



Use of a phage cocktail to reduce the numbers of seven *Escherichia coli* strains belonging to different STEC serogroups applied to fresh produce and seeds

Yiran Ding¹  | Yuchen Nan¹ | Yang Qiu¹ | Dongyan Niu² | Kim Stanford³  | Rick Holley¹ | Tim McAllister^{1,2,3,4} | Claudia Narváez-Bravo¹

¹Food and Human Nutritional Sciences, University of Manitoba, Winnipeg, Manitoba, Canada

²Ecosystem & Public Health, University of Calgary, Calgary, Alberta, Canada

³Department of Biological Sciences, University of Lethbridge, Lethbridge, Canada

⁴Agriculture and Agri-Food Canada, Lethbridge Research and Development Centre, Lethbridge, Alberta, Canada

Correspondence

Claudia Narváez-Bravo, University of Manitoba, Food and Human Nutritional Sciences, Winnipeg, MB, Canada.
Email: claudia.narvaezbravo@umanitoba.ca

Funding information

Ontario Ministry of Agriculture, Food and Rural Affairs

Abstract

The aims of this research were to evaluate the effectiveness of a phage cocktail at reducing seven Shiga toxin-producing *Escherichia coli* (STEC) serogroups on different food matrices: mung bean sprouts (MBP), lettuce, and mung bean seeds (MBS) and to test the phage cocktail effectiveness to reduce *E. coli* O157 on Romaine and iceberg lettuce. To study the effect of the type of food matrix on the STEC phage cocktail effectiveness, a mixture of seven highly sensitive STEC strains designated as phage propagation strains (PPS) were used to adulterate Romaine lettuce, MBP, and MBS matrices at a concentration of 10^5 logs CFU/g. A subsample of the treated MBS was germinated to assess STEC survival. Recovered STEC strains were confirmed using latex agglutination and PCR. To test the phage cocktail effectiveness to reduce *E. coli* O157:H7 on Romaine and iceberg lettuce, a mixture of four STEC strains (different than phage propagation strains, non-PPS) at both low (10^3 CFU/g) and high (10^5 CFU/g) concentrations were used to spike the samples in scaled up trials for the purpose of potential commercialization. Phage treatments including a combination of STEC phage cocktail and chlorinated water treatment were then applied to lettuce in a simulated scaled-up trial. STEC was assessed on the treated samples at different storage time and temperatures (0, 24, 48, and 72 hr at 2, 10 and 25°C). In the food matrix trial, the combination of STEC phage cocktail and chlorinated water-reduced PPS ($p < 0.001$) STEC on lettuce by 2.1 \log_{10} CFU/g and on MBP by 2.2 \log_{10} CFU/g. However, isolates from all 7 STEC serogroups remained viable after phage treatment in both lettuce and MBP; particularly those associated with serogroup O111, O121, O103, and O145, while only a few colonies of serogroup O26, O45, and O157 were detected. Lettuce adulterated with low levels of 4 non-PPS *E. coli* O157:H7 (10^3 CFU/g) achieved a reduction of 2.6–3.2 logs. While a reduction 1.7–2.3 logs was achieved by the phage cocktail when lettuce was inoculated with 10^5 CFU/g. Overall phage performance was more effective at 2 and 10°C and improved over storage time up to 72 hr. However, for MBS, the

This is an open access article under the terms of the [Creative Commons Attribution-NonCommercial](https://creativecommons.org/licenses/by-nc/4.0/) License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

© 2023 The Authors. *Journal of Food Safety* published by Wiley Periodicals LLC.

phage cocktail was not able to kill any of the STEC populations as all of them recovered during germination.

1 | INTRODUCTION

Shiga toxigenic *Escherichia coli* (STEC) is widely recognized as major causes of foodborne illness (Luna-Gierke et al., 2014; Rangel et al., 2005). *E. coli* O157 was the first *E. coli* serogroup associated with Shiga-toxigenic-related foodborne outbreaks. However, more than 50 non-O157 STEC serogroups have been reported to be responsible for human illness. In North America, the most commonly reported non-O157 STEC are O26, O145, O103, O111, O121 and O145. These STEC serogroups, along with O157, are known as the “top seven” (Fratamico et al., 2017; Heymann, 2008; Luna-Gierke et al., 2014). From 2011 to 2021, the Centers for Disease Control and Prevention (CDC) indicated that pathogenic *E. coli* caused 34 outbreaks, 1,420 cases, and 12 deaths in the United States (CDC, 2022). From these recalls and outbreaks occurring in the last decade, 4 of them have been linked to Romaine lettuce, 5 of them have been linked to leafy greens, and 4 of them were linked with raw clover and alfalfa sprouted seeds. In January 2019, a foodborne outbreak linked to *E. coli* O157:H7 on Romaine lettuce was reported, causing illness in 167 individuals, of which 85 were hospitalized and 15 developed the hemolytic uremic syndrome. In April 2020, *E. coli* O103 in clover sprouts caused illness in 51 people, with 3 hospitalizations (CDC, 2020). This series of outbreaks have impacted the produce industry with implications for international trade as the Canadian Food Inspection Agency requested suppliers to certify that *E. coli* concentrations were below detectable levels or provide proof that the imported lettuce did not originate from counties in the Salinas Valley of California, USA (CFIA, 2020).

The consumption of fresh produce is steadily increasing as consumers seek to decrease meat consumption and increase their intake of dietary fiber (Tobler et al., 2011). As the demand for products has grown, foodborne illness outbreaks have been increasingly linked to contaminated fresh produce (Lynch et al., 2009). This is particularly concerning and difficult to prevent as leafy greens and sprouts are usually consumed raw. Chemical disinfectants suitable for fresh produce, such as chlorinated water, have been shown to be insufficient to eliminate STEC on fresh produce and sprouts (Boyacioglu et al., 2013).

Bacteriophages can control a variety of pathogenic bacteria associated with vegetable and meat products, with some strains approved for use both in Canada and the United States (Moye et al., 2018; Sharma, 2013). Lytic phages kill bacteria by commandeering their metabolic machinery to produce phage progeny that is released through the lysis of the host cell (Abedon, 2011a, 2011b). Phages are highly specific to unique surface lipopolysaccharides and proteins on the surface of host bacteria and are generally considered harmless to humans, animals, or plants (Sharma et al., 2008). Regarding STEC, there are commercial phage cocktails (EcoShield and Finalyse)

approved for their use in food (Jassim & Limoges, 2017). EcoShield showed effectiveness in lettuce by reducing 2.22 log CFU/cm² of *E. coli* O157:H7 when a phage cocktail was sprayed on lettuce (Ferguson et al., 2013). Spraying cattle with Finalyse is approved to reduce *E. coli* O157:H7 contamination before slaughter; the maximum reduction was 1.3 logs at 1 hr (Mies & Davis, n.d.). However, there are still gaps of information regarding phage effectiveness on food products, specifically addressing factors such as temperature, delivery method, contact time, and food type, information on the impact of these variables on phage effectiveness are urgently needed (Hong et al., 2016; Soffer et al., 2017; Zhang et al., 2019). For instance, fresh produce is processed and stored at low temperatures to maintain freshness and extend shelf-life. Low temperature inhibits bacterial growth but may also limit the effectiveness of phages as replication requires the host to be metabolically active (Obeso et al., 2008). However, there is evidence that phages can reduce host cell viability even without the release of phage progeny (Abedon, 2011a, 2011b). Some phages may also remain infective even within the cold chain, although the mechanisms whereby these phages cause host cell death is unclear (Huang et al., 2018). New natural and organic interventions to reduce foodborne pathogens are needed by the fresh produce industry, thus a new phage product capable to effectively reduce the risk of “top 7” STEC contamination will be a novel post-harvest intervention in fresh produce industries. Although phages have been shown to control *E. coli* O157:H7 on produce, there is limited information on the ability of phages to target other STEC serogroups and the effect of the food matrix, temperature, and storage on phage efficacy. The objectives of this research were to: (i) to test individual phage efficacy against STEC strains at various concentrations in an in vitro format; (ii) Evaluate the food matrix effect on a STEC phage cocktail effectiveness to reduce the “top 7” STEC using phage propagation strains (PPS) on Romaine lettuce, mung bean sprouts (MBP), and mung bean seeds (MBS) at 2, 10, and 25°C for 0, 24, 48, and 72 hr; (iii) Compare the effectiveness of a phage cocktail against STEC in combination with chlorinated water; (iv) and to test STEC phage cocktail effectiveness to reduce non-PPS O157:H7 on Romaine and iceberg lettuce at low (10³ CFU/g) and high (10⁵ CFU/g) inoculation levels.

2 | MATERIALS AND METHODS

2.1 | STEC strains and phage propagation

Seven Shiga toxigenic *E. coli* strains were used to propagate the phages, and they are referred to in this paper as phage propagation strain (PPS) (Table 1). These PPS strains were also used to test the effect of the food matrixes (lettuce, sprouts and seeds) on the phage cocktail's effectiveness in reducing STEC. It was hypothesized that by

TABLE 1 STEC phage master stocks.

Phage official designation	Phage type	Phage family	STEC host serotypes used for phage propagation	STEC strains used for phage propagation (PPS)	STEC target (s) serogroup (s)	Phage titer (PFU/ml)
vB_EcoM_AYO26A	T4	Myoviridae	O26:H11	EC19960464	O26, O103, O145, and O157	7.55×10^8
vB_EcoS_AXO45B	T5	New taxonomy Tequintavirus	O45:H2	EC19940040	O45	5.7×10^9
vB_EcoS_AXO103A	T5	Siphoviridae	O103:H2	EC20010670	O26 and O103	2.3×10^9
vB_EcoM_AXO111A	Vil	Myoviridae	O111:NM	EC20030053	O26, O111, and O121,	3.5×10^8
vB_EcoM_AXO121A	rV5	Myoviridae	O121:H19	EC20040083	O103, O121, and O157	1.7×10^{10}
AYO145A	O1	Myoviridae	O145:NM	EC20020231	O145	2.5×10^{10}
vB_EcoS_AKfV33	T5	Siphoviridae	O157:H7	R508	O157	1.6×10^{11}

using PPS strains, which are highly susceptible to the phages, it could be possible to determine the food matrix effect on phage performance. On the other hand, to determine STEC phage cocktail efficacy on lettuce, four *E. coli* O157:H7 strains not used for phage propagation (non-PPS) (1931 from hamburger, 1934 from beef, 162-84 from humans and CO283 from cattle feces) were used. All STEC strains and phages were obtained from the culture collection of the University of Manitoba, Food & Human Nutritional Sciences, Winnipeg, Manitoba and the Alberta Agriculture and Agriculture and Agri-Food Canada in Lethbridge, AB, Canada. Bacterial cultures were maintained in Trypticase soy broth, TSB (BD Difco, Franklin Lakes, NJ) containing 15% glycerol and stored at -80°C . Each culture was thawed and streaked on to fresh TSB and MacConkey agar, MAC (Criterion Dehydrated Culture Media, Hardy Diagnostics, Santa Maria, CA) followed by incubation at 37°C for 24 hr. Bacterial suspensions were prepared by transferring one colony from each STEC serogroup into 10 ml TSB (BD Difco) and incubated overnight at 37°C to a density of 10^8 CFU/ml. The cultures were diluted as needed for phage propagation (10^4 CFU/ml), in an in vitro microplate assay (10^7 CFU/ml), food matrix trials, and scaled-up experiments (10^5 CFU/ml). The concentration of the mixture/individual bacteria culture was tested by reading OD at 600 nm wavelength and confirmed by enumeration on McConkey agar plates.

