CANADIAN FOOD SAFETY FUND

RESEARCH

Project: Bacteriophage cocktail to control Shiga-toxigenic *E. coli* on lettuce

Author: Claudia Narvaez-Bravo

Date: December 2020

University of Manitoba

CPMA | CANADIAN PRODUCE MARKETING ASSOCIATION

UNIVERSITY OF MANITOBA

Faculty of Agricultural & Food Sciences
Food & Human Nutritional Sciences

209 Human Ecology Building
Winnipeg, Manitoba
Canada R3T 2N2
Tel 204-474-6411
Fax 204-474-7593
Fhns.generalenquiries@umanitoba.ca

December 1, 2020

To: Jeff Hall
CPMA Food Specialist

Project: Bacteriophage cocktail to control Shiga-toxigenic *E. coli* on lettuce

Final Report. Research overview and summary:

From vegetables to meat products, the food we eat remains under constant threat of contamination. As the demand for fresh fruits and vegetables has grown, foodborne illness outbreaks have been increasingly linked to contaminated fresh produce. This is particularly concerning and difficult to prevent as leafy greens are usually consumed raw. The use of chemical antimicrobial agents such as chlorinated water alone is insufficient to eliminate STEC associated with fresh produce and seeds. Thus, there is a need to develop new safe, green, or GRASS (Generally Recognize as Safe) and effective antimicrobial alternatives. Bacteriophages are being investigated as a potential biocontrol technology to reduce foodborne pathogens on ready-to-eat foods and vegetables. Bacteriophages are viruses with specificity to attack and kill bacteria. Bacteriophage name originated from English (bacterium) and Greek (phage which means to "eat"); thus, defined as a virus that eats bacterium. Phages do not infect plants, animals, or human cells. Phages are abundant in nature and are part of the natural microflora in humans, plants, and animals.

Phage biocontrol is increasingly accepted as a natural and green technology. The application of bacteriophages to decontaminate food, ranging from vegetables to meat products, has been approved by regulatory agencies such as the US Food and Drug Administration and the US Department of Agriculture as well as Health Canada. Some examples of commercial phage products are Ecolicide for *E. coli* O157:H7, EcoShield (*E. coli* O157:H7), ListShield (*L. monocytogenes*)

(https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=172.785) and SalmoFresh (*Salmonella*)(https://www.fsis.usda.gov/wps/wcm/connect/bab10e09-aefa-483b-8be8-809a1f051d4c/7120.1.pdf?MOD=AJPERES) (http://www.intralytix.com/index.php?page=news&id=63)

The phage cocktail tested in this research showed itself to be effective at reducing *E. coli* O157:H7 (2-3 logs) on fresh lettuce. When the phage cocktail was compared with chlorinated water, STEC phages were more efficient at eliminating *E. coli* O157:H7. Among the future direction of the application of phages is the potential commercialization of this phage cocktail. Our goal is to have a product that can reduce *E. coli* O157:H7 burden is a safe way and at a reasonable cost.

UNIVERSITY OF MANITOBA

Faculty of Agricultural & Food Sciences
Food & Human Nutritional Sciences

209 Human Ecology Building
Winnipeg, Manitoba
Canada R3T 2N2
Tel 204-474-6411
Fax 204-474-7593
Fhns.generalenquiries@umanitoba.ca

Regarding knowledge transfer, an abstract from this research was submitted to the VTEC 2021 Symposium that will be held in Banff May 9-, 2021 (https://vtec2021.org/). We are also preparing a paper for publication, the targeted journal is the International Journal of Food Microbiology.

Some related papers published by our research group.

1. *Emelia Hornam Adator*; Meining Cheng*; Rick Holley; Tim McAllister; Claudia Narvaez (2018). Ability of Shiga Toxigenic Escherichia coli to survive within dry biofilms and transfer to fresh lettuce. International Journal of Food Microbiology. Int J Food Microbiol. 269:52-59.*
2. *Zhang X, Niu YD, 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. Int. J. Food Microbiol. Sep 16;305:108250. doi: 10.1016/j.ijfoodmicro.2019.108250*
3. Claudia Narvaez-Bravo and Tim McAllister. (2017). Bacteriophages at the forefront of Search for Alternative Antimicrobials http://www.foodqualityandsafety.com/article/bacteriophages-forefront-search-alternative-antimicrobials/. Food Quality and Safety Magazine.

