PFU/mL) at 2, 10 and 25 °C.

Temperature	2 °C	10 °C	25 °C	SEM	P value
Reduction (log_{10} CFU/g)	3.3652^a	3.2797^a	2.4543^b	0.13	< 0.0001

$^{a, b}$. Least square means within a row lacking a common superscript letter differ ($P < 0.05$)

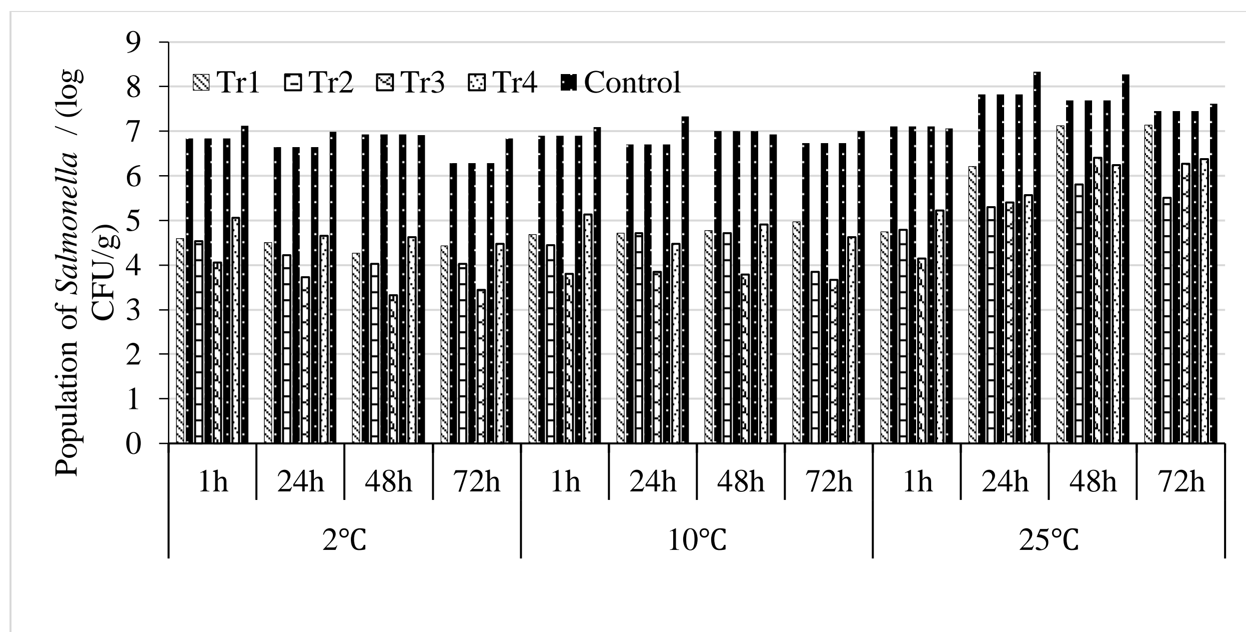

Figure 3-8. Population of *Salmonella* for scaled-up sprouts treated by SalmoFresh™ (10^8 PFU/ml) delivered through immersion. Each treatment group at 2, 10 and 25 ºC was treated for 1, 24, 48 and 72 h and compared relative to a positive control group.

Table 3-12. Treatment effects on *Salmonella* reduction on scaled-up sprout samples.

Treatment	Tr1*	Tr2*	Tr3*	Tr4*	SEM	P value
Reduction (log_{10} CFU/g)	1.83^{a}	2.36^{b}	2.69^{c}	2.16^{b}	0.08	< 0.0001

$^{a,b:}$ Least square means within a row lacking a common superscript letter differ (P < 0.05)

* Tr1: The inoculated lettuce leaves/sprouts/seeds samples washed with chlorinated water (150 ppm for lettuce and sprouts, 1000 ppm for seeds); Tr2: The inoculated lettuce leaves/sprouts/seeds samples treated with SalmoFresh™ and STEC bacteriophage cocktail combination (108 PFU/mL); Tr3: The inoculated lettuce leaves/sprouts/seeds samples washed with chlorinated water (150 ppm for lettuce and sprouts, 1000 ppm for seeds) before treatment by SalmoFresh™ and STEC bacteriophage cocktail combination; Tr4: The inoculated lettuce leaves/sprouts/seeds samples treated by SalmoFresh™ only.

Table 3-13. Temperature and time effects on *Salmonella* reduction (log_{10} CFU/g) scaled-up sprout samples.

	2 °C	10 °C	25 °C	SEM	P value
1 h	2.34^{a}	2.43^{a}	2.37$^{a/d}$	0.08	< 0.0001
24 h	2.46^{ab}	2.42^{a}	2.34$^{a/d}$		
48 h	2.87^{b}	2.47^{a}	1.44$^{b/e}$		
72 h	2.33^{a}	2.5^{a}	1.18$^{b/e}$		

$^{a, b}$: distinct letters for the effect of different treatment times at the same temperatures, indicate differences (P<0.05).

$^{d, e}$: distinct letters for the effect of different temperatures within the same times, indicate differences (P<0.05).

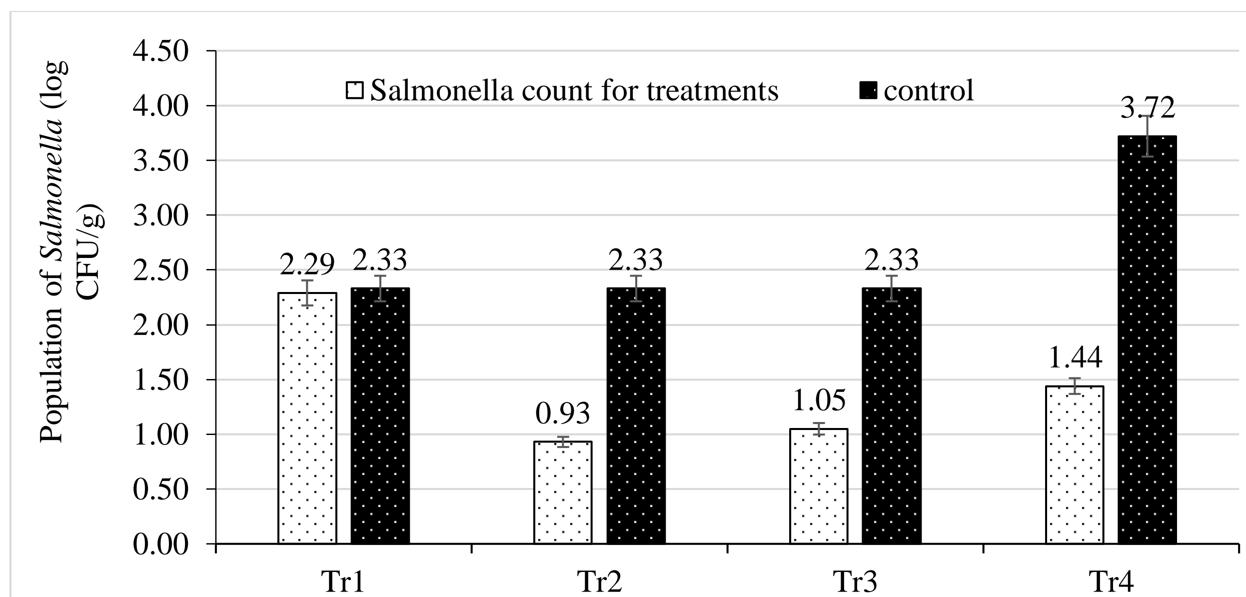

Figure 3-9. *Salmonella* population for scaled-up immersion-inoculated mung bean seeds (10^5 CFU/mL) treated by immersion in SalmoFresh™ (10^8 PFU/ml) for 72 h at 25°C.