Phages used for this study were isolated from beef cattle feces from previous studies by Alberta Agriculture and Forestry in Lethbridge (Table 1) (Wang et al., 2015). A total of 37 lytic phages with activity against serotypes O26:H11, O45:H2, O103:H2, O111:NM, O121:H19, O145:NM, and O157:H7 were isolated. Phage genome size was measured by pulsed-field gel electrophoresis and ranged from 38 to 177 kb, and the phages were assigned to families based on morphology as visualized using transmission electron microscopy and genomic sequencing. Host range and lytic capability of the phages were tested using a microplate phage virulence assay at 2, 10, and 25°C . Phage candidates used in this study were selected based on their: (i) lytic activity, (ii) effectiveness over a broad temperature range, (iii) cross-infectivity against multiple strains of the targeted 7 STEC and the absence of unwanted genes (antimicrobial resistance and virulence). Phages were individually propagated as previously

described (Niu et al., 2009) from phage master stock and the phage concentration for each working solution was quantified by soft agar overlay plaque assay (Kropinski et al., 2009). Propagated phages exhibiting titers ranging from 10^8 to 10^{10} PFU/ml were used in this study. The phage cocktail consisted of seven phages (Table 1) and was prepared by mixing 20 ml of each phage filtrate (10^8 – 10^{10} PFU/ml) in a sterile bottle prior to use. The microplate phage virulence assay, as described in the following sections, was then used to measure the multiplicity of infection (MOI) of the phage cocktail.

2.2 | In vitro microplate phage virulence assay

The microplate phage virulence assay was used to determine the lytic activity of STEC phages (Niu et al., 2009). Seven phages were combined and tested as a cocktail against each *E. coli* PPS. Each phage was also tested individually against its PPS (Table 1). Briefly, 20 μl of each lytic phage candidate or phage cocktail were serially diluted (10^{-1} – 10^{-8}) individually in a 96-well microplate (Thermo Fisher Scientific) containing 180 μl TSB supplemented with 10 mM MgSO_4 (1%) (mTSB) per well. Wells were inoculated with 20 μl of 10^7 CFU/ml bacterial culture ($\text{OD}_{600} = 1.1$ – 1.2) of each STEC serogroup. Positive controls with STEC cultures and negative control with only mTSB and mTSB plus STEC phage were included. The microplate was incubated at 2, 10, and 25°C for 5 hr. After incubation, the microplate (25°C) was visually observed for clear wells, and then a microplate reader (BioTek Instruments, Winooski, VT) was used to measure the optical density (OD) at 630 nm of each well. The MOI for each phage-host assay was calculated based on three repetitions with six observations in total ($\text{MOI} = \text{initial phages concentration; PFU/ml}$) of the highest dilution showing lytic activity (highest dilution with clear wells)/initial number of bacteria host concentration (CFU/ml) (Zhang et al., 2019). The experiment was repeated three times, the data were collected from 12 observations for each STEC serogroup at three temperatures.

The microplate virulence assay was effective at showing the effectiveness of bacteriophages to reduce STEC based on the optical density reading at 25°C . However, STEC serogroups are mesophilic and are not able to grow or grow slowly at 2 and 10°C , thus clear

wells were not conclusive. To confirm the STEC survival from microplates incubated at 2 and 10°C, spot plate technique was performed as previously described (Zhang et al., 2019). Briefly, 2 µl of sample from each well from the microplate was transferred to MacConkey agar. Plates were incubated at 37°C for 24 hr and colonies observed were considered as conformation of STEC survival. However, spot plate method only confirmed if STEC was completely killed by the phage at different concentrations; STEC reduction after phage treatment was investigated during in vitro trials in the following sections. Phages were not removed to test the effectiveness of remaining phages particles on STEC during storage.

2.3 | Effectiveness of the STEC phage cocktail on different produce matrixes controlling STEC strains used for phage propagation (PPS)

Romaine lettuce heads and fresh MBP were purchased from a local grocery store and kept at 4°C before use. Outer leaves and the stems were removed from 3 cm the base of the head, and then lettuce leaves were peeled from the head. Whole seed sprouts with cotyledon, stem, and root were selected. On the other hand, all MBS (Mumm's Sprouting Seeds, Shellbrook, SK, Canada) used in this study were visually inspected to ensure they were not damaged.

The lettuce leaves, MBP and MBS were washed three times with tap water pre-chilled at 10°C and excess water was removed using a sterilized salad spinner. They were packaged in plastic bags and kept at 4°C, and MBS were kept at room temperature until needed.

Romaine lettuce, MBP, and MBS matrixes were spiked with a STEC bacterial mixture prepared with PPS strains. The PPS-STECS was delivered by immersion since it was found to be a more efficient method based on pre-testing data (data not shown).

In the food matrix experiment, 600 g lettuce, 300 g MBP, and 30 g of MBS were collected for each trial. Produce was then inoculated by immersion in a 10⁵ CFU/ml of the STEC bacterial mixture for 30 min and excess liquid was removed using a sanitized salad spinner. Prior to treatment with chlorinated water or the phage cocktail, 10 g of leaves, MBP, and MBS that were only inoculated with the STEC mixture were collected and placed into a Whirl-Pak bag as a positive control. After inoculation, samples were then assigned in duplicate to one of three treatments: T1: STEC inoculated (adulterated samples) + chlorinated water wash. T2: STEC inoculated + phage cocktail. T3: STEC inoculated + chlorinated water + rinse + phage cocktail. Commercial liquid bleach containing sodium hypochlorite (Old Dutch®, Montreal, QC, Canada) was used to prepare chlorinated water at 150 (for lettuce and MBP) and 1,000 (for MBS) ppm. The concentration of the chlorinated water was measured using water quality test strips (HACH Co. ETS, Elkhart, IN). For treatments with chlorinated water (T1, T3), lettuce leave and MBP were washed with 10°C chlorinated water (150 ppm), followed by rinsing three times with chilled water to remove residual chlorine, a procedure that mimicked industry practice. MBS were immersed in 1,000 ppm chilled chlorinated water for 3 min and rinsed with chlorine-free water at room temperature.

For treatments with phage cocktail (T2, T3), lettuce and MBP were immersed in the STEC phage cocktail (>10⁸ PFU/ml) for 15 min while MBS were immersed in the phage cocktail for 1 hr at room temperature.

After treatment, 10 g of lettuce and MBP from each treatment was collected and placed in sterile bags (Seward Laboratory Systems, Inc., Bohemia, NY) and stored at 2, 10, and 25°C for 1, 24, 48, and 72 hr. MBS were dried in a biosafety cabinet at room temperature for a minimum of 1 hr and then stored at 25°C for 72 hr in a dark room. Half portion of the treated MBS were germinated to lab germinated (LG) sprouts by soaking in sterile water for 24 hr and then sprayed 2 times/day with sterilized distilled water for 3 days at 37°C. At the end of each storage period, the lettuce, MBP, MBS, and LG sprouts were placed in Whirl-Pak bags with 90 ml BPW and homogenized using a stomacher for 2 min (Intersciences, Inc., Markham, ON, Canada). For treatments with chlorinated water (T1, T3), 90 ml of D/E Neutralizing Broth (BD Difco) were substituted for BPW to neutralize the chlorine. For bacteria enumeration, 10-fold serial dilutions (10⁻¹–10⁻⁶) were prepared for each sample type and plated on Rainbow and MacConkey agar plates. Plates were incubated at 37°C for 24 hr. Colonies from MacConkey plates were counted to determine the total reduction in STEC caused by treatments. To estimate the reductions and survivability for each STEC serogroup, colonies with different colors from Rainbow plates were counted. Five colonies from each color were randomly picked and tested with latex agglutination and confirmed by specific multiplex PCR for their serogroups (DeRoy et al., 2011). Immunomagnetic separation (IMS) was used to recover STEC strains below detectable levels following manufacturer recommendations (Dynabead; Dynal Wirral, UK). All experiments were performed as independent trials and replicated three times.

2.4 | Targeting O157:H7 reduction on Romaine and iceberg lettuce using non-phage proration O157 strains (non-PPS) at high and low inoculation levels

Data obtained from preliminary experiments using PPS strains showed that the STEC phage cocktail was more effective at reducing *E. coli* O157:H7 strain on Romaine lettuce when compared with MBP and MBS. It was also challenging to differentiate STEC serogroups on Rainbow agar, and it required heavy work using serological test and PCR. Therefore, lettuce was chosen as the food matrix to further test the effectiveness of the STEC phage cocktail. For this purpose, a mixture of four strains of non-PPS *E. coli* O157:H7 at high (10⁵ CFU/g) and low (10³ CFU/g) concentrations was used. In this experiments we included Romaine and iceberg lettuce as they are the most popular lettuce types for consumption in North America, 62% of the lettuce production in the United States is the crisp head type (iceberg) (Mou, 2008). Romaine lettuce is often associated with outbreaks, so there is the question regarding potential differences in the two leafy greens concerning STEC predisposition for colonization. It is important to mention that the concentration of STEC contamination found

in fresh produce industry production is likely to be lower than 5 logs, probably in the range of 2–3 logs CFU/g (Althaus et al., 2012). Thus, to test the capability of this phage cocktail at reducing STEC high and low STEC concentrations, 10^5 and 10^3 CFU/g of *E. coli* O157:H7 were chosen to be inoculated on lettuce samples. STEC phage cocktail and lettuce were prepared as described in the previous sections. Commercial liquid bleach containing sodium hypochlorite was used to prepare chlorinated water at 150 ppm (measured using water quality test strips). The phage cocktail was applied through immersion. Four treatments were applied to lettuce leaves samples: T1: STEC inoculated (adulterated samples) + chlorinated water wash. T2: STEC inoculated + phage cocktail. T3: STEC inoculated + chlorinated water + rinse + phage cocktail. T4: STEC inoculated + phage cocktails + chlorinated water + rinse. T4 is introducing a change to the order of the phage intervention, where the STEC phage cocktail was applied on lettuce first, before washing with chlorinated water and finalizing with a fresh cold-water rinse. T4 will show if the chlorinated water wash will interfere with the phage cocktail efficacy. Another reason for added T4, was to test a synergistic antimicrobial effect, if phages cause damage (holes) in the bacterial cell envelope, this could enhance the bactericidal effect of the chlorinated water. While T3, where the chlorinated water wash was applied first, followed by a fresh cold-water rinse and then the phage cocktail was applied, in this case T3 will likely allow the phage to remain in the packaged produce during storage and reduce STEC over time.

After treatment, Romaine and iceberg lettuce leaves were packaged in sterile bags and stored at 2°C for 1, 24, 48, and 72 hr. At the end of each storage period, conventional, and molecular microbiology techniques as previously described in food matrix trial were used for STEC enumeration, screening, and confirmation. All experiments were performed as independent trials and replicated three times.

Samples where *E. coli* O157 was not detected in agar plates were further analyzed for *E. coli* O157 survival via enriching 1 ml of liquid from the stomacher bag in 9 ml of trypticase soy broth (TSB) (BD Difco) for 24–48 hr at 37°C. Any growth observed in TSB was plated on MacConkey plate for *E. coli* O157 detection. Suspected colonies were confirmed using latex agglutination test for *E. coli* O157

serogroup and PCR if required. There was no further step taken to remove the background microflora as MacConkey is a selective and differential media for *E. coli*. In addition, there was no background microflora showing similar phenotype to *E. coli* on MacConkey plates.

2.5 | Statistical analysis

All statistical analyses were conducted using the Statistical Analysis System (SAS), version 9.2. The MOIs from *in vitro* experiments were analyzed following the mixed linear procedure (for fixed and random effects). Data were analyzed using SAS with each produce type analyzed separately. The model used was a completely randomized design with factorial arrangement where the replications of treatments were assigned at random to independent experimental subjects. Treatments, storage time, temperature, and their interactions were considered as fixed effects.

Differences were considered significant where $p < 0.05$. Reduction in *E. coli* population was calculated as the difference between treated and untreated *E. coli* counts. Log reduction = $\log N_0/N$ (CFU/g), where N_0 is the initial population and N is the population at a time interval where sample was collected.