Industry / academia collaborations are essential for the continuation of food safety research targeting the risks associated with fresh fruits and vegetables. Research projects, like this one, will lead to affordable, accessible solutions which reduce the risks associated with ready-to-eat commodities.

We would like to thank the CPMA membership which made this work possible and we look forward to partnering with CPMA again on future projects.

Claudia Narvaez

Claudia Narvaez-Bravo, PhD
Associated Professor
Food Science Program Chair
IFT Coordinator
Food and Human Nutritional Sciences Department
University of Manitoba
Winnipeg
204-474-6658
Claudia.narvaezbravo@umanitoba.ca

Canadian Produce Marketing Association

Project: Bacteriophage cocktail to control Shiga-toxigenic *E. coli* on lettuce

Final Report –September 30, 2020.

Project main goal: To determine the effectiveness of a STEC bacteriophage cocktail to decrease STEC on Romaine and Iceberg lettuce.

Introduction:

From vegetables to meat products, the food we eat remains under constant threat of contamination. As the demand for fruits and vegetables had grown, foodborne illness outbreaks have been increasingly linked to contaminated fresh produce. This is particularly concerning and difficult to prevent as leafy greens are usually consumed raw. The use of chemical antimicrobial agents such as chlorinated water alone is insufficient to eliminate STEC associated with fresh produce and seeds. Thus, there is a need to develop new safe, green, and effective antimicrobial alternatives. Bacteriophages are being investigated as a potential biocontrol technology to reduce foodborne pathogens on ready-to-eat foods and vegetables. Bacteriophages are viruses with specificity to attack and kill bacteria (Zhang et al., 2019). This curious name originated from English (bacterium) and Greek (phage in "eat"); thus, defined as a virus that eats bacterium. They do not infect plants, animals, or human cells. Phages are abundant in nature and are part of the natural microflora in humans, plants, and animals (Narvaez-Bravo & McAllister, 2017).

Phage biocontrol is increasingly accepted as a natural and green technology. Bacteriophages' application to decontaminate food, ranging from vegetables to meat products, has been approved by regulatory agencies such as the US Food and Drug Administration and the US Department of Agriculture as well as Health Canada (CFIA, 2019; FDA, 2013). Some examples of commercial phage products are Ecolicide for *E. coli* O157:H7, EcoShield (*E. coli* O157:H7), ListShield (*L. monocytogenes*) and SalmoFresh (*Salmonella*).

The phage cocktail tested in this research showed to be effective at reducing *E. coli* O157:H7 (2-3 logs) on fresh lettuce. When the phage cocktail was compared with chlorinated water, STEC phages were more efficient at eliminating *E. coli* O157:H7. Among the future direction is the potential commercialization of this phage cocktail; our goal is to have a product that can reduce *E. coli* O157:H7 burden is a safe way and at a reasonable cost.

Project Specific Outcomes:

1. To determine the effectiveness of the STEC bacteriophage cocktail when used to control STEC (*E. coli* O157:H7) on Romaine and Iceberg lettuce.

Methods:

A STEC phages cocktail was tested on produce adulterated with a O157:H7 cocktail in high (10^5 CFU/g) and low (10^3 CFU/g) concentrations. Four treatments were applied to Romaine and iceberg lettuce leafs samples:

T1: samples STEC inoculated (adulterated samples) + chlorinated water wash.
T2: samples STEC inoculated + phages cocktails.
T3: samples STEC inoculated + chlorinated water + rinse + phages cocktails.
T4: samples STEC inoculated + phages cocktails + chlorinated water.