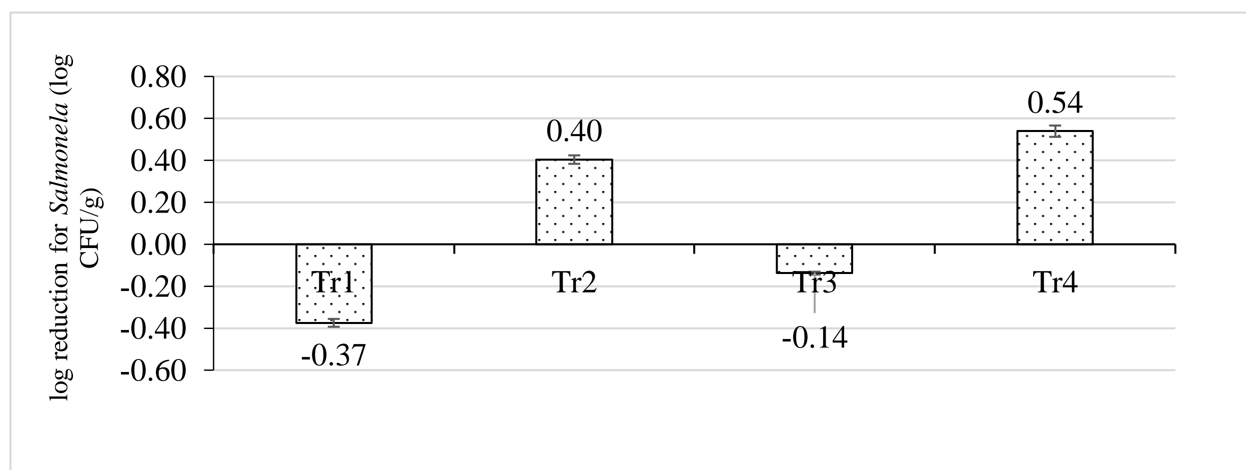

Figure 3-10. *Salmonella* reduction for the germinated mung bean seeds irrigated with sterile water. The seeds were spiked with *Salmonella* (10^5 CFU/ml) and treated by immersion in SalmoFresh™.

3.5. Conclusion

SalmoFresh™ exhibited effectiveness in eliminating/reducing *Salmonella* attached to fresh produce, but less effective on mung bean seeds and germinated sprouts when applied alone or in

combination with an STEC bacteriophage cocktail and chlorinated water. The reduction was not meaningful when SalmoFresh™ was spray-delivered. SalmoFresh™ and chlorinated water alone or in combination with STEC bacteriophage were not adequate to reduce *Salmonella* on contaminated seeds and their effectiveness was even lower after germination. When applied to scaled-up lettuce and sprouts by immersion, the bacteriophage cocktail achieved a greater overall reduction of 2 to 4 log_{10}. Treatment groups that received SalmoFresh™ in combination with the STEC bacteriophage cocktail and chlorinated water achieved the highest reduction for both lettuce and sprouts (3.81 and 2.69 logs). Treatments that received SalmoFresh™ only (lettuce: 2.41 log reduction; sprouts: 2.16 log reduction) or in combination with STEC bacteriophage (lettuce: 3.48 log reduction; sprouts: 2.36 log reduction) were similar or more effective than chlorinated water alone (lettuce: 2.41 log reduction; sprouts: 1.83 log reduction). Therefore, the combination of bacteriophage cocktails and traditional disinfectant (chlorinated water) is the most effective way to reduce *Salmonella* on fresh produce surfaces. The susceptibility of surviving colonies to bacteriophage from the treatment groups was tested with microplate assay, and no evidence of resistance to bacteriophage was observed. However, for mung bean seeds, bacteriophage in combination with chlorinated water did not significantly reduce *Salmonella.* This pathogen was recovered in numbers up to 4 log_{10} CFU/g after the contaminated seeds were germinated.

In summary, SalmoFresh™ could be used as a potential pathogen reduction intervention alone or in combination with STEC bacteriophage cocktail to reduce *Salmonella* on lettuce and sprouts. However, limitations for SalmoFresh™ exist. Firstly, the effectiveness for bacteriophage is limited when applied to mung bean seeds both prior to and after germination. Secondly, SalmoFresh™ only target at 15 different *Salmonella* serotypes (Typhimurium, Enteritidis, Heidelberg, Newport, Hadar, Kentucky, Thompson, Georgia, Agona, Grampian, Senftenberg,

Alachua, Infantis, Reading, and Schwarzengrund.) (Intralytix., 2015), therefore, if produce is contaminated by other *Salmonella* serotypes, the commercial bacteriophage may not be as effective. In this way, hurdle technology needs to be applied together with the biocontrol intervention to make sure the produce are safe for consumers.

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4. . CHAPTER 4. OBJECTIVE II

The Effectiveness of Using a STEC Bacteriophage Cocktail to Prevent *E coli* O157:H7 Biofilm Formation on Romaine Lettuce.

4.1. Abstract

Introduction: Biofilms are a growing concern for the food processing industry. *E. coli* O157:H7 is a critical pathogen related to foodborne illnesses as it can persist in many foods and produce biofilms, structures composed of an extracellular polysaccharide matrix that enables colonization of surfaces and provides protection. Current strategies have limited effectiveness in preventing biofilm formation on lettuce surfaces. Bacteriophages are known to produce enzymes that degrade this extracellular matrix. *E. coli* O157:H7 can be destroyed by lytic bacteriophages, which makes them a potential intervention to prevent biofilm formation.

Purpose: i) To evaluate the biofilm forming abilities of 4 *E. coli* O157:H7 strains *in vitro*; ii) To determine the ability of an STEC bacteriophage cocktail to prevent *E. coli* O157:H7 biofilm formation on intact Romaine lettuce pieces and cut leaf surfaces at different temperatures and times and iii) to evaluate if the presence of cofactors can enhance the ability of bacteriophages to prevent *E. coli* O157:H7 biofilm formation.

Methods: The ability of 4 *E. coli* O157:H7 strains to form biofilms *in vitro* was tested using a crystal violet technique at different temperatures (25 and 37 ºC) and times (24, 48 and 72 h). To test the ability of STEC bacteriophage to prevent *E. coli* O157:H7 attachment on biotic

surfaces, intact and damaged lettuce pieces were obtained and pre-treated with an STEC bacteriophage cocktail (with or without cofactors), and then inoculated with the 4 *E. coli* O157:H7 strains. Samples were incubated for 24 h at 2, 10 and 25 ºC. Lettuce pieces were washed twice with sterile water to remove non-attached cells. *E. coli* O157:H7 reduction/survival was determined by total *E. coli* numbers using MacConkey Agar with Sorbitol (SMAC).

Results: All strains were weak to moderate biofilm formers. *In vitro* biofilm formation abilities differed among *E. coli* O157:H7 strains ($P<0.0001$). Strain R508 was the only strain that formed an intermediate biofilm when grown at 25 ºC for 72 h, exhibiting a stronger ($P<0.0001$) biofilm forming capacity than the other strains (1931, 161-84 and EO122). Pre-treatment with an STEC bacteriophage cocktail and cofactors reduced ($P<0.0001$) *E. coli* O157:H7 attachment from 0 to 3.5 logs CFU/cm^2 at 2, 10 and 25 °C with an overall reduction of 1.31 logs CFU/cm^2. The highest reduction (3.42 log) was achieved at 25 °C using bacteriophages plus cofactors for *E. coli* O157:H7 strain EO122. However, when no cofactors were applied, the overall reduction of *E. coli* O157:H7 attachment (on average 0.75 $\log_{10}$ CFU/cm^2) was lower ($P<0.0001$) than for bacteriophage treatments with cofactors.

Conclusion: All 4 *E. coli* O157:H7 strains were able to form biofilms *in vitro*. The STEC bacteriophage cocktail largely prevented *E. coli* O157:H7 attachment and therefore biofilm formation on fresh produce. When bacteriophages were applied with cofactors, the cocktail was even more effective at reducing biofilm formation.

4.2. Introduction

On average, STEC have been reported to cause 265,000 human illnesses annually, resulting in 3600 hospitalizations and 61 deaths in the United States (Bryan et al., 2015; Scallan et al., 2011). From 2006 to 2013, the incidence of laboratory confirmed *E. coli* O157:H7 infections in the United States was 1.15 cases per 100,000, a rate similar to non-O157 STEC (1.17 cases per 100,000) (Crim et al., 2014). The Economic Research Service (ERS) of the U.S. Department of Agriculture estimated that STEC O157:H7 outbreaks have a yearly cost of $405 million associated with premature deaths ($370 million), medical care ($30 million), and lost productivity ($5 million) (Frenzen et al., 2005).

E. coli O157:H7 is responsible for most of the severe foodborne diseases (i.e., hemorrhagic diarrhea and kidney failure) linked to STEC (Bosilevac & Koohmaraie, 2011; Luna-Gierke et al., 2014). Human infections by non-O157 STEC, with the most common serogroups of O26 (190; 21%), O103 (178; 20%) and O111 (106; 12%), are steadily increasing in the year of 2017 comparing to the year from 2013 to 2016 (Marder et al., 2017). In the past, consumption of undercooked ground beef was the most frequent cause of STEC outbreaks in Canada (Bosilevac & Koohmaraie, 2011). However, unpasteurized dairy products (Guh et al., 2010), and juice (Cody et al., 1999), as well as fresh produce have also been reported to be primary sources of STEC (Breuer et al., 2001; Hilborn et al., 1999; Sharapov et al., 2016).