3 | RESULTS

3.1 | In vitro microplate STEC phage virulence assay

For phage prorogation strains, an interaction ($p = 0.003$) between phage lytic activity and temperature indicated that lytic activity against STEC improved as temperature increased. At 25°C, both individual STEC phages and the phage cocktail were effective against targeted host(s), with at least a 5 \log_{10} CFU/ml reduction in host concentrations. Five phages (AYO26A, phage O45, AXO103A, AXO121A, and AKFV33) were able to eliminate their targeted STEC host when they were individually tested at 25°C (Table 2). More

TABLE 2 STEC survival as detected using a spot-plate method after treatment with individual phage or a phage cocktail at 2, 10, or 25°C for 5 hr in vitro.

	Temperature (°C)	STEC serogroups						
		O26	O45	O103	O111	O121	O145	O157
Individual. Phage	2°C	N	D	N	D	D	N	N
	10°C	N	D	N	D	D	N	N
	25°C	N	N	N	D	N	D	N
Phage cocktail	2°C	N	D	N	D	D	N	N
	10°C	N	D	N	D	D	N	N
	25°C	N	N	N	D	D	D	N

Note: One phage was used against its target serogroup at each temperature for treatment with individual phage. Phage cocktail was mixed with 20 μ l of phage AKFV33, AYO26A, phage O45, AXO103A, AXO111A, AXO121A, and AYO145A at high titer ($>10^8$ PFU/ml). Abbreviations: D, STEC survival detected from spot plating; N, No STEC survival detected from spot plating.

details related to OD reading for microplate incubated at 25°C can be found in Figure S1. At 2 and 10°C, there was no detectable growth of STEC after 5 hr, and as a result OD was not a helpful indicator of phage effectiveness. Consequently, the spot plate technique was used at lower temperatures and showed that 4 STEC serogroups (O26, O103, O145, and O157) were eliminated when exposed to the phage cocktail or their individual targeted phages at 2 and 10°C (Table 2). Phages O45 and AXO121A failed to completely kill their PPS host at 2 and 10°C, whereas bacteria survivals from serogroup O111 were observed at any of the temperatures evaluated. Bacterium survivors detected through spot plate technique indicated phage 45, AXO121A, and AXO111A are less effective compared with other phages. They were effectively reducing their PPS host but could not eliminate them. It was found that the overall MOI was higher at 2 and 10°C (MOI = 5.5×10^0 PFU/CFU) than at 25°C (MOI = 2×10^{-2} PFU/CFU) ($p = 0.001$) for STEC O26:H11, O111:NM, O121:H19, O145:NM, and O157:H7 (Table 3). The higher MOI indicated that a larger number of phage particles (both as individual and/or phage cocktail) were needed to eliminate STEC (10^7 CFU/ml) at temperatures lower than 25°C.

Regarding non-PPS strains, *E. coli* O157:H7 strains 1931, 161-84, 1934 and CO-238 (Table 4), had MOI >100 after being treated with phages O45, AXO103A, and AXO111A; those three phages were not effective against the four strains tested in this study. Phage AKFV33 was the most effective, as it had activity against all four tested O157 strains at all incubation temperatures. Phage AYO26A and AXO121A were effective against strains 161-84, 1934, and CO-283 at all tested temperatures (2, 10, 25, and 37°C), and effective in reducing *E. coli* O157 (1931) at 25 and 37°C (Table 4). Phage AYO145A was only effective at 25 and 37°C, which could help in conditions of temperature abuse. Overall, the MOI for AKFV33 ranged from 2 to 2×10^{-6} . Phage AYO26A and AXO121A were more effective against O157:H7 strains 161-84, 1934, and CO-238 (MOI = 40-53, 4-50, respectively). Interestingly strain 1931 was insensitive to AYO26A at 2 and 10°C. In contrast, phage AYO145A exhibited lytic activity against all 4 *E. coli* O157:H7 strains only at 25 and 37°C. In general, the MOI was higher for phages at 2 and 10°C than at 25 and 37°C. Overall, the MOI of phage AKFV33 for all 4 non-PPS (MOI = 1×10^{-2} to 19) was

TABLE 3 Multiplicity of infection (MOI) of STEC phages against specific PPS STEC strains (PFU/CFU) effected by temperature.

STEC strains	Temperature (°C)		
	2	10	25
O26:H11	3.4×10^9	3.4×10^{1a}	2×10^{-2b}
O45:H2	4×10^{-2a}	4×10^{-2a}	4×10^{-2b}
O103:H2	8×10^{-2a}	8×10^{-2a}	9×10^{-2b}
O111:NM	2.9×10^{0a}	2.9×10^{0a}	2×10^{-2b}
O121:H19	5×10^{-1a}	5×10^{-1a}	5×10^{-4b}
O145:NM	4×10^{-1a}	8×10^{-1a}	4×10^{-4b}
O157:H7	8×10^{-1a}	7×10^{-1a}	4×10^{-4b}

Note: Means in a row with different superscripts differ, p value = 0.01.

higher than the MOI for the O157:H7 strain (R508) used for phage propagation (MOI = 4×10^{-4} to 8×10^{-1}) at 2, 10 and 25°C, except for CO-238 at 10°C (Tables 3 and 4).

3.2 | Effectiveness of STEC of phage cocktail on produce matrixes using phage propagation (PPS) STEC strains

Use of the phage cocktail (T2) to reduce STEC on produce resulted in the highest reduction for MBP ($1.71 \log_{10}$ CFU/g), followed by MBS

TABLE 4 Multiplicity of infection (MOI) of STEC phages required for lysis of 4 non-PPS *E. coli* O157:H7 strains (PFU/CFU) effected by temperature.

Phage strain ID	2°C	10°C	25°C	37°C
1931				
AYO26A	R	R	2	3
O45	R	R	R	R
AXO103A	R	R	R	R
AXO111A	R	R	R	R
AXO121A	R	R	20	30
AYO145A	R	R	1×10^{-1}	1×10^{-4}
AKFV33	21	19	1×10^{-2}	2×10^{-6}
161-84				
AYO26A	40	42	2	2
O45	R	R	R	R
AXO103A	R	R	R	R
AXO111A	R	R	R	R
AXO121A	17	25	15	9×10^{-4}
AYO145A	R	R	1×10^{-1}	6×10^{-3}
AKFV33	3	2	2×10^{-2}	2×10^{-6}
1934				
AYO26A	44	47	2	9
O45	R	R	R	R
AXO103A	R	R	R	R
AXO111A	R	R	R	R
AXO121A	4	14	12	2×10^{-4}
AYO145A	R	R	2×10^{-1}	2×10^{-5}
AKFV33	11	17	1×10^{-2}	2×10^{-6}
CO-283				
AYO26A	45	53	5×10^{-1}	5
O45	R	R	R	R
AXO103A	R	R	R	R
AXO111A	R	R	R	R
AXO121A	26	50	1	6×10^{-4}
AYO145A	R	R	1×10^{-1}	4×10^{-6}
AKFV33	2	4×10^{-2}	2×10^{-2}	5×10^{-6}

Note: R indicates the STEC strain is resistant to phage, MOI >100.

(1.69 \log_{10} CFU/g), and lettuce (1.56 \log_{10} CFU/g). However, STEC reduction decreased from 1.69 (MBS) to 0.61 (LG sprouts) \log_{10} CFU/g, which indicates the phage cocktail was less effective in controlling STEC during germination (Figure 1).

Significant differences were found between treatments for total STEC reduction ($p < 0.0001$) (Figure 1). The combination of chlorinated water with phage cocktail treatment (T3) achieved the highest overall reductions in STEC total counts on lettuce and sprouts with 2.1 and 2.2 \log_{10} CFU/g reductions respectively. The application of only the STEC phage cocktail (T2) achieved 1.6 \log_{10} CFU/g reductions on lettuce and 1.7 \log_{10} CFU/g on MBP, which was not as effective at reducing STEC as T3. The STEC phage cocktail alone (T2) showed the highest reduction (1.7 \log_{10} CFU/g) of STEC on MBS. In comparison, the reductions were lower in beans treated with 1,000 ppm chlorinated water (T1) (1.2 \log_{10} CFU/g) and the combination of phage cocktail and chlorinated water (T3) (1.5 \log_{10} CFU/g). In LG sprouts, regardless of the treatment, the reductions in total STEC numbers were less than 1 \log_{10} CFU/g. Although the STEC phage

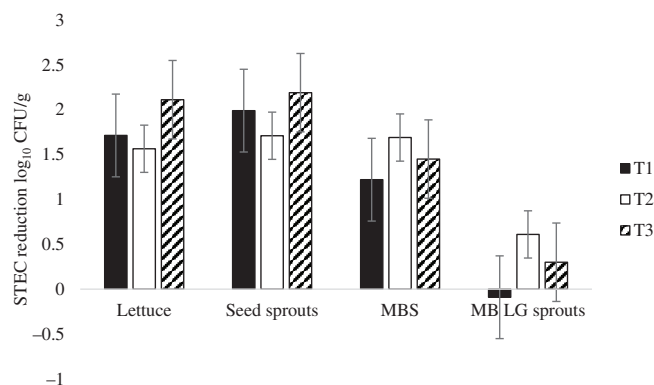


FIGURE 1 STEC reduction (\log_{10} CFU/g) on lettuce, mung bean sprouts (MBP), MBS, and LG sprouts immersed in 10^5 CFU/ml STEC after treatment with chlorinated water (T1); STEC phage cocktail (T2); STEC phage cocktail plus chlorinated water (T3) ($p < 0.0001$), 150 ppm chlorinated water was used for lettuce and MBP, 1,000 ppm chlorinated water was used for MBS.

cocktail was effective against all STEC serogroups on MBS either alone or when combined with 1,000 ppm chlorinated water wash, it did not prevent the regrowth of STEC during germination. After germination, none of the treatments was able to reduce more than 0.6 logs of STEC on lab germinate sprouts (Figure 1).

While 5 out of 7 STEC serogroups were eliminated *in vitro* (STEC O26, O45, O103, O121, and O157), isolates from all 7 STEC serogroups remained viable after phage treatment in food matrixes trials. There were no significant differences observed between treatments in lettuce ($p = 0.82$) (Figure 2a). In MBP, serogroups O111, and O121 were the dominating serogroups surviving from the treatments, while STEC O26, O45, O103, O145, and O157 were found in relatively lower numbers (<10 colonies) on plates, and treatment with phage cocktail was able to reduce 3.1–5.2 \log_{10} CFU/g of those serogroups by itself or when combined with chlorinated water. The reduction (\log_{10} CFU/g) of STEC after treatments was higher than the initial inoculation concentration, indicating the treatments effectively reduced the STEC number to low/undetectable levels, where the STEC number in positive control grew greater than inoculation concentrations during the storage period. The combination treatment was the most effective ($p < 0.001$), reducing 4/7 serogroups (O26, O45, O111, O145) in MBP (Figure 2b).

In MBS, there was a significant difference ($p < 0.05$) among treatments in their ability to reduce numbers of serogroups O157, O26, O111, O121, and O145 (Figure 3a), but no significant difference ($p > 0.05$) were found among treatments for control of O45 and O103. STEC phage cocktail alone (T2) was more effective against serogroups O157, O26, and O145 ($\geq 3 \log_{10}$ CFU/g), while less effective for control of O111 and O121. Chlorinated water alone (T1) was the most effective at reducing serogroup O121, with a 3.7 \log_{10} CFU/g reduction. The highest reduction of serogroup O111 was 2.74 \log_{10} CFU/g with STEC phage cocktail combined with chlorinated water treatment (T3).

Serogroup O26, O103, O121, O145, and O157 isolates were the principal survivors in LG sprouts; particularly for serogroups O26 and O103, where there was low/no reduction either by the STEC phage cocktail, chlorinated water, or the treatment combination (Figure 3b).