Treatments were packaged in sterile bags and stored at 2°C for 1, 24, 48 and 72 hours. At the end of each storage period, conventional and molecular microbiology techniques were be used for STEC screening and confirmation.

The original proposal included 7 days storage; however, we modified the proposal and are now testing for up to 72 h. The reason for the change is that in a pre-testing, we did not observe significant differences in reduction over time beyond 72h. Another change to the proposal is that we added one more treatment, where phages are inoculated first followed by the chlorinated water treatment. The reason for this change is that some phages can cause damage (holes) to the bacterial cell envelope, which could enhance the bactericidal effect of the chlorinated water.

STEC bacterial cocktails were prepared for low and high inoculation levels (10^5 & 10^3 CFU/ml). The cocktails consisted of 4 *E. coli* O157H7 strains:

Culture ID	Serotype	Source	Virulence factors
1931	O157:H7	Hamburger	Stx1, Stx2, eae, hlyA
1934	O157:H7	Beef	Stx1, Stx2, eae, hlyA
161-84	O157:H7	Human	Stx1, Stx2, eae, hlyA
CO283	O157:H7	Cattle feces	Stx1, Stx2, eae, hlyA

MILESTONES

Milestone	Description of activities	Target completion	Actual completion	Brief explanation
Phage cocktail preparation	Seven phages were grown (propagated) individually (10^8 PFU/mL) and pooled for use in mixtures (total phage titer of 10^9 PFU/mL).	July 2019-September 2019	October 2019	Seven phages were propagated individually and later pooled into STEC phage cocktails. Each time each phage is propagated, its lytic activity and phage titer is tested using microplate assays and overlays, respectively. Phages must be in the concentration of phage 10^8-10^9 PUF/ml). Each propagation aims to produce about 1 Lt of phage.

Adulterated (spiked with STEC) produce trials. Romaine High inoculation and storage.	Romaine lettuce was inoculated with STEC *E. coli* cocktail (10^5 CFU/g). Samples were assigned to each of 4 treatments: T1, T2, T3 & T4. Samples were stored at 2 °C and bacterial counts were performed at 0, 24, 48 and 72 h. Data were collected in each of the Treatments. Each treatment was repeated 3 times.	October 2019 - December 2019	December 2019	Three repetitions were performed and completed. Data collection and statistical analysis were also completed.
Adulterated (spiked with STEC) produce trials. Romaine low inoculation and storage.	This section is underway with inoculation of STEyyyyC E. coli cocktail (10^2 CFU/g). Samples will be assigned to each of 4 treatments: T1, T2, T3 & T4. Samples will be storage at 2 °C and bacterial counts will be performed at 0, 24, 48 and 72 h. Data collection and analysis will be completed. Each treatment will be repeated 3 times.	January 2020-February 2020.		Delayed due to Covid 19.
Adulterated (spiked with STEC) produce trials. Iceberg High inoculation and storage.	Produce was inoculated with STEC E. coli cocktail (10^5 CFU/g). Samples were assigned to each of 4 treatments: T1, T2, T3 & T4. Samples were stored at 2 °C and bacterial counts will be performed at 0, 24, 48 and 72 h. Data collection and analysis will be completed. Each treatment will be repeated 3 times.	March 2020-April 2020		Delayed, due to covid 19.
Adulterated (spiked with STEC) produce trials. Iceberg low inoculation and storage.	Produce will be inoculated with STEC E. coli cocktail (10^2 CFU/g). Samples will be assigned to each of 4 treatments: T1, T2, T3 & T4. Samples will be storage at 2 °C and bacterial counts will be	May 2020-June 2020		Delayed, due to Covid 19. The project was finalized in August 2020

	performed at 0, 24, 48 and 72 h. Data collection and analysis will be completed. Each treatment will be repeated 3 times.			

RESULTS.