In the US, from 1982 to 2002, produce was linked to 21% (38 of 183) of foodborne outbreaks, in which 34% of the total of 5269 cases were caused by *E. coli* O157:H7 (Matthews, 2006). Restaurants and other food service facilities/activities are the primary sources of *E. coli* O157:H7 produce-related foodborne illnesses (40%), with cross-contamination during food preparation being responsible for 47% of outbreaks (Matthews, 2006).The processing steps used

to prepare ready-to-eat fresh produce such as cutting, slicing, peeling and shredding can expose interior cellular surfaces and provide nutrients that support the rapid growth of background microflora (Kozak et al., 2013). The Public Health Agency of Canada (PHAC) reported that among the 11,600 hospitalizations caused by foodborne illnesses in Canada, *E. coli* O157:H7 was responsible for 245 (6%) cases and 8 deaths each year (PHAC, 2016). Since the trend for increasing consumption of fresh fruits and vegetables is expected to continue to at least 2020 (Lin, 2004), outbreaks linked to produce will almost certainly continue to occur. This is also important for Canada as its greenhouse vegetable production was reported to be worth $1.2 billion in 2006 (Kozak et al., 2013).

E. coli O157:H7 can form biofilms on the surface of fresh produce (Silagyi et al., 2009), a factor that is undoubtedly responsible for its frequent linkage to foodborne illness outbreaks. Biofilms can be described as sessile microbial communities that grow on surfaces and are embedded in an exopolysaccharide (EPS) matrix with a heterogeneous gel-like architecture (Gómez-López, 2012). The architecture is a highly hydrated three-dimensional structure consisting of dense areas interspersed with pores and channels (Allison, 2000; Garrett et al., 2008; Gómez-López, 2012). The resistance of biofilms to antimicrobial biocides, environmental and oxidative stressors, disinfectants and sanitizers used in the food industry make them a severe food safety problem (Almasoud et al., 2015; Silagyi et al., 2009). The formation of biofilms by pathogens on the surface of fresh produce can cause post-processing cross-contamination, resulting in a direct negative impact on safety and public health (Gómez-López, 2012; Kumar & Anand, 1998).

Biofilm formation occurs in several distinct steps: 1) the initial process involves reversible attachment through van der Walls forces, electrostatic forces and hydrophobic interactions. Once attached, bacterial cells proliferate and accumulate in multi-layer cell clusters; 2) the second step

involves the production of surface polysaccharides and the formation of extracellular polymeric matrix that promote irreversible attachment and act as a bridge between bacterial cells and the substratum; 3) the third step involves the establishment of a biofilm architecture and 4) single cells are dispersed to enable the biofilm to spread over the produce surface and colonize downstream environments (Gómez-López, 2012). The target for my research was to prevent the formation of biofilms on lettuce by impeding the initial attachment phase. Avoidance of this first step is crucial because if the bacteria are not able to attach, biofilm formation on lettuce will not occur. In natural environments such as on plant leaves, biofilms can represent 80% of the total microbial population (Jahid & Ha, 2012). In the laboratory, biofilm formation has been reported to occur on the surface of lettuce (Jahid et al., 2015; Niemira & Cooke, 2010; Patel & Sharma, 2010), spinach (Niemira & Cooke, 2010), cabbage (Patel & Sharma, 2010) and sprouts (Fransisca et al., 2011). Examining aggregated *E. coli* O157:H7 on the surface of lettuce using a confocal laser electron microscope, Seo and Frank (1999) found that cells were entrapped and located 20 to 100 µm below the surface of the EPS matrix in stomata and cut edges with preferential colonization of the cut edges.

Sanitation programs have been applied in many food processing industries to decrease the risk of biofilm contamination (Jahid & Ha, 2012), using both physical and chemical approaches (Gibson et al., 1999; Jahid & Ha, 2012). Physical mechanisms include high-pressure spray and mechanical floor scrubbing systems that involve a high degree of mechanical action. These approaches have been reported as the most effective at removing naturally occurring food factory biofilms (Gibson et al., 1999). Chemical disinfectants such as chlorine and hydrogen peroxide have also been reported to effectively reduce bacterial numbers associated with produce (Jahid & Ha, 2012).The efficacy of disinfectants is influenced by several factors including the duration

between contamination and sanitizer application, contact time, internalization of bacteria within produce, temperature, pH, bacterial load, and the maturity of formed biofilms (Jahid & Ha, 2012). Standard sanitizers aim to achieve a 5 log reduction within a contact time of 30 sec on food contact surfaces, while irreversibly inactivating or destroying all specified microorganisms within 10 min (Pfuntner, 2011). The efficacy of a disinfectant depends on the type and load of microorganisms that are associated with the produce (Jahid & Ha, 2012). One of the shortcomings of using chlorine is that it can form carcinogenic compounds when used on fresh vegetables (Jahid & Ha, 2012). Therefore, research on other effective biocontrol methods such as bacteriophages to reduce biofilm formation is gaining popularity.

Bacteriophages are viruses that occur in large numbers and are ubiquitous in nature. They are also present in many different food commodities such as cheese, sausages and a variety of ready-to-eat foods (Sharma et al., 2009). Lytic bacteriophages replicate through a lytic cycle followed by cell lysis and liberation of new bacteriophage progeny (Kutter & Sulakvelidze, 2004). Many commercial bacteriophages such as BacteriophageGuard Listex, EcoShield™, and ListShield™ have already been developed for use in the food industry (Taylor et al., 2015). SalmoFresh™, a *Salmonella* bacteriophage cocktail has been certified as Generally Recognized As Safe (GRAS) by the FDA (Intralytix, 2015). Lytic bacteriophages replicate through a lytic cycle by injecting their genome into the host cell after absorption, followed by cell lysis and liberation of new bacteriophage progeny (Kutter & Sulakvelidze, 2004). In this way, bacteriophages can continuously reproduce themselves by attacking host cells and causing bacterial cell lysis in a process known as 'lysis from within' (LI) (Abedon, 2011). The limitation for LI is that most bacteria cease or exhibit low metabolic activities at typical cold storage temperatures which can impede the ability of bacteriophage to complete the lytic cycle (Ferguson et al., 2013). In contrast,

the ‘lysis from without’ (LO) mechanism can kill bacterial cells without completion of the formal lytic cycle, but rather through the adherence of numerous bacteriophage which cause cell wall damage. (Abedon, 2011; Ferguson et al., 2013). When the number of bacteriophages attaching to the cell wall is above a threshold level (MOI > 50), the bacterial cell wall can be destroyed within 10 min by extracellular agents (Abedon, 2011). Through the process of LO, bacteriophage can kill pathogens at lower temperatures (Abedon, 2011).

Another primary concern for using bacteriophages as a tool in food safety is the emergence of bacteriophage-resistant bacteria (Demerec & Fano, 1945). The alteration or deletion of the receptors on the outer membrane of the bacterial cell including *OmpA, OmpC, OmpF* or lipopolysaccharide can enable the bacteria to evade bacteriophage attack (Kudva et al., 1999; Tanji et al., 2004). Because bacteria are likely to develop resistance to only a single bacteriophage, the use of bacteriophage cocktails is often recommended as an approach to delay the development or prevent the emergence of resistant bacteria (Fischer et al., 2013). Bacteriophages have the potential to control biofilms through several mechanisms. Firstly, bacteriophages can rapidly multiply during the lytic cycle and reach numbers that are sufficient to disrupt biofilms. This self-replicating phenomenon has the potential for a single bacteriophage to degrade the biofilm matrix as bacteriophage progeny infect adjacent bacterial cells within the biofilm (Fu et al., 2010). Secondly, some bacteriophages can produce polysaccharide-degrading enzymes that degrade the EPS matrix of the biofilm (Fu et al., 2010).

Bacteriophage can be extremely specific in their host range owing to the structural peculiarities of receptors on bacteria cell surfaces (Rakhuba et al., 2010). Receptors located on the bacterial surface include: pili, flagella, lipopolysaccharide, surface proteins, teichoic acids and capsules (Clokie & Kropinski, 2008a). Lindberg (1973) and Rakhuba et al. (2010) reported that

receptors for Gram-negative bacteria (*E. coli* and *Salmonella*) consist of protein, LPS, and phospholipid complexes. Cations such as Ca^{2+} and Mg^{2+} are important cofactors that bacteriophages require to complete their lytic cycle (Clokie & Kropinski, 2008a). Although the binding of some bacteriophages (i.e., coliphages) with the LPS-protein complex is reversible, when Mg^{2+} acts as a cofactor and interacts with the complex, it can promote irreversible bacteriophage attachment (Rakhuba et al., 2010). In the case of T5 bacteriophage, the receptor has been identified as a lipoprotein molecule with high molecular weight (Douglas, 2013). In T-even group bacteriophages (T2, T4, and T6), absorption to the target host has been found to be impacted by a number of cofactors (Brenner et al., 1962). For example, T4 and T6 bacteriophages require L-tryptophan as a cofactor to unwind the tail fibers from the sheath surface before interacting with the bacterial-host surface (Anderson, 1945; Brenner et al., 1962; Douglas, 2013). Since cofactors are critical for bacteriophage attachment, I also examined the effectiveness of bacteriophage at preventing biofilm formation on lettuce in the presence and absence of Mg^{2+}, Ca^{2+} and L-tryptophan cofactors.