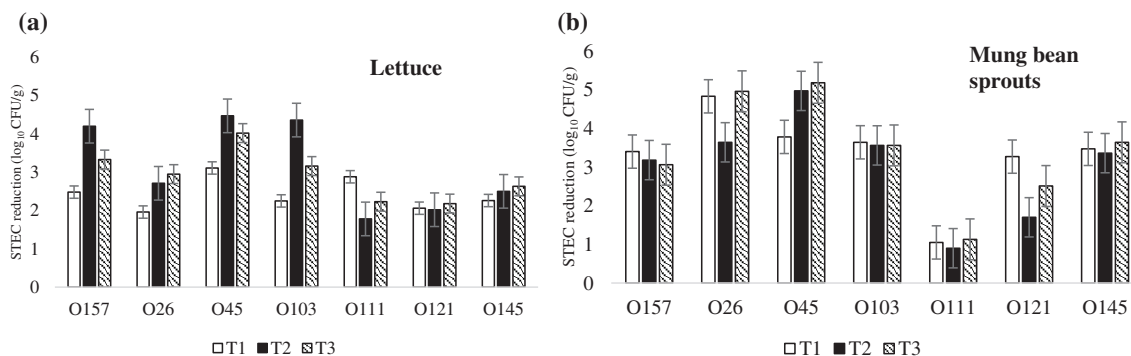


FIGURE 2 (a, b) STEC serogroup reductions on lettuce ($p = 0.82$); and mung bean sprouts ($p < 0.001$) adulterated through immersion with 10^5 CFU/ml STEC and treated with 150 ppm chlorinated water (T1); STEC phage cocktail (T2); STEC phage cocktail plus 150 ppm chlorinated water (T3).

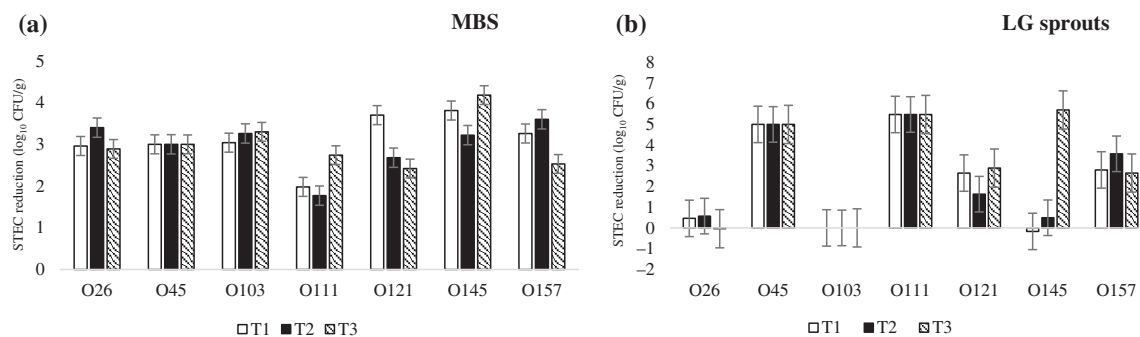


FIGURE 3 (a, b) STEC serogroup reductions on MBS; and LG sprouts adulterated through immersion with 10^5 CFU/ml STEC and treated with 1,000 ppm chlorinated water (T1); STEC phage cocktail (T2); STEC phage cocktail plus 1,000 ppm chlorinated water (T3) ($p < 0.05$). SEROGROUPS O45 and O111 were below the countable range in LG sprouts after all treatments.

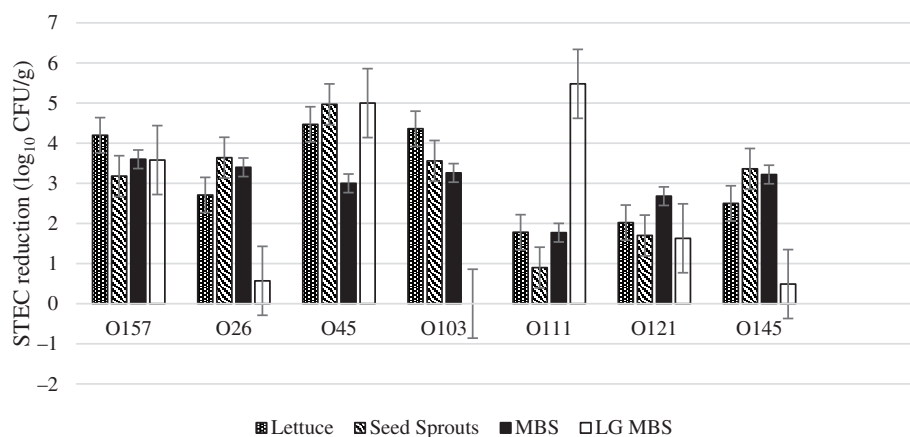


FIGURE 4 Individual STEC strain reduction (Log_{10} CFU/g) in lettuce, mung bean sprouts (MBP), MBS, and LG MBP inoculated with 10^5 CFU/ml STEC bacteria followed by immersing with a phage cocktail regardless storage temperature and time.

Serogroups O45 and O111 were below the countable range after all treatments, but they were detected through PCR/IMS, indicating that a $5 \log_{10}$ CFU/g reduction was achieved.

The phage cocktail was more effective at reducing STEC O157, O26, O45, O103, and O145 (2.5 – $5.0 \log_{10}$ CFU/g) on lettuce and MBP than serogroups while poorly effective on O111 O121 ($\leq 2 \log_{10}$ CFU/g), which is the opposite to MBS and LG sprouts, where phages were most effective at reducing STEC O121 on MBS ($3.4 \log_{10}$ CFU/g), and O111 on LG sprouts ($5.5 \log_{10}$ CFU/g) (Figure 4).

3.3 | Effectiveness of a phage cocktail treatment (T2) at controlling phage propagation STEC strains (PPS) on produce stored for different periods and temperatures

3.3.1 | Lettuce

A significant temperature-by-storage time interaction ($p < 0.0001$) was found in lettuce. The phage cocktail was more effective at controlling total numbers of STEC at 2 and 10°C than 25°C (Table 5). The highest reduction achieved in STEC total numbers was $2.8 \log_{10}$ CFU/g at 2°C after 72 hr. During storage, the reduction achieved by the STEC phage cocktail was increased at 2°C and remained relatively

TABLE 5 Effect of temperature \times time interaction (SEM 0.25; p value < 0.0001) on reduction of total numbers of STEC as a result of immersion of lettuce/sprouts in a phage cocktail (\log_{10} CFU/g).

Time (hr)	Lettuce			Sprouts		
	Temperature					
	2°C	10°C	25°C	2°C	10°C	25°C
1	2.3 ^a	2.3 ^a	2.5 ^a	2.40 ^a	2.35 ^a	2.45 ^a
24	2.4 ^a	2.4 ^a	0.9 ^b	2.30 ^a	2.09 ^{a,b}	1.20 ^b
48	2.6 ^a	2.2 ^a	0.8 ^b	2.40 ^a	1.98 ^b	1.11 ^b
72	2.8 ^a	2.3 ^a	0.4 ^b	2.27 ^a	1.35 ^b	0.59 ^b

Note: Reduction achieved by phage cocktail only (T2). Least square means lacking a common letter differ (p value < 0.0001).

stable at 10°C . At 25°C , the phage cocktail reached a $2.5 \log_{10}$ CFU/g reduction in total STEC within 1 hr, but the STEC population returned to levels that were similar to the control sample after 24 hr (Table 5). Phage cocktail was not able to control the growth of STEC population at 25°C overtime as they continued to grow after 1 hr.

Regarding the individual serogroups, the phage cocktail exhibited high activity against all 7 serogroups at 2°C , causing reductions between 2.3 and $6.4 \log_{10}$ CFU/g after 72 hr of storage (Figure 5a). At 10°C , the phage cocktail remained effective against isolates from

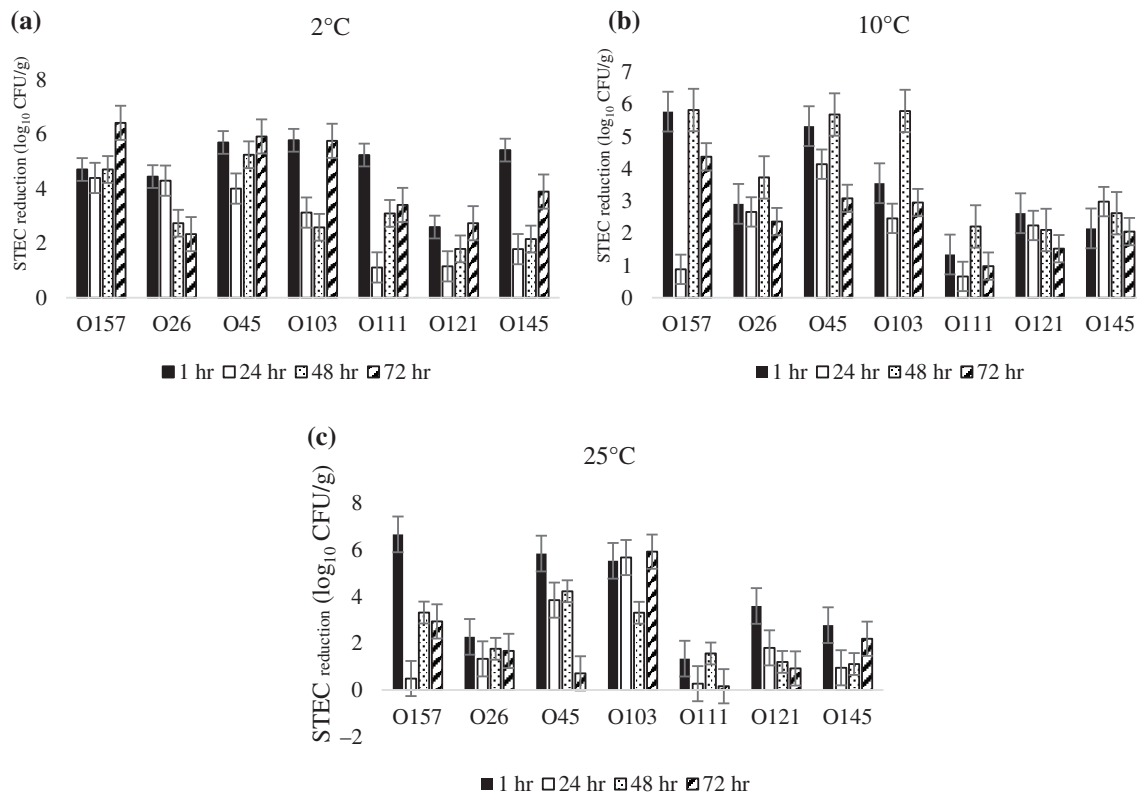


FIGURE 5 (a–c) Reduction of individual STEC serogroups on lettuce immersed in 10^5 CFU/ml STEC bacteria, treated with a phage cocktail and stored at 2, 10, and 25°C for 72 hr.

serogroup O157, O26, O45, O103, and O145 with at least 2.1 \log_{10} CFU/g reduction at the end of the 72 hr storage period, but had less effect on O111 and O121 as there were lower reductions in these serogroups (1.0 and 1.5 logs CFU/g respectively) (Figure 5b). At 25°C, STEC numbers increased after 1 hr in all serogroups except for STEC O103 where the phage cocktail controlled its numbers over 72 hr storage (Figure 5c).

3.3.2 | MBP

As expected, an interaction was found between treatment and temperature, as well as between temperature and time. At temperatures $\leq 10^\circ\text{C}$, the phage cocktail caused a similar reduction in STEC during storage and consistently controlled STEC (Table 5). The highest reduction of total STEC numbers, with the phage cocktail, was 2.4 \log_{10} CFU/g both at 2 and 10°C within 1 hr. Similar to lettuce, the highest reduction at 25°C (2.45 \log_{10} CFU/g) after phage cocktail treatment was at 1 hr, but it was only effective in the short term; reduction in STEC decreased as sprouts were stored longer (≤ 1 hr).