High E. coli O157:H7 inoculation trials

Romaine lettuce

Overall, from all the tested treatments, the ones showing better reductions (Table 1) were T3 and T4, about 2 logs. When applying chlorinated water (T1) or the phage cocktail (T2), slightly lower reductions were observed, 1.95 and 1.67 logs, respectively. No significant differences were found regarding the order in which the phage was applied (before or after the chlorinated water wash). Regarding storage time, no time x treatment interaction was found (P=0.9). In some cases, slightly better O157 reductions were observed at 72 h for treatments T3 and T4, but over time, the reductions were very similar for all treatments (Fig. 1).

Iceberg Lettuce

Overall, T1, T3 and T4 showed better O157 reductions, about 2 logs (Table 1). When the phage cocktail was applied alone (T2), it achieved a slightly lower reduction (1.67 logs) ($P < 0.05$). Similar to Romaine lettuce, O157 reductions over time were not significantly different at 1h, 24h, 48h and 72h (Fig. 2).

Previous studies conducted in our lab using the same phage cocktail and lettuce spiked at 10^5 CFU/g showed a more significant reduction (about 3-5 logs CFU/g). The differences in the O157:H7 strains could explain the lower efficacy found in this study compared with previous studies:H7 strains used to prepare the bacterial cocktail. In previous studies, we only used one strain, while in the current study, we are using four.

Overall the collected data on O157:H7 (10^5 CFU/g) adulterated lettuce indicates that the STEC phage cocktail, when used in combination with chlorinated water, was able to reduce at least 2.4 logs of *E. coli* O157:H7 on fresh Romaine and iceberg lettuce leaves.

Low *E. coli* O157:H7 inoculations trials

Romaine Lettuce

All tested treatments showed similar *E. coli* O157:H7 reductions for all four treatments (Table 2). Interestingly, the reductions achieved were better than those observed in the high inoculation trials, about 3 logs. Regarding storage time, treatments T1, T2 and T4 showed to be more effective, since *E. coli* O157 was only detected and enumerated at 1 h or 48 hours (Fig. 2). All the treatments effectively reduced O157 after 72 h, since no *E. coli* O157:H7 was recovered at this time point.

Iceberg Lettuce

Similar to the results obtained for Romaine lettuce, all tested treatments showed similar *E. coli* O157:H7 reductions for icerbeg lettuce samples. The reductions (2.6 logs) were slightly better than those observed

in the high inoculation trials (2 logs); regarding storage time, T1 (chlorinated water) showed itself to be the less effective treatment since *E. coli* O157 was present at 1h, 24 and 48 h. Overall, all the treatments were equally effective at reducing O157 after 72 h, since no *E. coli* O157:H7 was recovered.

Significant differences were found regarding the type of lettuce, being the treatments more effective at reducing *E. coli* O157:H7 on Romaine lettuce than on iceberg lettuce (P <0.001).

The collected data on O157:H7 (10^3 CFU/g) adulterated lettuce indicate that the STEC phage cocktail when used in combination with chlorinated water, was able to reduce at least 3 logs of *E. coli* O157:H7 on fresh Romaine and iceberg lettuce leaves.

***E. coli* O57:H7 detection:**

Samples where *E. coli* O157 was not detected in agar plates were further analyzed for *E. coli* O157 survival. For survival, we used non-enrichment (directly from Buffer peptone water) and enrichment (trypticase soy broth). Results for Romaine (Table 3) showed a higher survival rate on adulterated lettuce after being treated with chlorinated water (T1). After exposure to chlorinated water, the total recovery percentage (Table 3) was as follows: 1h - 100% recovery, 24 h - 83.3 %, after 48 h - 33.3 % and after 72 h -50% recovery. When only the phage cocktail was applied, *E. coli* O157:H7 cells were recovered only after 1 h (33.3%). After 24, 48 and 72h, no *E. coli* O157:H7 cells were detected, which indicated that the STEC phage cocktail, when applied by itself, is the most efficient treatment when looking at limiting *E. coli* O157 ability to recover after a pathogen reduction intervention. T3 and T4 were also better than chlorinated water at keeping *E. coli* O157:H7 from recovery. These findings suggest that the phage cocktail is causing more cell damage than chlorinated water. Similar results were observed for iceberg lettuce (Table 4), where more *E. coli* O157:H7 cells recovered after being exposed to chlorinated water when comparing it with the other treatments. T2 was the more effective, followed by T3 and T4.