In previous research, pre-treatment with bacteriophages successfully reduced the formation of biofilms on solid surfaces such as Mg^{2+}, Ca^{2+} and L-tryptophan (Curtin & Donlan, 2006; Fu et al., 2010). Curtin and Donlan (2006) reported that bacteriophage cocktails with divalent cations (Mg^{2+} and Ca^{2+}) achieved a 4.47 $\log_{10}$ CFU/cm^2 reduction of catheter-associated *Staphylococcus epidermidis* within biofilms, while Fu et al. (2010) reported a 4.37 $\log_{10}$ CFU/cm^2 reduction of *Pseudomonas aeruginosa* biofilms on abiotic catheter surfaces. Therefore, we hypothesize that STEC bacteriophage in the presence of cofactors has the potential to prevent *E. coli* O157:H7 biofilm formation on intact lettuce and cut surfaces.

The purpose of this research is to determine the effectiveness of an STEC bacteriophage cocktail at preventing biofilm formation when applied with or without Mg^{2+}, Ca^{2+} and L-tryptophan as cofactors to control *E. coli* O157:H7 on the intact and cut surfaces of Romaine lettuce.

4.3. Methods and Material

4.3.1. Bacterial strains and growth conditions

Strains used in the study originated from ground beef, beef cattle or human feces (Table 4-1). Isolates were streaked on SMAC agar (Criterion™ Dehydrated Media, Hardy Diagnostics, Santa Maria, CA, US) plates and incubated at 37 ºC for 24 h. An isolated colony of each strain was inoculated into 10 mL of TSB (Criterion™, Hardy Diagnostics) and incubated at 37 ºC overnight. Culture concentration was adjusted to 10^5 CFU/mL (OD_{600} =~ 0.070) using a spectrophotometer (GENESYS 20, Thermo Fisher Scientific, Inc., Rochester, NY, US).

Table 4-1. *E. coli* O157:H7 strain information.

Serotype	Strain No.	Origin
E. coli O157:H7	EO-122	Bovine feces
E. coli O157:H7	R508	Bovine feces
E. coli O157:H7	1931	Ground beef
E. coli O157:H7	161-84	Human feces

4.3.2. STEC bacteriophage cocktail

The STEC bacteriophage cocktail used for this study contained bacteriophages isolated from beef feces from feedlots in Alberta, Canada (Table 4-2.). Fecal samples (47 cattle per trailer)

were collected every two weeks from May 2013 to August 2013 as described by Stanford et al. (2010). This previous study isolated 37 lytic bacteriophages against non-O157 STEC (O26, O45, O103, O111, O121, O145) and O157:H7. The lytic capability and host range of the bacteriophages was assessed (Niu et al., 2012; Wang et al., 2015) and the STEC bacteriophage cocktail was propagated prior to its employment in the present study. The 10^8 PFU/ml STEC bacteriophage cocktail was diluted (1:10) using sterile deionized water with or without a mixture of cofactors (3mM $MgSO_4$ + 4mM $CaCl_2$ + 15mg/L L-tryptophan) (Curtin & Donlan, 2006; Fu et al., 2010). The ability of the bacteriophage to impede biofilm formation was assessed with (WCO) and without cofactors (NCO).

The STEC bacteriophage cocktail preparations are mixtures of virulent lytic bacteriophages and would not enter the lysogenic phases in prophage status.

Table 4-2. Taxonomy, genome size and morphology of 7 STEC bacteriophage stocks.

Taxonomy		**Serotypes**	**Strain**	**Estimated genome size (kb)**	**Head dimensions (nm)**	**Tail dimensions (nm)**		**References**
Genus	**Family**					**Length**	**Width**	
T4	*Myoviridae*	O26	EC19960464	177	115-117	109-118	21-24	(Wang et al., 2015)
N/A	N/A	O45	EC19940040	N/A	N/A	N/A	N/A	
T5	*Siphoviridae*	O103	EC20010670	126	82-85	178-196	10	
ViI	*Myoviridae*	O111	EC20030053	151	92-95	100-102	16-20	
rV5	*Myoviridae*	O121	EC20040083	140	93	122	17	
O1	*Myoviridae*	O145	EC20020231	68-89	78-79	113-114	17-18	
T5	*Siphoviridae*	O157	R508	108	78	185	8.5	(Niu et al., 2012)

4.3.3. Biofilm formation microplate assay *in vitro*

The *in vitro* biofilm formation assay was conducted in 96 well microplates (Nunc™, Roskilde, Denmark) following Wang et al. (2016) with some minor modifications. Starter cultures for the 4 individual *E. coli* O157:H7 strains were incubated in Luria–Bertani broth (Oxoid™, Basingstoke, UK) without salt (LB-NS) at 37 ºC overnight prior to a 1:100 dilution in 9 mL of LB-NS. Diluted culture (100 µL) of each strain was then transferred into 6 replicate wells. Plates were then incubated at 25 and 37 ºC for 24, 48, or 72 h to enable biofilm development. After incubation, the supernatant was removed from each well and washed three times with 200 µL sterile water. The remaining biofilm attached to each well was fixed with 250 µL of absolute methanol (ACS reagent, ≥99.8%, ACROS Organics, Morris Plains NJ, US) for 15 min. Plates were emptied, air-dried and stained with 200 µL 1% crystal violet (Sigma, St. Louis, MO, US) for 15 min and then washed three times with sterile water prior to air-drying. Biofilm-bound dye was dissolved in 200 µL 33% glacial acetic acid (≥99.5% pure, ACROS Organics), and the optical density at 630nm (OD_{630nm}) was measured using a microplate reader (Model ELx800; BioTek Instruments Inc., Winooski, VT, US).

Based on the biofilm OD_{630nm} result, the 4 *E. coli* O157:H7 strains were classified as no biofilm ($OD \leq OD_c$), weak ($OD_c < OD \leq 2OD_c$), intermediate ($2OD_c < OD \leq 4OD_c$), or strong biofilm ($OD > 4OD_c$) producers (Wang et al., 2016). A cut-off OD (OD_c) of 0.0406 was defined based on it being three standard deviations above the mean values of OD for the negative controls (Stepanović et al., 2000).

4.3.4. STEC bacteriophage prevention of biofilm formation on intact and cut surfaces of lettuce pieces

4.3.4.1. Lettuce surfaces preparation

Bacterial growth and lettuce pieces were prepared as described by Patel and Sharma (2010) and Sadekuzzaman et al. (2017) with some minor modifications. Romaine lettuce (Dole™ pre-packed romaine hearts,) was purchased from local retail markets (Winnipeg, MB, Canada), and the two outer layers of leaves were removed before being washed three times under cold tap water to reduce background flora. For intact surfaces (I), a 2 cm diameter sterilized cork borer was used to cut disc-shaped lettuce pieces. For cut surfaces (C), 2 cm × 0.5 cm rectangular strips were cut from the center vein at the base of the lettuce head with a sterilized scalpel, followed by removing the upper and lower epidermis of the leaf with sterile tweezers. All the prepared lettuce pieces were temporarily stored at 4 ºC in 100×15 mm^2 Petri dishes (FisherBrand®, Pittsburg, PA, US) covered with water-soaked Kimwipes® (Kimberly-Clark Global Sales, Inc., Roswell, GA, US.) to reduce evaporation prior to inoculation. Background microflora on lettuce pieces was reduced by exposing them to UV light in a biosafety cabinet for 1 h.

4.3.4.2. STEC bacteriophage cocktail effectiveness to prevent biofilm formation by 4 individual *E. coli* O157:H7 strains on prepared intact lettuce and cut surfaces.