At 2°C, phage cocktail resulted in a > 2 \log_{10} CFU/g reduction in serogroups O157, O103, O111, and O145 over 72 hr, and achieved a ≥ 5 log CFU/g reduction of *E. coli* O26 and O45 (Figure 6a). At 10°C, the phage cocktail remained efficacious against serogroups O157, O45, O103, O145, and O111 during storage, but it was less

effective against O26 (3.8–2.13 \log_{10} CFU/g reduction) and O121 (3.53–1.78 \log_{10} CFU/g reduction) (Figure 6b). At 25°C, the phage cocktail caused a > 5 \log_{10} CFU/g reduction in STEC O26, O45, 103, and O145 over 72 hr of storage (Figure 6c).

3.4 | *E. coli* O157:H7 reduction on Romaine and iceberg lettuce using non-phage proration O157 strains (non-PPS) at high and low inoculation levels

There were significant differences found between all the tested treatments on both romaine and iceberg lettuce at high inoculation level ($p < 0.001$) (Table 6). Chlorinated water (T1) and phage cocktail combined with chlorinated water (T3, T4) had about 2 \log_{10} CFU/g reductions, while phage cocktail alone (T2) achieved 1.7–1.8 \log_{10} CFU/g reductions for both romaine and iceberg lettuce, respectively. Overall, the collected data on O157:H7 (10^5 CFU/g) adulterated lettuce indicated that the STEC phage cocktail combined with chlorinated water was the most effective treatment, which reduced *E. coli* O157:H7 by 2.3 logs on fresh Romaine lettuce, and 2.2 logs on iceberg lettuce. Phage cocktail alone was not an ideal treatment to control *E. coli* O157:H7 at high concentrations.

Unlike the high inoculation level trial, all tested treatments showed similar *E. coli* O157:H7 reductions for all four treatments regardless of lettuce types at low inoculation level (10^3 CFU/g)

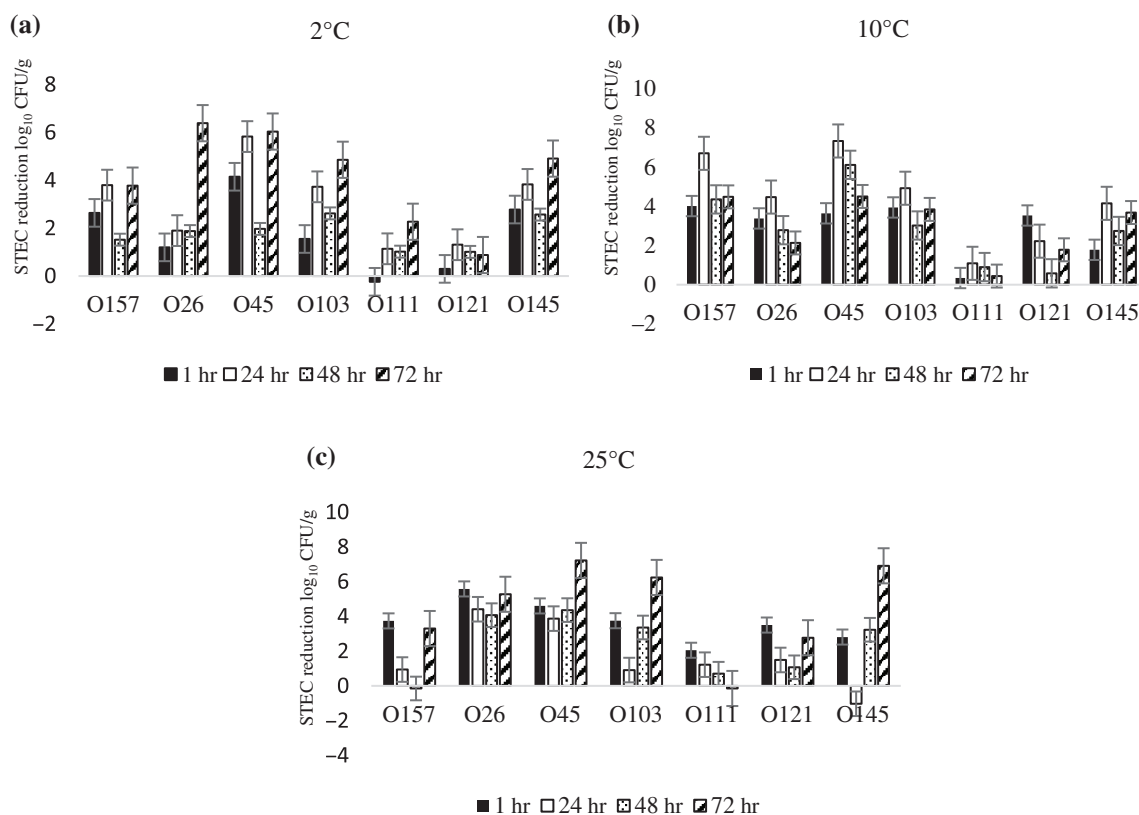


FIGURE 6 (a–c) Reduction of individual STEC serogroups on mung bean sprouts immersed in 10^5 CFU/ml STEC bacteria, treated with a phage cocktail and stored at 2, 10, and 25°C for 72 hr.

TABLE 6 Treatment effect on STEC O157 reduction on Romaine and iceberg lettuce using non-phage prorogation O157 strains (non-PPS) at high and low inoculation levels.

	Treatment	T1	T2	T3	T4	SEM	p-Value
High inoculation	Reduction (\log_{10} CFU/g) on iceberg	1.95 ^{ab}	1.67 ^a	2.15 ^{bc}	2.09 ^{bc}	0.13	<0.0001
	Reduction (\log_{10} CFU/g) on Romaine	2.07 ^{bc}	1.82 ^a	2.34 ^c	2.32 ^{bc}		
Low inoculation	Reduction (\log_{10} CFU/g) on iceberg	2.61 ^a	2.64 ^a	2.74 ^a	2.69 ^a	0.12	<0.0001
	Reduction (\log_{10} CFU/g) on Romaine	3.22 ^b	3.23 ^b	3.11 ^b	3.23 ^b		

Note: a, b, c: Least square means without a common superscript letter indicate difference ($P < 0.05$).

Abbreviations: T1, adulterated lettuce samples washed with chlorinated water (150 ppm) three times; T2, adulterated lettuce samples washed with STEC phage cocktail (10^8 PFU/ml); T3, adulterated lettuce samples washed with chlorinated water (150 ppm) three times before the STEC phage cocktail treatment; T4, adulterated lettuce samples washed with chlorinated water (150 ppm) three times after the STEC phage cocktail treatment.

(Table 6). The phage cocktail was effective at reducing the number of *E. coli* O157:H7, either alone or combined with chlorinated water with >2.6 and >3.1 \log_{10} CFU/g reductions on iceberg and Romaine lettuce, respectively. Interestingly, the reductions achieved were better than those observed in the high inoculation trials; only 44–46% of STEC was reduced in high inoculation trials, while 90–100% of STEC were reduced in low inoculation trials (percentage of STEC reduced = STEC reduction/STEC inoculated). In addition, no significant difference in phage efficacy was found regarding the order in which the phage was applied (before or after the chlorinated water wash) for both high and low inoculation levels ($p > 0.05$).

Significant differences ($p < 0.0001$) were found according to the type of lettuce at both high and low inoculation levels; interestingly, all the treatments were more effective at reducing *E. coli* O157:H7 on Romaine than on iceberg lettuce (Table 6). Regarding storage time, no time \times treatment interaction was found at high inoculation levels ($p = 0.9$) (Figure S2A). At low inoculation levels (10^3 CFU/g), T1, T2, and T4 were more effective in romaine lettuce since *E. coli* O157 was only detected and enumerated at 1 hr or 48 hr. Treatment 3 showed itself to be the less effective treatment in iceberg lettuce since *E. coli* O157 was present at 1 hr, 24 hr, and 48 hr. All the treatments effectively reduced O157 in Romaine and iceberg lettuce after 72 hr, as no *E. coli* O157:H7 was recovered (Figure S2B).

3.5 | *E. coli* O157:H7 detection followed from low inoculation study

Samples, where *E. coli* O157 was not detected in agar plates, were further analyzed for *E. coli* O157 survival in the low inoculation study, whereas *E. coli* O157 was detected from all plates from the high inoculation study. After exposure to chlorinated water, recovery rates of *E. coli* O157:H7 were: 1 hr—100%, 24 hr—83.3%, after 48 hr—33.3%, and 72 hr—50% (Table 7). When only the phage cocktail was used, *E. coli* O157:H7 cells were recovered at 33.3% after 1 hr and were not isolated thereafter. The combination treatment with phage cocktail and chlorinated water (T3 and T4) was also more effective at keeping *E. coli* O157:H7 from recovering than using chlorinated water alone. It was found that only 1/36 samples (2.8%) had bacterial cells that were able to recover when treated with phage cocktail alone (T2) with storage time \leq 24 hr and there were no bacterial cells recovered at 48 and 72 hr. Using chlorinated water treatments without phages (T1) allowed for 66.7% (16/24) *E. coli* O157:H7 recovery in romaine lettuce and 54.2% (13/24) bacterial cells recovery in iceberg lettuce (Table 7). Recovery percentage [% (n/N)] was calculated according to the number of positive sample (n) divided by the total sample size (N) at 1, 24, 48, and 72 hr. These findings suggest that the phage cocktail is causing more cell damage than chlorinated water. The phage cocktail was able to completely kill *E. coli* O157:H7, followed by a phage cocktail combined with chlorinated water treatments.

4 | DISCUSSION

The lack of effective pathogen reduction interventions for fresh produce is a concern worldwide. Shiga-toxigenic *E. coli* (STEC) outbreaks linked to contaminated produce are rising in North America (Lynch et al., 2009). The use of lytic phages as a post-harvest intervention to eliminate STEC could be a safe and effective way to reduce STEC on leafy greens and other vegetables as a hurdle intervention. However, there are still information gaps regarding the efficacy of phage at controlling pathogens within produce-processing environments.

Our results showed that a higher MOI of phages was required to eliminate PPS STEC as well as for non-PPS *E. coli* O157:H7 strains as temperature decreased. Similar results were found when phage product (SalmoFresh) was applied to *Salmonella* (10^8 CFU/ml) *in vitro* under different temperatures (2, 10, 25°C); more phages (higher MOI) were required at lower temperatures for all five strains tested ($p < 0.05$) (Zhang et al., 2019). Result obtained *in vitro* shows the STEC phage cocktail has the potential to reduce STEC (both PPS and non-PPS strains) when applied at refrigeration temperatures; such results set the path for testing phages effectiveness on refrigerated produce.

Treatment of lettuce, MBP, and MBS adulterated with PPS STEC, with the phage cocktail reduced STEC to various degrees (1–5 log₁₀ CFU/g) at storage temperatures of 2, 10, and 25°C at 1, 24, 48, and 72 hr. The phage cocktail was most effective at reducing STEC at lower temperatures (2 and 10°C) after 72 hr of storage, which is surprising, as the optimal phage activity has been reported at 30–45°C. Storage in

TABLE 7 Recovery percentage of O157 on romaine and iceberg lettuce after 72 hr of storage at 2°C with and without enrichment.