Key findings

- The collected data indicate that the STEC phage cocktail, when used in combination with chlorinated water, was able to reduce at least 2.4 logs of *E. coli* O157:H7 on fresh Romaine and iceberg lettuce leaves when *E. coli* O157 contamination levels were 10^5 CFU/g.
- All of the treatments were more effective at reducing E. coli O157:H7 in produce adulterated with lower *E. coli* O157:H7 contamination levels (10^3 CFU/g). The reduction range was 2.6-3.0 logs.
- The treatments were more effective at reducing *E. coli* O157:H7 on Romaine lettuce than on icerbeg lettuce (P <0.001).
- In those treatments where the STEC phage cocktail was used alone (T2), it was found that E. coli O157:H7 cells were not able to recover after 24 h enrichment. Therefore T2 was the most efficient treatment at eliminating *E. coli* O157:H7; while chlorinated water treatments allowed for *E. coli* O157:H7 recovery. Data indicated that the phage cocktail is a more practical intervention regarding limiting bacterial survival and recovery.

Table 1. Treatment effect on STEC O157 reduction on high-inoculation lettuce trials

Treatment	T1	T2	T3	T4	SEM	P-value
Reduction (log10 CFU/g) on Iceberg	1.95ab	1.67a	2.15bc	2.09bc	0.13	<.0001
Reduction (log10 CFU/g) on Romaine	2.07bc	1.82a	2.34c	2.32bc		

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

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 2. Treatment effect on STEC O157 reduction on low-inoculation lettuce trials.

Treatment	T1	T2	T3	T4	SEM	P-value
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		

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

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 3. Recovery percentage of O157:H7 on romaine lettuce after 72 hours storage at 2 °C with and without enrichment.

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

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

[a.] If the O157 was not recovered, 1 ml of the homogenized sample was transferred into 9 ml modified TSB for 24 hours enrichment at 37 °C.

Table 4. Recovery percentage of O157 on iceberg lettuce after 72 hours of storage at 2 °C with and without enrichment.

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

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

[a.] If the O157 was not recovered, 1 ml of the homogenized sample was transferred into 9 ml modified TSB for 24 hours enrichment at 37 °C.

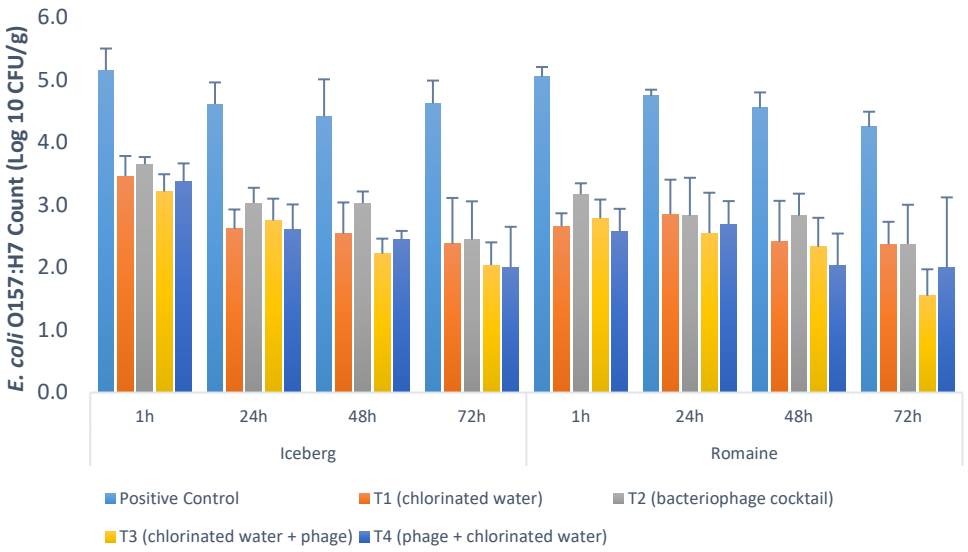

Figure 1. STEC O157:H7 counts (log10 CFU/g) on high inoculated iceberg lettuce and Romaine lettuce after exposure to the different treatments. Each treatment group was stored at 4 °C for 1, 24, 48 and 72 h. Treatment had a significant effect on STEC O157:H7 reduction ($P < 0.001$).