The STEC bacteriophage cocktail consisted of lytic bacteriophages against non-O157 STEC (O26, O45, O103, O111, O121, O145) and O157:H7. Prepared lettuce surfaces for the treatment groups (n = 288) were submerged in the STEC bacteriophage cocktail (10^7 PFU/ml) with or without cofactors for 2 h at 2, 10, 25 ºC. Subsequently, the controls exposed to *E. coli*

O157:H7, no exposure to phage cocktail n = 48 and treatments (n = 288) were immersed in the culture of 4 *E. coli* O157:H7 strains (10^5 CFU/ml) for 24 h at 2, 10 and 25 ºC to enable biofilm formation on the surface of lettuce. Negative controls (neither treated by bacteriophage nor exposed to *E. coli* O157:H7, n = 48) were also included. Planktonic cells (loose or unattached cells) associated with the samples were removed by two washes with sterile water. To determine the number of attached cells, previously washed lettuce samples were placed in sterile bags (Whirl-Pak®, Nasco, Fort Atkinson, WI, US), with 50 mL of 0.1% peptone water (Difco, Detroit, MI., US) (1:10 dilutions) and stomached (stomacher blender, Interscience, France) for 3 min. Homogenized samples (1.5 mL) were taken from each sterile bag into 2 mL micro-centrifuge tubes. Non-adherent bacteriophages were removed from homogenates by centrifuging (ThermoFisher mySPIN 6, cat. no. 75004061) for 2 min at 12000 × g. The supernatant containing bacteriophages was removed, and the cell pellet was re-suspended in 1.5 mL 0.1% peptone water. Samples were serially diluted from 10^{-1} to 10^{-7} by adding a 1 mL sample from centrifuge tubes to 9 mL diluent (0.1% peptone water) in tubes and spread-plated on SMAC agar for enumeration.

4.3.5. Statistical analysis

Data were analyzed as a completely random design with a split plot arrangement using SAS version 9.2 (SAS Institute Inc., Cary, NC, US). In the whole plot portion two treatments (NCO and WCO) were included, and in the sub-plot, it was assigned to surface (D and I); while temperature (2, 10, and 25 ºC) was in the sub-sub-plot. Thus, treatment, surface, temperature and their interactions were considered as fixed variables. Least square means were separated (F test, $P < 0.05$) using least significant differences generated through the PDIFF option.

A bar chart of the OD values for the biofilm formation assay in microplate wells was established to determine the weak, intermediate and strong biofilm formers. The population as well as the reduction in *E. coli* O157:H7 was determined during the initial exploration of the data. Tables for the interaction of treatment, surface status, and temperatures were constructed based on the SAS output to show the significance of different treatments.

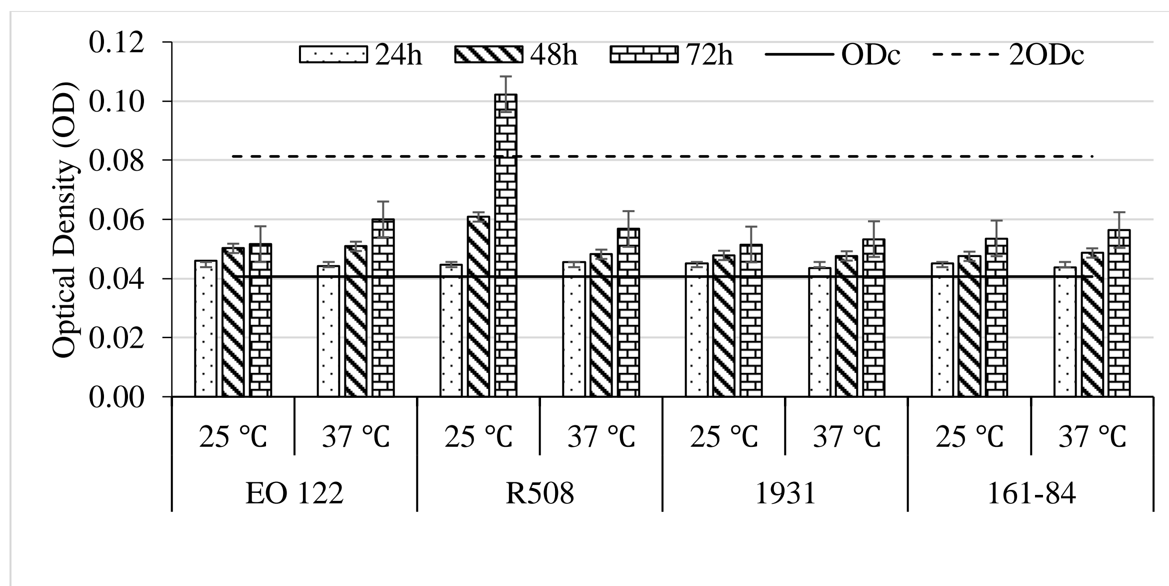
4.4. Result and discussion

4.4.1. Biofilm formation assay

All of the *E. coli* O157:H7 strains examined were capable of forming at least weak biofilms (Figure 4-1.). Strain R508 was the only strain that formed an intermediate biofilm when grown at 25 ℃ for 72 h, exhibiting a stronger ($P<0.0001$) biofilm forming capacity than the other strains (1931, 161-84 and EO122). This finding contrasts with those of Vijay et al. (2015), where Enteroaggregative *Escherichia coli* isolates from humans more readily formed biofilms than isolates from animals. As for our results, although strain 161-84 was isolated from human feces and R508 from bovine feces, strain 161-84 exhibited a weaker biofilm forming capacity than R508.

All 4 strains of *E. coli* O157:H7 more readily ($P<0.0001$) formed biofilms at 25 ℃ than 37 ℃. Nesse et al. (2014) also found that *E. coli* O103:H2 less readily formed biofilms on polystyrene surfaces at 37 ℃ than at 20 ℃. This may contribute to the positive relationship between *E. coli* O157:H7 curli production and the attachment to polystyrene surfaces. Patel et al. (2011) and Prigent-Combaret et al. (2000) reported that the greater curli expression of *E. coli* O157:H7 at 22-26 ℃ can lead to better biofilm formation than at 37 ℃.

Biofilm formation by all *E. coli* O157:H7 strains clearly increased with incubation time (Figure 4-1.). Fouladkhah et al. (2013) also reported that *E. coli* O157:H7 biofilms formed over 7 d were more developed than those formed over a day at 15 and 25 °C. After 72 h, biofilm formation by *E. coli* O157:H7 was greater (P<0.0001) than at 24 or 48 h.



*ODc is the cut off value for OD calculated by three standard deviation above the mean values of OD for the negative controls. 2ODc=ODc×2

Figure 4-1. Biofilm formation by 4 *E. coli* O157:H7 strains on microplates.

4.4.2. STEC bacteriophage cocktail preventing biofilm formation on intact lettuce and cut surfaces

The overall reduction in *E. coli* O157:H7 numbers in biofilms after treatment with bacteriophage with NCO and WCO treatments were 0.76 and 1.33 $\log_{10}$ CFU/cm^2, respectively (Figure 4-2.). To be precise, the average reductions in biofilms as a result of treatment with bacteriophage were 1.5 log for EO122, 1.23 log for R508, 1.20 log for 1931, and 1.26 log for 161-84 (treatment with phage and cofactors-WCO); 0.86 log for EO 122, 0.84 log for R508, 0.58 log for 1931, and 0.75 log for 161-84 (treatment with phage but no cofactors-NCO). The highest

reduction (3.42 $\log_{10}$ CFU/cm^2) was achieved at 25 °C for strain EO122 after pre-treatment with the STEC bacteriophage cocktail and cofactors. The effectiveness of the STEC phage cocktail with cofactors to prevent *E. coli* O157:H7 biofilm formation was substantial, even though the reduction on the surface of lettuce was less than when *P. aeruginosa* phage M4 was used to control *Pseudomonas aeruginosa* on solid abiotic surfaces (4.37 log reduction) (Fu et al., 2010). Because the surface of lettuce is structurally complex and differs substantially from solid abiotic surfaces, it is likely that the mechanism of bacterial attachment and biofilm formation differs between these. The lettuce surface is covered by a hydrophobic cuticle layer, and hydrophobic interactions play a major role in the attachment of bacteria to the lettuce surface (Hassan & Frank, 2003). The junction of lettuce cells has been found to be the main site of colonization of *E. coli* O157:H7 that enter into lettuce cells (Takeuchi & Frank, 2000). Differences in surface chemistry may account for the differential responses observed between abiotic and biotic surfaces.