Storage time (hr)	Treatment	Percent recovery without enrichment, % (n/N)		Percent recovery with enrichment, ^a % (n/N)		Total recovery rate % (n/N)	
		Romaine	Iceberg	Romaine	Iceberg	Romaine	Iceberg
1	T1	33.33(2/6)	16.67(1/6)	100.00(4/4)	80.00(4/5)	100.00(6/6)	83.33(5/6)
	T2	33.33(2/6)	0.00(0/6)	0.00(0/4)	0.00(0/6)	33.33(2/6)	0.00(0/6)
	T3	50.00(3/6)	0.00(0/6)	0.00(0/3)	0.00(0/6)	50.00(3/6)	0.00(0/6)
	T4	0.00(0/6)	16.67(1/6)	33.33(2/6)	0.00(0/5)	33.33(2/6)	16.67(1/6)
24	T1	0.00(0/6)	16.67(1/6)	83.33(5/6)	40.00(2/5)	83.33(5/6)	50.00(3/6)
	T2	0.00(0/6)	16.67(1/6)	0.00(0/6)	0.00(0/5)	0.00(0/6)	16.67(1/6)
	T3	16.67(1/6)	0.00(0/6)	0.00(0/5)	0.00(0/6)	16.67(1/6)	0.00(0/6)
	T4	0.00(0/6)	16.67(1/6)	16.67(1/6)	0.00(0/5)	16.67(1/6)	16.67(1/6)
48	T1	0.00(0/6)	16.67(1/6)	33.33(2/6)	40.00(2/5)	33.33(2/6)	50.00(3/6)
	T2	0.00(0/6)	0.00(0/6)	0.00(0/6)	0.00(0/6)	0.00(0/6)	0.00(0/6)
	T3	16.67(1/6)	16.67(1/6)	0.00(0/5)	0.00(0/5)	16.67(1/6)	16.67(1/6)
	T4	33.33(2/6)	0.00(0/6)	0.00(0/4)	0.00(0/6)	33.33(2/6)	0.00(0/6)
72	T1	0.00(0/6)	0.00(0/6)	50.00(3/6)	33.33(2/6)	50.00(3/6)	33.33(2/6)
	T2	0.00(0/6)	0.00(0/6)	0.00(0/6)	0.00(0/6)	0.00(0/6)	0.00(0/6)
	T3	0.00(0/6)	0.00(0/6)	0.00(0/6)	0.00(0/6)	0.00(0/6)	0.00(0/6)
	T4	0.00(0/6)	0.00(0/6)	16.67(1/6)	0.00(0/6)	16.67(1/6)	0.00(0/6)

Note: Recovery percentage [% (n/N)] was calculated according to the number of positive sample (n) divided by the total sample size (N).

^aIf the O157 was not recovered, 1 ml of the homogenized sample was transferred into 9 ml modified TSB for 24 hr enrichment at 37°C.

colder temperatures is typically not preferred as it could reduce the activity of phage as they depend on the metabolic activity of the host to complete their lytic cycle (Boyer & McKinney, 2018). At low temperature, *E. coli* growth and metabolism is generally limited as membrane fluidity is decreased and membranes become rigid, which makes it more difficult for phage replication when bacteria growth is reduced to a minimal pace (Graumann & Marahiel, 1996; Parola et al., 1990). The findings of the present study are encouraging since the recommended shelf-life for fresh lettuce and sprouts at 4°C is 1 week (168 hr), and 3–5 days, respectively (Obeso et al., 2008), and the STEC phage cocktail was still effective at refrigeration temperatures during washing and storage. Some of the phages might be active at these lower temperatures; however, the mechanism is unknown. In addition, the STEC phage cocktail was effective against non-PPS STEC on lettuce stored at 2°C as well, especially at low inoculation levels (10³ CFU/g). It is possible that those phages release lytic enzymes such as endolysins which are capable of degrading peptidoglycan to the bacterial cell wall, which also results in a rapid lysis of the bacterial cell at low temperatures (Hermoso et al., 2007; Shannon et al., 2020). Phage endolysins may have remained active during storage at low temperatures and could have contributed to the observed results found. The investigation of reasons for phage activity at lower temperatures is essential since most fresh produce is stored at ≤4°C prior to sale. More research is required to understand the mechanism(s) that the phages use to reduce targeted bacterial populations at refrigeration temperatures.

Overall, phages exhibited the potential to control all targeted O157:H7 non-PPS with MOI from 10⁻⁶ to 21 at all tested temperatures in vitro at 2, 10, 25 and 37°C. Similar results have been reported by others, where some phages were effective under a wide range of temperatures. For example, Hudson et al. reported that phage FAHEc1 effectively infected multiple *E. coli* O157 strains under temperatures ranging from 5°C (4 log₁₀ reductions) to 37°C (>2.7 log₁₀ reduction) (Hudson et al., 2013). Results from this current study show the phage cocktail can be used as an effective antibacterial method for reducing *E. coli* O157:H7 under different temperatures, including those conditions of temperature abuse.

Besides the impact of different temperatures, phage effectiveness may be influenced by different food matrixes. In this study, it was hypothesized that the use of PPS STEC would shed some light on the food matrix's role in phage efficacy. We observed that phage performance varied according to the food matrix, even with the same host. For example, *E. coli* O157:H7 showed the best reduction on romaine lettuce (4.2 logs), followed by MBS (3.6 logs), LG sprouts (3.6 logs), and MBP (3.2 logs). The structures of lettuce and sprouts influenced the effectiveness of the phage cocktail. The phage cocktail itself (T2) was more efficient at reducing STEC in MBP as compared to lettuce ($p < 0.0001$). These results could be due to the fact that the lettuce was cut and that some STEC cells entered the into internal plant tissues, restricting the contact of phage with host bacterial cells. STEC outbreaks have often been linked to shredded produce which promotes the internalization of foodborne pathogens within plant tissues (Beuchat, 1999). Previous research showed that phage treatment has lower efficacy on foods with an uneven surface than those with an

even surface due to the limited diffusion and contact between bacteria and phage particles (Guenther et al., 2009). The leaf surface is less complicated than the interior structure of a leaf, where the bacteria internalized and are less accessible to phages, reducing their effectiveness at decontaminating produce (Solomon et al., 2003; Stine et al., 2005). This finding is supported by Snyder et al., who found in their research that immersion of the fresh produce in the phage treatment was more effective in removing the cells attached to the undamaged leaf as opposed to the cells which potentially internalized into the tissues through the cut surface (Snyder et al., 2016). More research is needed to understand specific mechanisms limiting phages' ability to reach bacteria within the tissue.

Bacterial cells are often found residing in the crevices of the seed coat, as well as in the cotyledon, which can shield bacteria from sanitizers (Bang et al., 2011). Sprouting occurs as a result of exposure of the seed to higher temperatures and moisture, and these conditions, combined with nutrient release from the seed, create conditions that are ideal for the rapid growth of *E. coli* O157:H7 (FSAI, 2011; Stewart et al., 2001). In the present study, the phage cocktail was effective to some degree but is unlikely to eliminate STEC on MBS. During sprout germination, any pathogens that survive in the seeds can grow rapidly (FSAI, 2011), and this was not prevented by phage cocktail or chlorinated water. Perhaps phage-based interventions have most merit as part of a multi-hurdle approach but may lack efficacy if they are used as the sole intervention.

Regarding trials using STEC non-PPS, significant differences were found between lettuce type ($p < 0.01$), where non-PPS *E. coli* O157:H7 was reduced slightly more in Romaine than in iceberg lettuce at both high and low inoculation levels. These differences in reduction were small, about 0.6 logs; however, it is unclear what these differences in reduction could be attributed. *E. coli* O157:H7 outbreaks have been more related to Romaine than iceberg lettuce, perhaps due to the shape of Romaine lettuce head, where the leaves are more exposed to the environment than iceberg lettuce; increased exposure could lead to bacterial contamination in the field (Mou, 2008). From 2008 to 2018, there were 48 outbreaks related to leafy green products in Canada and United States, 37.5% of the outbreaks occurred in romaine lettuce, while only 14.6% were linked to iceberg lettuce (Coulombe et al., 2020). However, of all outbreaks associated with iceberg and Romaine lettuces, 28.6% (iceberg) and 11.1% (Romaine) were caused by shredded or ready-to-eat products. A potential reason is that iceberg lettuce leaves are more fragile than romaine lettuce leaves, and it is possible that during the experimental conditions, the iceberg lettuce leaves were structurally more damaged, increasing the risk that STEC becomes internalized within plant tissues. However, according to results from previous studies, no differences were found in *E. coli* O157:H7 attachment and survivability on Romaine and iceberg at 4°C and 15°C (Patel et al., 2011; Tian et al., 2012). More research is needed to understand potential differences in phage effectiveness in Romaine compared to iceberg lettuce.

Nevertheless, we found that the STEC phage cocktail was very effective at reducing STEC when in low inoculation levels (10³ CFU/g) on both romaine and iceberg lettuce; thus, it seems that the lettuce

matrix may not be the critical factor that inhibited the effectiveness of the phage cocktail. The concentration and strains of STEC inoculated on the fresh produce may play a more critical role on STEC reductions after treated by bacteriophages. The phage cocktail was more effective at reducing non-PPS *E. coli* O157:H7 when lettuce was adulterated with low O157 levels (10^3 CFU/g) than high levels (10^5 CFU/g). Similar results were shown elsewhere when the ability of a phage cocktail to reduce a mixture of *E. coli* O157:H7 strains was tested on baby romaine lettuce and baby spinach leaves (Viazis et al., 2011). The *E. coli* strains were spot inoculated onto the leaves at low (10^4), medium (10^5), and high concentrations (10^6 CFU/ml) followed by applied phage cocktail at a concentration of approximately 10^6 PFU/piece. Similar to our result, the efficacy of the phage cocktail also increased as the bacterial concentrations decreased in their study. A previous survey was conducted among produce from the United States and Mexico and found *E. coli* level of all *E. coli* serogroups, including both generic and pathogenic *E. coli* ranged in concentration from 1.0 to 4.0 \log_{10} CFU/g (Bhagwat, 2003; Johnston et al., 2005; Johnston et al., 2006; Snyder et al., 2016). The concentration of *E. coli* O157:H7, which caused outbreaks in produce, can vary, but is generally believed to be less than our inoculated concentration (10^3 CFU/g) based on the result from a previous microbiological survey (Bohaychuk et al., 2009). Accordingly, we hypothesized that the phage cocktail will be more effective at eliminating *E. coli* O157:H7 at STEC concentrations lower than 10^3 CFU/g in fresh produce. There is a potential for this phage cocktail to be an effective post-harvest disinfection agent in real-world produce production.

It is important to mention that bacterial sensitivity to phages varies among different *E. coli* O157:H7 strains. When phage cocktail was used on romaine lettuce spiked at 10^5 CFU/g of inoculum, a more significant reduction was found in PPS (4.2 logs CFU/g) than non-PPS (1.7 logs CFU/g) *E. coli* O157:H7 strains. Furthermore, the MOIs for O157:H7 non-PPS were higher than for the PPS at all tested temperatures, indicating that more phage particles are needed to eliminate O157 with lower sensitivity to phage. In support of our study, Wang et al. (2015) reported phage lytic capabilities of 11 non-O157 STEC phages, phages AYO26A showed lytic capabilities in different levels to 4 STEC O26 strains tested. The MOI ranged from 6×10^{-6} to 1; and phage AXO121A showed lytic capabilities to four different STEC O121 strains with MOI from 10^{-3} to 10^{-5} (Wang et al., 2015).