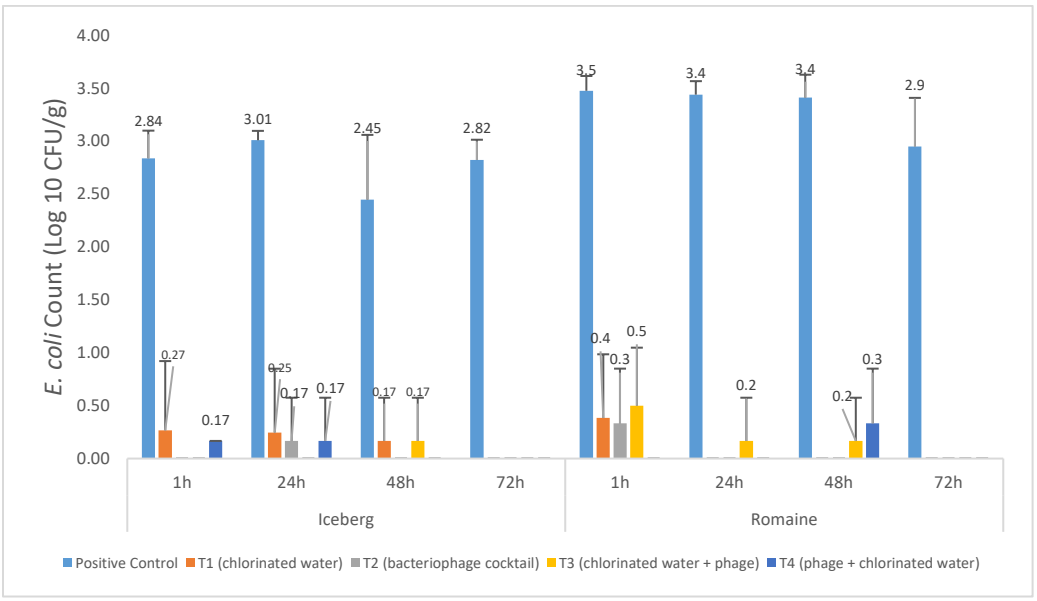

Figure 2. STEC O157:H7 count on low inoculated iceberg lettuce and romaine lettuce after treated by chlorine water and bacteriophage cocktail individually and combined. Each treatment group at 4 °C was stored for 1, 24, 48 and 72 h and compared with the positive control group. The sample type had a significant effect on STEC O157:H7 reduction ($P < 0.0001$).

CFIA. (2019). *Control measures for Listeria monocytogenes in ready-to-eat foods*. https://www.inspection.gc.ca/preventive-controls/listeria-monocytogenes/eng/1518103693274/1528201904208: Goverment of Canada Retrieved from https://www.inspection.gc.ca/preventive-controls/listeria-monocytogenes/eng/1518103693274/1528201904208

FDA. (2013). *Agency Response Letter GRAS Notice No. GRN 000435*. https://wayback.archive-it.org/7993/20171031005936/https://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm345473.htm: U.S Department of health and Human Service Retrieved from https://wayback.archive-it.org/7993/20171031005936/https://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm345473.htm

Narvaez-Bravo, C., & McAllister, T. (2017). Bacteriophages at Forefront of Search for Alternative Antimicrobials. *Food Quality & Safety Farm to Fork safety*. Retrieved from https://www.foodqualityandsafety.com/article/bacteriophages-forefront-search-alternative-antimicrobials/

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. *Int J Food Microbiol, 305*, 108250. doi:10.1016/j.ijfoodmicro.2019.108250