Effect of cofactors on the effectiveness of STEC bacteriophage cocktail. The overall reduction (0.76 log) in biofilms was lower ($P<0.0001$) for bacteriophage with NCO than for bacteriophage WCO groups (1.33 log) (Tables 4-4.). This can be explained by the fact that cofactors assist bacteriophage with attaching to the surface of the bacterial cell, the first step in biofilm formation (Rakhuba et al., 2010). The cofactor Mg^{2+} enables the bacteriophage to interact with the bacterial cell complex and results in irreversible bacteriophage attachment (Rakhuba et al., 2010). Clokie and Kropinski (2008a) also indicated that bacteriophage require cations such as Ca^{2+} and Mg^{2+} as cofactors to complete their lytic cycle. L-tryptophan is an organic cofactor which has been reported to interact with bacteriophage during activation to enable attachment to the host cell (Anderson, 1945). L-tryptophan can interact with endogenous tryptophan residues on the base plate or tail sheath of bacteriophage, resulting in the active configuration of the tail fiber and

promoting attachment to the bacteria cells (Brenner et al., 1962). Other amino acids have also been shown to enhance bacteriophage activity, but were less effective than L-tryptophan (Storms et al., 2012). With the application of Mg^{2+}, Ca^{2+} and L-tryptophan in WCO groups, bacteriophage more readily prevented the formation of biofilms as compared to NCO groups. After attachment, polysaccharide-degrading enzymes produced by bacteriophages can also help degrade the EPS matrix of the biofilm (Fu et al., 2010). The level of bacterial reduction number in biofilm with WCO and NCO groups for strain EO122 (3.4 and 2.91 logs) and 161-84 (1.61 and 1.52 logs), respectively, did not differ on lettuce at 25 ºC. However, as for strains R508 and 1931, WCO groups had a higher ($P<0.05$) level (2.73 and 2.18 logs) of bacterial reduction in biofilms than NCO (1.44 and 0.86 logs) on intact lettuce surfaces at 25 ºC. These results suggest that the ability of cofactors to enhance bacteriophage activity may differ among strains of *E. coli* O157:H7.

Temperature effect on the effectiveness of STEC bacteriophage cocktail. At 25 ºC all strains exhibited a higher ($P<0.0001$) level of reduction (1.72 $\log_{10}$ CFU/cm^2) as compared to 2 and 10 ºC (0.84 and 0.56 $\log_{10}$ CFU/cm^2; Figure 4-2). Since LO is likely responsible for the destruction of bacteria at 2 and 10 ºC, this might suggest that LO is not occurring efficiently when bacteriophages are pre-applied to produce. When the lettuce pieces were removed from the bacteriophage cocktail, only a few phage particles from the solution may have remained on the lettuce surface. This can present an obstacle to LO as this process requires a large number of bacteriophage (MOI > 50) to attach to the bacteria in order to cause sufficient damage to the bacterial cell wall (Abedon, 2011). Takeuchi and Frank (2000) showed that *E. coli* O157:H7 cells penetrated the surface of cut lettuce to a greater degree at 4 °C than 25 °C. This could reduce the extent to which these pathogens came in contact with bacteriophage, lowering the effectiveness of the cocktail at cold storage temperatures.

Effect of integrity of intact surfaces versus cut surfaces on the effectiveness of STEC bacteriophage cocktail. The overall reduction of bacteria in biofilms on intact and cut surfaces of the lettuce was 1.44 and 1.04 log_{10} CFU/cm^2, respectively (Figures 4-2., 4-3.). This indicated that the effect of bacteriophage pre-treatment at reducing *E. coli* O157:H7 was almost the same, but slightly lower than when the bacterium was associated with the cut versus intact surface of lettuce. These findings agree with those reported by Takeuchi and Frank (2000). The low reduction for both types of surfaces (I and C) could be attributed to the heterogeneous structure of biofilms (Takeuchi & Frank, 2000). Differences in surface area and hydrophobicity of intact vs. cut surfaces can explain the larger attached bacteria population on cut surfaces than intact surfaces. The greater surface area and congregation of *E. coli* O157:H7 along the edge of cut surfaces can result in increased attachment of bacteria (Patel et al., 2011). Therefore, the number of *E. coli* O157:H7 attaching to cut edges was greater than on intact surfaces. Previous studies reported a positive relationship between surface hydrophobicity (surface free energy) and the degree and strength of attachment of bacteria to abiotic surfaces (Gallardo-Moreno et al., 2002; Ukuku & Fett, 2002). Factors such as protein/polysaccharide ratio and the orientation and type of hydrocarbons on cell envelopes can affect cell surface hydrophobicity to a large extent (Hassan & Frank, 2004). It seems probable that the lower hydrophobicity of the waxy intact surfaces of the cuticle lowers the attachment strength of *E. coli* O157:H7 as compared to the hydrophilic cut edges (Hassan & Frank, 2003; Takeuchi & Frank, 2000). In this way, more loosely attached *E. coli* O157:H7 cells with weak attachment strength are on intact surfaces which can be easily washed off. These factors would partially explain the larger bacterial populations observed on control and treatment groups of cut surfaces compared to intact surfaces. Moreover, the release of nutrients from damaged plant cells would increase the growth of bacteria on cut surfaces.

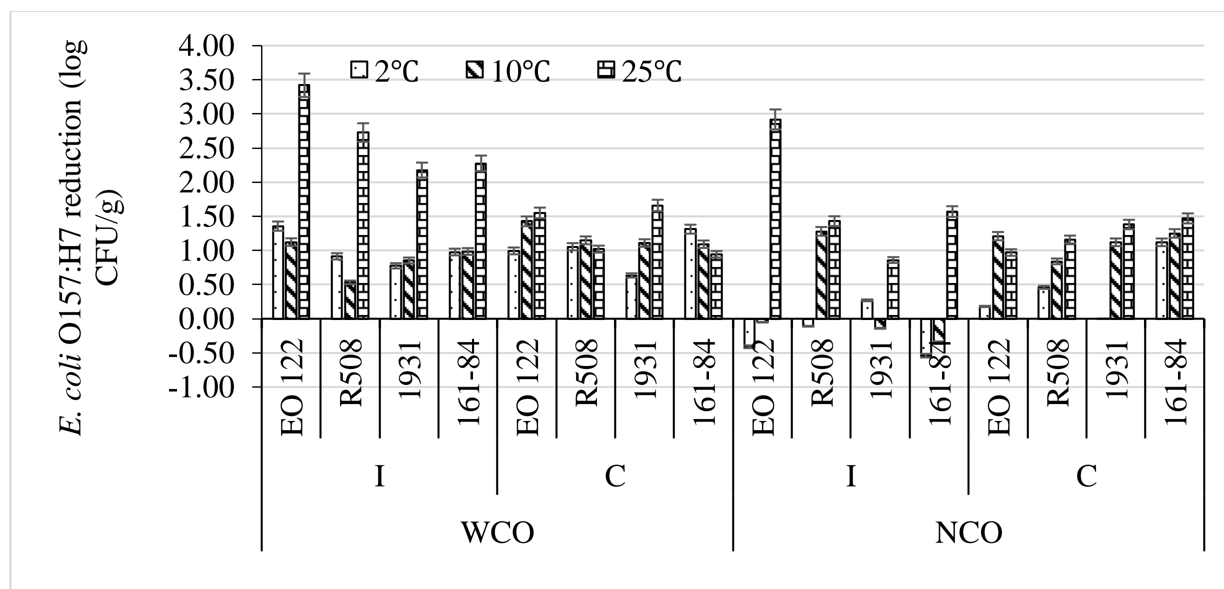


Figure 4-2. Reduction in *E. coli* O157:H7 numbers in biofilms on the surface of intact (I) and cut (C) lettuce after treatment with bacteriophage plus cofactors (WCO) and without (NCO) cofactors at different temperatures.

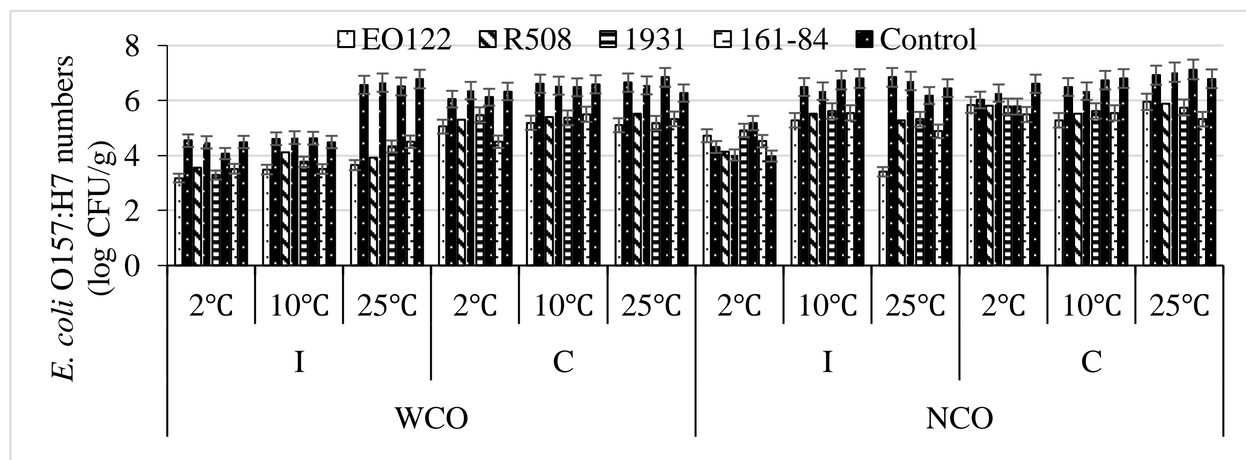


Figure 4-3. *E. coli* O157:H7 population for positive control and treatment groups when applying bacteriophage with cofactors (WCO) and bacteriophage with no cofactors (NCO) to prevent biofilm formation at different temperature on intact (I) and cut (C) lettuce surfaces.