When looking at the phage cocktail effectiveness in different STEC serogroups, it was found that not all serogroups had the same sensitivity to the phages included in the phage cocktail, even on the same food matrix. For example, phages were very effective for *E. coli* O157:H7 with the best reduction on romaine lettuce (4.2 logs), but for *E. coli* O26 only reduced it by 2.7 logs. It is important to mention that phages used in the present study were selected with a bias toward their effectiveness against O157, but with exception of phage AKFV33, only phages AYO26A, and AXO121A could lyse the 4 common STEC O157:H7 strains. Multiple phages used in the current study were effective against serogroup O26 (AKFV33, AYO26A, AXO103A, and AXO111A) and O103 (AYO26A, AXO103A, and AXO121A) (Wang et al., 2015), which may explain why the phage cocktail had the highest effectiveness

against these serogroups. Bacteriophage are known to be able to infect only a narrow range of the bacterium, and the host range is determined by the specificity in the nature and structural peculiarities of bacteria cell surface receptors (Rakhuba et al., 2010). We hypothesized that when multiple phages are applied, some of them may be able to attach to receptors of multiple STEC serogroups, and this could increase phage efficiency. The combination of phages can target multiple STEC strains or cover multiple species that are associated with enteric diseases; a bacterium that is resistant to one phage type can be killed by another type (Chan & Abedon, 2012). Genetic similarities found in those more effective phages are very limited, phage AXO103A and AKFV33 are T5-like phages in *Siphoviridae* family. Phage AXO45B is a T5-like phage belongs to *Myoviridae* family. Phage AYO26A is a T4-like phage belongs to *Myoviridae* family. Phage AXO111A, AXO121A, and AYO145A are also in *Myoviridae* family but different in phage type (VII, rV5, O1, respectively) (Wang et al., 2015). Previous research shows the ability of phages to work in cocktails is enhanced if the phages are less closely related to each other, the diversities of the phage families of the phages chosen in the cocktail of the current study may promote the effectiveness of the phage cocktail (Yan D Niu et al., 2021). Although the phage mechanisms for STEC intervention was not analyzed in this research, we hypothesized that T4 and T5 phages may have more lytic activities than the other phage types involved in this study because phage AYO26A, AXO45B, AXO103A, and AKFV33 showed more effectiveness on their host serogroups. However, mechanisms involving underlying interactions between *E. coli* and anti-STECS phages are specific for each phage, new knowledge related to genetic factors of different phage types is needed and will be valuable to define criteria to select appropriate phage candidates as effective biocontrol agents. These questions remain unknown because the lack of information on phage genus, pharmacokinetic aspects, or the phage susceptibility type.

Our results obtained showed that the effectiveness of a phage cocktail to control PPS/non-PPS STEC on fresh produces improved when combined with chlorinated water, indicating the possibility of using this phage cocktail in the presence of such a sanitizer. The combination treatment of chlorinated water with phage cocktail achieved the highest reductions in both PPS and non-PPS STEC at high inoculation levels (10^5 CFU/g) in lettuce, as well as in MBP (PPS STEC). The use of wash water containing sodium or calcium hypochlorite is the primary method of preventing pathogen contamination during produce production. Chlorinated water has been proven effective at reducing pathogenic *E. coli* attached to surfaces, cut edges or stomata in produce (Sharma et al., 2009). Chlorine causes irreversible oxidation of sulphhydryl groups of enzymatic sites in the cytoplasmic membrane, causing membrane damage (Estrela et al., 2002) which could make the cell envelope more unstable and susceptible to disinfectants. However, the combination treatments, including the phage cocktail were more effective than using chlorinated water alone in reducing STEC; likely because the potency of active chlorine can diminish rapidly when it comes in contact with seeds soaking in water due to the high levels of organic matter. High organic material can neutralize chlorine-based sanitizers (Taormina & Beuchat, 1999). A previous study reported using 50 $\mu\text{g/ml}$ sodium hypochlorite (NaOCl) and a commercial phage cocktail known

as EcoShield treating lettuce leaves contaminated with *E. coli* O157:H7 that were stored at 4°C for 24 hr, the combination of NaOCl and EcoShield achieved 1.54 log₁₀ CFU/cm² reduction in *E. coli* O157:H7, which was more effective than either NaOCl (1.38 log₁₀ CFU/cm²) or EcoShield (1.32 log₁₀ CFU/cm²) alone (Ferguson et al., 2013). The combination treatment resulted in a 1.54 log₁₀ CFU/cm² reduction in *E. coli* O157:H7, which was more effective than either NaOCl (1.38 log₁₀ CFU/cm²) or EcoShield (1.32 log₁₀ CFU/cm²) alone; (Ferguson et al., 2013). These previous findings align with the findings presented here and provide further evidence that chlorinated water and STEC phage cocktail can be used as hurdle technology by the fresh produce industry to reduce STEC populations. Future research should consider testing chlorine-resistant STEC strains to assess phage efficacy.

In order to answer the question from the industry regarding phage compatibility with chlorinated water interventions, different treatments were tested during the commercial simulation trials using lettuce adulterated with non-PPS *E. coli* O157:H7 strains. No significant differences were found regarding the order in which the phage was applied (before or after the chlorinated water wash). The residual chlorine remains on lettuce leaves did not reduce the lytic potential of the phages on *E. coli* O157:H7 or inactivate the phage cocktail. This is encouraging since it simplifies the use of phages by the food industry to use phages as a hurdle approach along with chlorine.

When examining STEC survival after exposure to the antimicrobial treatments, in trials testing the phages against non-PPS *E. coli* O157:H7 strains on lettuce, it was found that more *E. coli* O157:H7 cells recovered from chlorinated water treatment compared with phage cocktail treatment. The higher survival of *E. coli* O157:H7 with chlorinated water may cause by the organic substances from the damaged or cut surface of the leafy green, which will inactivate the oxidative effect of the free chlorine in chlorinated water (Marriott et al., 2018). Even though the interaction between the effectiveness of phage cocktail and organic substances was not tested in this study, there is no evidence that the phage cocktail is affected by leafy green organic substances compared with the chlorine disinfectants used in the food industry. The lower O157 recovery rate achieved by treatments with phages from this study indicated that the phage cocktail is a more practical intervention regarding limiting pathogens' survival and recovery compare with traditional chlorinated water treatment alone.

The phage cocktail remained effective against both PPS and non-PPS STEC strains that survived. This suggested that none of those isolates developed resistance to phage, and therefore, reasons for STEC survival remain unclear. Another theory is that under refrigeration, the STEC mutation rate and mechanism to overcome phage attacks should be lower since bacterial metabolism is reduced.

5 | CONCLUSIONS

Overall, the present study demonstrates the effectiveness of using a phage cocktail to reduce STEC in different foods, including lettuce,

MBP, and MBS. The reduction was affected by a time and temperature interaction; phages were effective at 2, 10, and 25°C in vitro, phage showed reductions of 5 log₁₀ CFU/ml for the 7 phage-host STEC serotypes. At 2 and 10°C, phages were effective over 72 hr in both lettuce and MBP even when bacterial metabolism was limited by low temperatures.

The STEC phage cocktail was effective against isolates from 7 STEC serogroups, particularly on lettuce and MBP, mainly for serogroups O26, O45, and O157.

It was observed that phage performance varied according to the food matrix; the effectiveness of some phages was affected by the specific food matrix, even if the targeted bacteria was highly sensitive to the phage (in vitro). There was 84% of PPS *E. coli* O157:H7 reduced on romaine lettuce by phage cocktail, 72% reduced on MBS and LG sprouts, and only 64% reduced on MBP. Phage cocktail was not effective at fully eliminating pathogen contamination from the LG sprouts. In trials simulating commercial conditions, the STEC phage cocktail was able to reduce 2.6–3.0 logs of non-PPS *E. coli* O157:H7 on fresh Romaine and iceberg lettuce leaves at low inoculation levels (10³ CFU/g). The findings of the present research could represent an important step towards understanding phage applications to enhance food safety on fresh-cut leafy produce. Our STEC phage cocktail was effective at reducing O157:H7 when present in low levels, in combination with chlorinated water and under refrigeration. Phages also showed maximal inactivation of non-PPS *E. coli* O157:H7 and the sustainable suppression of their regrowth. The promising results from this study provide more possibility of commercialization of this phage cocktail as a post-harvest disinfection treatment in fresh produce industries in the future.

AUTHOR CONTRIBUTIONS

Claudia Narváez-Bravo developed this project and obtained the funding. Tim McAllister provided the phages. Claudia Narváez-Bravo supervised the students and lab work. Yiran Ding executed the study and performed the experiment with the help from Yuchen Nan and Yang Qiu. Yiran Ding drafted the manuscript with the helps from Kim Stanford, Rick Holley, Tim McAllister, and Claudia Narváez-Bravo. Yiran Ding, Yuchen Nan, Yang Qiu, Dongyan Niu, Kim Stanford, Rick Holley, Tim McAllister, and Claudia Narváez-Bravo made a significant and direct contribution to this project and approved it for publication.

ACKNOWLEDGMENTS

The authors offer special thanks to the Ontario Ministry of Agriculture, Food and Rural Affairs and the Canadian Produce Marketing Association for funding the project.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Yiran Ding  <https://orcid.org/0000-0003-2333-5682>

Kim Stanford  <https://orcid.org/0000-0001-8784-0042>

REFERENCES

- Abedon, S. T. (2011a). Lysis from without. *Bacteriophage*, 1(1), 46–49. <https://doi.org/10.4161/bact.1.1.13980>
- Abedon, S. T. (2011b). Phage therapy pharmacology: Calculating phage dosing. *Advances in Applied Microbiology*, 77, 1–40.
- Althaus, D., Hofer, E., Corti, S., Julmi, A., & Stephan, R. J. (2012). Bacteriological survey of ready-to-eat lettuce, fresh-cut fruit, and sprouts collected from the Swiss market. *Journal of Food Protection*, 75(7), 1338–1341.
- Bang, J., Kim, H., Kim, H., Beuchat, L. R., & Ryu, J.-H. (2011). Inactivation of *Escherichia coli* O157:H7 on radish seeds by sequential treatments with chlorine dioxide, drying, and dry heat without loss of seed viability. *Applied and Environmental Microbiology*, 77(18), 6680–6686.
- Beuchat, L. R. (1999). Survival of enterohemorrhagic *Escherichia coli* O157:H7 in bovine feces applied to lettuce and the effectiveness of chlorinated water as a disinfectant. *Journal of Food Protection*, 62(8), 845–849.
- Bhagwat, A. A. (2003). Simultaneous detection of *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella* strains by real-time PCR. *International Journal of Food Microbiology*, 84(2), 217–224.
- Bohaychuk, V., Bradbury, R., Dimock, R., Fehr, M., Gensler, G., King, R., ... Romero Barrios, P. (2009). A microbiological survey of selected Alberta-grown fresh produce from farmers' markets in Alberta, Canada. *Journal of Food Protection*, 72(2), 415–420.
- Boyacioglu, O., Sharma, M., Sulakvelidze, A., & Goktepe, I. (2013). Biocontrol of *Escherichia coli* O157:H7 on fresh-cut leafy greens. *Bacteriophage*, 3(1), e24620.
- Boyer, R., & McKinney, J. (2018, June 16). Food Storage Guidelines For Consumers. *Virginia cooperative extension*. <https://pubs.ext.vt.edu/348/348-960/348-960.html>
- CDC. (2020). *E. coli* (*Escherichia coli*). Outbreak of *E. coli* infections linked to clover sprouts. <https://www.cdc.gov/ecoli/2020/o103h2-02-20/index.html>
- CDC. (2022). Reports of selected *E. coli* outbreak investigations. <https://www.cdc.gov/ecoli/outbreaks.html?msclkid=465737c9d07511ec8328bf95bd995cdd>
- CFIA. (2020). New import requirement: Romaine from parts of California must be tested for *E. coli*. <https://www.canada.ca/en/food-inspection-agency/news/2020/09/new-import-requirement-romaine-from-parts-of-california-must-be-tested-for-e-coli.html>
- Chan, B. K., & Abedon, S. T. (2012). Phage therapy pharmacology phage cocktails. *Advances in Applied Microbiology*, 78, 1.
- Coulombe, G., Catford, A., Martinez-Perez, A., & Buenaventura, E. (2020). Outbreaks of *Escherichia coli* O157:H7 infections linked to Romaine lettuce in Canada from 2008 to 2018: An analysis of food safety context. *Journal of Food Protection*, 83(8), 1444–1462.
- DebRoy, C., Roberts, E., Valadez, A. M., Dudley, E. G., & Cutter, C. N. (2011). Detection of Shiga toxin-producing *Escherichia coli* O26, O45, O103, O111, O113, O121, O145, and O157 serogroups by multiplex polymerase chain reaction of the *wzx* gene of the O-antigen gene cluster. *Foodborne Pathogens and Disease*, 8(5), 651–652.
- Estrela, C., Estrela, C. R., Barbin, E. L., Spano, J. C., Marchesan, M. A., & Pecora, J. D. (2002). Mechanism of action of sodium hypochlorite. *Brazilian Dental Journal*, 13(2), 113–117.
- Ferguson, S., Roberts, C., Handy, E., & Sharma, M. (2013). Lytic bacteriophages reduce *Escherichia coli* O157:H7 on fresh cut lettuce introduced through cross-contamination. *Bacteriophage*, 3(1), e24323. <https://doi.org/10.4161/bact.24323>
- Fratamico, P. M., Bagi, L. K., & Abdul-Wakeel, A. (2017). Detection and isolation of the "top seven" Shiga toxin-producing *Escherichia coli* in ground beef: Comparison of RapidFinder kits to the U.S. Department of Agriculture microbiology laboratory guidebook method. *Journal of Food Protection*, 80, 829–836. <https://doi.org/10.4315/0362-028X.Jfp-16-296>
- FSAI. (2011). Guidelines on safe production of ready-to-eat sprouted seeds (sprouts).
- Graumann, P., & Marahiel, M. A. (1996). Some like it cold: Response of microorganisms to cold shock. *Archives of Microbiology*, 166(5), 293–300.
- Guenther, S., Huwlyer, D., Richard, S., & Loessner, M. J. (2009). Virulent bacteriophage for efficient biocontrol of *Listeria monocytogenes* in ready-to-eat foods. *Applied and Environmental Microbiology*, 75(1), 93–100.
- Hermoso, J. A., García, J. L., & García, P. (2007). Taking aim on bacterial pathogens: from phage therapy to enzybiotics. *Current Opinion in Microbiology*, 10(5), 461–472.
- Heymann, D. L. (2008). *Control of communicable diseases manual*. American Public Health Association.
- Hong, Y., Schmidt, K., Marks, D., Hatter, S., Marshall, A., Albino, L., & Ebner, P. (2016). Treatment of salmonella-contaminated eggs and pork with a broad-spectrum, single bacteriophage: Assessment of efficacy and resistance development. *Foodborne Pathogens and Disease*, 13(12), 679–688. <https://doi.org/10.1089/fpd.2016.2172>
- Huang, C., Shi, J., Ma, W., Li, Z., Wang, J., Li, J., & Wang, X. (2018). Isolation, characterization, and application of a novel specific salmonella bacteriophage in different food matrices. *Food Research International*, 111, 631–641. <https://doi.org/10.1016/j.foodres.2018.05.071>
- Hudson, J., Billington, C., Cornelius, A., Wilson, T., On, S., Premaratne, A., & King, N. J. (2013). Use of a bacteriophage to inactivate *Escherichia coli* O157:H7 on beef. *Food Microbiology*, 36(1), 14–21.
- Jassim, S. A. A., & Limoges, R. G. (2017). *Bacteriophages: Practical applications for Nature's Biocontrol*: Springer International Publishing.
- Johnston, L. M., Jaykus, L.-A., Moll, D., Anciso, J., Mora, B., & Moe, C. L. (2006). A field study of the microbiological quality of fresh produce of domestic and Mexican origin. *International Journal of Food Microbiology*, 112(2), 83–95.
- Johnston, L. M., Jaykus, L.-A., Moll, D., Martinez, M. C., Anciso, J., Mora, B., & Moe, C. L. J. (2005). A field study of the microbiological quality of fresh produce. *Journal of Food Protection*, 68(9), 1840–1847.
- Kropinski, A. M., Mazzocco, A., Waddell, T. E., Lingohr, E., & Johnson, R. P. (2009). Enumeration of bacteriophages by double agar overlay plaque assay. *Methods in Molecular Biology*, 501, 69–76. https://doi.org/10.1007/978-1-60327-164-6_7
- Luna-Gierke, R. E., Griffin, P. M., Gould, L. H., Herman, K., Bopp, C. A., Strockbine, N., & Mody, R. K. (2014). Outbreaks of non-O157 Shiga toxin-producing *Escherichia coli* infection: USA. *Epidemiology and Infection*, 142(11), 2270–2280. <https://doi.org/10.1017/S0950268813003233>
- Lynch, M. F., Tauxe, R. V., & Hedberg, C. W. (2009). The growing burden of foodborne outbreaks due to contaminated fresh produce: Risks and opportunities. *Epidemiology and Infection*, 137(3), 307–315.
- Marriott, N. G., Schilling, M. W., & Gravani, R. B. (2018). Food contamination sources. In *Principles of food sanitation* (pp. 83–91). Springer.
- Mies, P., & Davis, M. The effects of a three-strain mixture of bacteriophage on *E. coli* O157:H7-inoculated hide squares over a 10-hour period.
- Mou, B. (2008). Lettuce. In *Vegetables I* (pp. 75–116). Springer.
- Moye, Z. D., Woolston, J., & Sulakvelidze, A. (2018). Bacteriophage applications for food production and processing. *Viruses*, 10(4), 1–22. <https://doi.org/10.3390/v10040205>
- Niu, Y. D., Johnson, R. P., Xu, Y., McAllister, T. A., Sharma, R., Louie, M., & Stanford, K. (2009). Host range and lytic capability of four bacteriophages against bovine and clinical human isolates of Shiga toxin-producing *Escherichia coli* O157:H7. *Journal of Applied Microbiology*, 107(2), 646–656. <https://doi.org/10.1111/j.1365-2672.2009.04231.x>
- Niu, Y. D., Liu, H., Du, H., Meng, R., El Mahmoud, S., Wang, G., ... Stanford, K. (2021). Efficacy of individual bacteriophages does not

- predict efficacy of bacteriophage cocktails for control of *Escherichia coli* O157. *Frontiers in Microbiology*, 12, 616712.
- Obeso, J. M., Martínez, B., Rodríguez, A., & García, P. (2008). Lytic activity of the recombinant staphylococcal bacteriophage Φ H5 endolysin active against *Staphylococcus aureus* in milk. *International Journal of Food Microbiology*, 128(2), 212–218.
- Parola, A. H., Ibdah, M., Gill, D., & Zaritsky, A. (1990). Deviation from homeoviscous adaptation in *Escherichia coli* membranes. *Biophysical Journal*, 57(3), 621–626. [https://doi.org/10.1016/s0006-3495\(90\)82578-9](https://doi.org/10.1016/s0006-3495(90)82578-9)
- Patel, J., Sharma, M., & Ravishakar, S. J. (2011). Effect of curli expression and hydrophobicity of *Escherichia coli* O157:H7 on attachment to fresh produce surfaces. *Journal of Applied Microbiology*, 110(3), 737–745.
- Rakhuba, D., Kolomiets, E., Dey, E. S., & Novik, G. J. (2010). Bacteriophage receptors, mechanisms of phage adsorption and penetration into host cell. *Polish Journal of Microbiology*, 59(3), 145–155.
- Rangel, J. M., Sparling, P. H., Crowe, C., Griffin, P. M., & Swerdlow, D. L. (2005). Epidemiology of *Escherichia coli* O157:H7 outbreaks, United States, 1982–2002. *Emerging Infectious Diseases*, 11(4), 603–609. <https://doi.org/10.3201/eid1104.040739>
- Shannon, R., Radford, D. R., & Balamurugan, S. J. (2020). Impacts of food matrix on bacteriophage and endolysin antimicrobial efficacy and performance. *Critical Reviews in Food Science and Nutrition*, 60(10), 1631–1640.
- Sharma, C. S., Mishra, V., Mohammed, A., & Babu, C. R. (2008). Phage specificity and lipopolysaccharides of stem-and root-nodulating bacteria (*Azorhizobium caulinodans*, *Sinorhizobium* spp., and *Rhizobium* spp.) of *Sesbania* spp. *Archives of Microbiology*, 189(4), 411–418.
- Sharma, M. (2013). Lytic bacteriophages: Potential interventions against enteric bacterial pathogens on produce. *Bacteriophage*, 3(2), e25518. <https://doi.org/10.4161/bact.25518>
- Sharma, M., Patel, J. R., Conway, W. S., Ferguson, S., & Sulakvelidze, A. (2009). Effectiveness of bacteriophages in reducing *Escherichia coli* O157:H7 on fresh-cut cantaloupes and lettuce. *Journal of Food Protection*, 72(7), 1481–1485.
- Snyder, A. B., Perry, J. J., & Yousef, A. E. J. (2016). Developing and optimizing bacteriophage treatment to control enterohemorrhagic *Escherichia coli* on fresh produce. *International Journal of Food Microbiology*, 236, 90–97.
- Soffer, N., Woolston, J., Li, M., Das, C., & Sulakvelidze, A. (2017). Bacteriophage preparation lytic for *Shigella* significantly reduces *Shigella sonnei* contamination in various foods. *PLoS One*, 12(3), e0175256. <https://doi.org/10.1371/journal.pone.0175256>
- Solomon, E. B., Pang, H.-J., & Matthews, K. R. (2003). Persistence of *Escherichia coli* O157:H7 on lettuce plants following spray irrigation with contaminated water. *Journal of Food Protection*, 66(12), 2198–2202.
- Stewart, D., Reineke, K., Ulaszek, J., Fu, T., & Tortorello, M. J. (2001). Growth of *Escherichia coli* O157:H7 during sprouting of alfalfa seeds. *Letters in Applied Microbiology*, 33(2), 95–99.
- Stine, S. W., Song, I., Choi, C. Y., & Gerba, C. P. (2005). Effect of relative humidity on preharvest survival of bacterial and viral pathogens on the surface of cantaloupe, lettuce, and bell peppers. *Journal of Food Protection*, 68(7), 1352–1358.
- Taormina, P. J., & Beuchat, L. R. (1999). Behavior of enterohemorrhagic *Escherichia coli* O157:H7 on alfalfa sprouts during the sprouting process as influenced by treatments with various chemicals. *Journal of Food Protection*, 62(8), 850–856.
- Tian, J. Q., Bae, Y. M., Choi, N. Y., Kang, D. H., Heu, S., & Lee, S. Y. (2012). Survival and growth of foodborne pathogens in minimally processed vegetables at 4 and 15 °C. *Journal of Food Science*, 77(1), M48–M50.
- Tobler, C., Visschers, V. H., & Siegrist, M. (2011). Eating green. Consumers' willingness to adopt ecological food consumption behaviors. *Appetite*, 57(3), 674–682. <https://doi.org/10.1016/j.appet.2011.08.010>
- Viazis, S., Akhtar, M., Feirtag, J., & Diez-Gonzalez, F. (2011). Reduction of *Escherichia coli* O157:H7 viability on leafy green vegetables by treatment with a bacteriophage mixture and trans-cinnamaldehyde. *Food Microbiology*, 28(1), 149–157.
- Wang, J., Niu, Y. D., Chen, J., Anany, H., Ackermann, H. W., Johnson, R. P., ... McAllister, T. A. (2015). Feces of feedlot cattle contain a diversity of bacteriophages that lyse non-O157 Shiga toxin-producing *Escherichia coli*. *Canadian Journal of Microbiology*, 61(7), 467–475. <https://doi.org/10.1139/cjm-2015-0163>
- Zhang, X., Niu, Y. D., Nan, Y., Stanford, K., Holley, R., McAllister, T., & Narváez-Bravo, C. (2019). SalmoFresh™ effectiveness in controlling salmonella on romaine lettuce, mung bean sprouts and seeds. *International Journal of Food Microbiology*, 305, 108250. <https://doi.org/10.1016/j.ijfoodmicro.2019.108250>

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Ding, Y., Nan, Y., Qiu, Y., Niu, D., Stanford, K., Holley, R., McAllister, T., & Narváez-Bravo, C. (2023). Use of a phage cocktail to reduce the numbers of seven *Escherichia coli* strains belonging to different STEC serogroups applied to fresh produce and seeds. *Journal of Food Safety*, e13044. <https://doi.org/10.1111/jfs.13044>