Table 4-3. The effect of interaction of STEC bacteriophage cocktail treatment × temperature × surface on log reduction (log_{10} CFU/g) of *E. coli* O157:H7 strain EO122 (P = 0.0012), R508 (P = 0.0004), 1931 (P = 0.04).

EO122				
Temperature	WCO		NCO	
	I	C	I	C
2 ℃	$1.36\pm0.15^{a,d,h}$	$0.99\pm0.15^{a,d,h}$	$-0.4\pm0.26^{a,d,g}$	$0.18\pm0.26^{a,d,g}$
10 ℃	$1.12\pm0.15^{a,d,h}$	$1.43\pm0.15^{ab,d,g}$	$-0.21\pm0.26^{a,d,g}$	$1.21\pm0.26^{b,e,g}$
25 ℃	$3.4\pm0.15^{b,d,g}$	$1.55\pm0.15^{b,e,g}$	$2.91\pm0.26^{b,d,g}$	$0.97\pm0.26^{b,e,g}$
R508				
Temperature	WCO		NCO	
	I	C	I	C
2 ℃	$0.92\pm0.14^{a,d,h}$	$1.05\pm0.14^{a,d,h}$	$-0.11\pm0.24^{a,d,g}$	$0.46\pm0.24^{a,d,g}$
10 ℃	$0.53\pm0.14^{a,d,h}$	$1.15\pm0.14^{a,e,g}$	$1.28\pm0.24^{b,d,g}$	$0.84\pm0.24^{ab,d,g}$
25 ℃	$2.73\pm0.14^{b,d,h}$	$1.02\pm0.14^{a,e,g}$	$1.44\pm0.24^{b,d,g}$	$1.16\pm0.24^{b,d,g}$
1931				
Temperature	WCO		NCO	
	I	C	I	C
2 ℃	$0.78\pm0.13^{a,d,g}$	$0.63\pm0.13^{a,d,h}$	$0.27\pm0.22^{ab,d,g}$	$0\pm0.22^{a,d,g}$
10 ℃	$0.86\pm0.13^{a,d,h}$	$1.11\pm0.13^{b,d,g}$	$-0.14\pm0.22^{a,d,g}$	$1.12\pm0.22^{b,e,g}$
25 ℃	$2.18\pm0.13^{b,d,h}$	$1.66\pm0.13^{c,e,g}$	$0.86\pm0.22^{b,d,g}$	$1.38\pm0.22^{b,d,g}$

[*a, b] distinct letters for different temperatures with the same surface and bacteriophage treatment, indicate differences (P<0.05)

[*d, e] distinct letters for different surface at the same temperature and bacteriophage treatment, indicate differences (P<0.05)

[*g, h,] distinct letters for different bacteriophage treatment with the same surface and temperatures, indicate differences (P<0.05)

Table 4-4. The effect of interaction of STEC bacteriophage cocktail treatment × temperature ($P<0.0001$) and surface × temperature ($P<0.0001$) on log reduction of strain 161-84.

161-84		
Temperature	WCO	NCO
2 ℃	$1.14 \pm 0.12^{a,e}$	$0.29 \pm 0.20^{a,d}$
10 ℃	$1.03 \pm 0.12^{a,e}$	$0.44 \pm 0.20^{a,d}$
25 ℃	$1.61 \pm 0.12^{b,d}$	$1.52 \pm 0.20^{b,d}$
Temperature	I	C
2 ℃	$1.22 \pm 0.17^{f,k}$	$0.22 \pm 0.17^{f,j}$
10 ℃	$1.17 \pm 0.17^{f,k}$	$0.32 \pm 0.17^{f,j}$
25 ℃	$1.2 \pm 0.17^{f,k}$	$1.92 \pm 0.17^{g,j}$

*a, b: distinct letters for different temperatures in the same bacteriophage treatment, indicate differences ($P<0.05$).
*d, e: distinct letters for different bacteriophage treatments at the same temperature, indicate differences ($P<0.05$).
*f, g, h: distinct letters for different temperatures with the same surface states, indicate differences ($P<0.05$).
*j,k: distinct letters for different surface states at the same temperature, indicate differences ($P<0.05$).

4.5. Conclusion

All the tested *E. coli* O157:H7 strains had weak to intermediate biofilm-forming potential. Biofilm formation was similar for all 4 strains of STEC, while R508 had a relatively greater biofilm-forming ability.

The pre-treatment of the STEC bacteriophage cocktail in the WCO group achieved a greater reduction of attached *E. coli* O157:H7 (1.33 $\log_{10}$ CFU/cm^2) compared to the NCO group (0.75 $\log_{10}$ CFU/cm^2). The highest reduction (3.42 log reduction) was obtained with EO122 on the surface of intact lettuce and when the STEC bacteriophage cocktail was applied with cofactors.

In commercial produce settings, the pathogen contamination level caused by cross-contamination is usually low. Since the WCO group can reduce the size of biofilms developed by 1.33 $\log_{10}$ CFU/cm^2. This makes it necessary to conduct a future test with a lower concentration of bacterial culture on lettuce surfaces for biofilm formation.

Although the STEC bacteriophage cocktail with cofactors can prevent *E. coli* O157:H7 biofilm formation on lettuce surfaces to some extent, the overall reduction of bacterial levels (1.33 logs) was much lower than the 5 log sanitation standard (Pfuntner, 2011). Therefore, further research needs to focus on how to improve the effectiveness of bacteriophage in preventing biofilm formation. Conventional disinfectants (i.e., chlorinated water) can be applied in combination with bacteriophage and cofactors to achieve higher levels of bacterial reduction. Moreover, the interaction between bacteria and the lettuce surfaces when forming biofilms also needs more research for a better understanding of the bacterial attachment mechanism.

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5. CHAPTER 5. CONCLUSION AND SUMMARY

Through the production of a range of virulence factors, Shiga-toxigenic *E. coli* (STEC) and *Salmonella* can cause severe foodborne diseases (Cliver et al., 2011; Majowicz et al., 2014). STEC and *Salmonella* are the causative agents of both outbreaks and sporadic cases of human illness ranging in severity from mild intestinal discomfort to hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) (Majowicz et al., 2014; Padola & Etcheverría, 2014). Fresh produce related food safety problems caused by pathogens are a serious health threat throughout the world. Over the last decade in North America, most produce linked foodborne-outbreaks were associated with *Salmonella* and STEC (Bryan et al., 2015; Lynch et al., 2009). My study has demonstrated that bacteriophage cocktails show promise as a biocontrol intervention against these pathogens in commercial fresh lettuce and sprouts, but less effective when applying to mung bean seeds and germinated sprouts.

The first objective of this research was to test the effectiveness of SalmoFresh™ at eliminating spot-inoculated *Salmonella* on lettuce pieces (3×3 cm^2) and sprouts. The overall reduction in *Salmonella* was 0.76 for lettuce and 0.83 for sprouts, while the highest reduction was 1.04 log_{10} CFU/g (2 ℃, 72 h) for lettuce and 1 log_{10} CFU/g (25 ℃, 48 h) for sprouts when SalmoFresh™ was spray-applied to the samples (MOI = 1). This result was similar to that previously reported where a 1 log_{10} CFU/leaf reduction of *E. coli* O157:H7 was obtained at 4 °C for spot-inoculated leafy greens after a 10 min exposure to a mixture of bacteriophage specific for 8 *E. coli* O157:H7 strains (Viazis et al., 2011). Another research group reported a higher reduction (1-2 log_{10} CFU/g) of *E. coli* O157:H7 on fresh-cut spot-inoculated lettuce surfaces after a lytic bacteriophage cocktail was applied and the produce was stored at 4 °C for up to 7 d (Sharma et al.,

2009). The low reduction in *Salmonella* after application of SalmoFresh™ showed that the *Salmonella* reduction on lettuce and sprouts was virtually negligible when phages were applied using sprayers.

The second objective of the research was to test the effectiveness of SalmoFresh™ alone or in combination with an STEC bacteriophage cocktail to reduce immersion-inoculated *Salmonella* on the surface of lettuce, sprouts, and mung bean seeds. For this part of the study, bacteriophages were delivered by immersion as it was hypothesized that this would be a more effective method of delivering bacteriophage to adulterated lettuce. Immersion also more closely resembles the industry practices used to wash leafy green vegetables, as the entire produce surface is exposed to antimicrobial treatment, whether it is phages or chlorinated water. When SalmoFresh™ was applied alone or in combination with an STEC bacteriophage cocktail, the data showed an overall reduction of 2 to 4 log CFU/g, 1 to 3 log CFU/g and 0.2 log CFU/g for lettuce, sprouts, and mung bean seeds, respectively. Ferguson et al. (2013) reported a 1.32 log reduction when contaminated fresh-cut lettuce samples were washed with a cocktail of three lytic phages specific for *E. coli* O157:H7. As for sprouts, Yang et al. (2013) reported a 3.41 log reduction in *Salmonella* on mung bean sprouts 4 d after treatment with a lytic *Salmonella* bacteriophage cocktail. Samples treated with SalmoFresh™ in combination with STEC bacteriophage cocktail, and 150 ppm chlorinated water achieved the highest reduction for both lettuce and sprouts (3.81 and 2.69 logs, respectively). When applying SalmoFresh™ alone (lettuce: 2.41 log reduction; sprouts: 2.16 log reduction) or in combination with STEC bacteriophage (lettuce: 3.48 log reduction; sprouts: 2.36 log reduction), reductions were similar or more effective than those obtained by chlorinated water alone (lettuce: 2.41 log-reduction; sprouts: 1.83 log reduction). Results indicated that the combination of the two bacteriophage cocktails plus traditional

disinfectant (chlorinated water) are more effective at reducing *Salmonella* on fresh produce surfaces. Another factor that must be considered for future research is the number of bacteria inoculated. Since the practical post-harvest pathogen cross-contamination level in food processing industries is usually low on fresh produce, conducting studies with low levels of pathogen inoculum would create a more realistic scenario. Researchers have reported that when the pathogen contamination level is lower than 4 log_{10} CFU/ml, treatment with bacteriophage combined with other interventions (i.e., organic acid) can reduce *Salmonella* to as low as < 1 log_{10} CFU/mL (Hara-Kudo & Takatori, 2011; Viazis et al., 2011).

For mung bean seeds, the overall effect of all treatments was not significant ($P = 0.2395$), although the highest reduction (2.28 log) was observed for the group treated only with SalmoFresh™. When the adulterated seeds were germinated, up to 10^4 CFU/g *Salmonella* were recovered. Ye et al. (2010) reported a similar result where mung beans inoculated with *Salmonella* and treated with *Salmonella*-infecting bacteriophages still maintained a population of 6 log CFU/g. The recovery of high *Salmonella* levels from sprouted seeds reflects the possibility the pathogen avoided the control measures through internalization in sprouts. Pathogens can enter the plant through natural (i.e., stomata, lenticels, sites of lateral root emergence) or damaged openings (i.e., cuts) on the plant, a process that is enhanced if *Salmonella* travels in water that enters into internal plant tissues (Deering et al., 2012). Other researchers have documented the internalization of *Salmonella* through stomata in lettuce leaves by flowing with washing water (Erickson et al., 2010; Li et al., 2008). There is little information regarding the internalization of foodborne pathogens on fresh produce and therefore further research focusing on preventing the internalization of pathogens in lettuce during the washing step is necessary. Moreover, research regarding the application of lytic bacteriophage during soaking and germination of seeds is needed to kill

foodborne pathogens before internalization into leaves or seeds occurs. Since cofactors can improve the effectiveness of bacteriophage by enhancing their attachment to bacterial cells, they should be applied in consort with phage.

Biofilms are a growing concern for the food processing industry. *E. coli* O157:H7 is also a critical pathogen related to foodborne illnesses as the organism can persist in many foods and produce biofilms (Fouladkhah et al., 2013). The ability of *E. coli* O157:H7 to form biofilms both on the surface and inside plant tissues enhances the persistence of these pathogens in fresh produce (Sadekuzzaman et al., 2017; Steenackers et al., 2012). Therefore, identifying effective bacterial control methods that disrupt biofilms on the surface of produce is an important approach to reducing outbreaks caused by *Salmonella* and STEC (Parish et al., 2003).

The third objective of the research was to use STEC bacteriophage cocktail alone or in combination with cofactors (Mg^{2+}, Ca^{2+} and L-tryptophan) to prevent *E. coli* O157:H7 (strain EO122, R508, 1931 and 161-84) attachment and biofilm formation on intact and cut lettuce surfaces. Cations such as Ca^{2+} and Mg^{2+} are important cofactors that bacteriophages require to complete their lytic cycle (Clokie & Kropinski, 2008a). Although the binding of some bacteriophages (i.e., coliphages) with LPS-protein complex is reversible, Mg^{2+} as a cofactor can interact with the complex and promote irreversible bacteriophage attachment (Rakhuba et al., 2010). In T-even group bacteriophages (T2, T4, and T6), absorption to the target host has been found to be impacted by a number of cofactors (Brenner et al., 1962). Therefore, bacteriophage applied together with cofactors such as Mg^{2+} and Ca^{2+} and L-tryptophan can aid in the attachment of phage to cell surfaces and therefore improve the ability of bacteriophage to prevent biofilm development (Rakhuba et al., 2010).

Although the STEC bacteriophage cocktail with cofactors can prevent *E. coli* O157:H7 biofilm formation on lettuce surfaces to some extent, the overall bacterial number reduction (1.33 logs) in biofilm was much lower than the 5 log sanitation standard (Pfuntner, 2011). Therefore, further research needs to focus on how to improve the effectiveness of bacteriophage in preventing biofilm formation. Conventional disinfectants (i.e., chlorinated water) can be applied in combination with bacteriophage and cofactors to achieve higher levels of bacterial reduction. Moreover, the interaction between *E. coli* O157:H7 and the lettuce surfaces when forming biofilms also needs more research for a better understanding of the bacterial attachment mechanism.

In summary, SalmoFresh™, a 6-strain *Salmonella* bacteriophage cocktail, can be effectively used as a biocontrol intervention to reduce *Salmonella* attached to fresh produce, especially if produce is contaminated by the 15 *Salmonella* serotypes (Typhimurium, Enteritidis, Heidelberg, Newport, Hadar, Kentucky, Thompson, Georgia, Agona, Grampian, Senftenberg, Alachua, Infantis, Reading, and Schwarzengrund.) that SalmoFresh™ targets at (Intralytix., 2015). However, if contamination is caused by other *Salmonella* serotypes other than the targeted ones, SalmoFresh™ may not be as effective. Therefore, hurdle technology needs to be applied together with biocontrol intervention to make sure the produce is safe for consumers. When applied to mung bean seeds and germinated sprouts, SalmoFresh™ did not significantly reduce *Salmonella*. Bacteriophage efficacy was enhanced or kept the same when phages were used in combination with chlorinated water. STEC bacteriophage cocktails can reduce *E. coli* O157:H7 biofilm formation (1 log reduction) when applied with the cofactor.

Further research is necessary to study: 1) fresh produce in large quantities with low bacterial inoculum treated with lytic bacteriophage; 2) to reduce/prevent the issue of pathogen

internalization of contaminated seeds during germination; 3) to improve the potential effect of biofilm control with bacteriophage applied in combination with cofactors.

For the first study, the recommended bacterial culture concentration would be < 3 log CFU/ml treated by 10^8 PFU/ml bacteriophage combined with chlorinated water. The low inoculum level will be closer to the environment of fresh produce processing industries, while the high phage concentration will ensure that the mechanism of 'lysis from without' at low temperatures. The result will be ideal if the final *Salmonella* population can be reduced to less than 1 log_{10} CFU/mL.

For the second study, instead of sterile water, high concentrations of bacteriophage in water could be used to soak the seed during germination. If the bacteriophage lysed pathogen cells during soaking and irrigation, it could be possible for the bacteriophage to kill the bacteria before internalization occurs. Cofactors could also be applied to improve bacteriophage attachment.

For the third study, bacteriophage applied with the cofactors in combination with chlorinated water might have the potential of reducing/preventing biofilm formation on biotic surfaces. Moreover, the interaction between pathogens and the biotic surfaces is also an area that requires critical study.

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