International Journal of Food Microbiology 305 (2019) 108250

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

SalmoFresh™ effectiveness in controlling *Salmonella* on romaine lettuce, mung bean sprouts and seeds

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ARTICLE INFO

Keywords:
SalmoFresh™
Lettuce
Sprouts
Mung bean seed
Lytic bacteriophage
Produce
Chlorination

ABSTRACT

The purpose of this study was to determine the effectiveness of a commercial *Salmonella* bacteriophage mixture (SalmoFresh™ 6-phage strains) and to compare its effectiveness with a chlorinated water treatment to reduce *Salmonella* on produce and seeds at different temperatures and storage times. Two sets of experiments were designed to test phage and chlorinated water effectiveness on produce at 2, 10 and 25 °C at different storage times (1, 24, 48 and 72 h). First, SalmoFresh™ was applied to the surface of lettuce, mung bean sprouts and mung bean seeds that were spot-inoculated with a five *Salmonella* strain mixture (Newport, Braenderup, Typhimurium, Kentucky, and Heidelberg, 10^5 CFU/mL) by spraying phages onto lettuce (n = 48 pieces, 3×3 cm^2 per treatment) and sprouts (n = 48 pieces per treatment). A second set of experiments (scaled-up) consisted in the application of phages by immersion to *Salmonella* adulterated lettuce (600 g), 300 g sprouts (300 g) or mung bean seeds (30 g) in a phage cocktail (10^8 PFU/mL) for 15 min (lettuce and sprouts) or 1 h (seeds). Another group of samples was washed with chlorinated water and yet another group was treated with a combination of chlorinated water followed by phage cocktail. Each experiment was repeated three times by quadruplicates. After the treatments for spot-inoculated and scaled-up experiments, lettuce and sprouts were separated into different lots (10 g/lot) and stored at 2, 10 and 25 °C; *Salmonella* was enumerated after 1, 24, 48 and 72 h. Adulterated phage-treated seeds were packaged and stored dry at 25 °C. *Salmonella* was enumerated after 72 h of storage. Groups of phage treated mung bean seeds (720 g) were germinated, and the reduction in *Salmonella* determined. Results of microplate virulence assays indicated that SalmoFresh™ reduced ($P = 0.007$) *Salmonella* by an average of 5.34 logs CFU/mL after 5 h at 25 °C. Spraying SalmoFresh™ onto lettuce and sprouts reduced *Salmonella* by 0.76 and 0.83 $\log_{10}$ CFU/g, respectively ($P < 0.01$). Immersion of produce in a phage solution was better at killing *Salmonella* $P < 0.05$) than spraying it onto the surface, reducing *Salmonella* by 2.43 and 2.16 $\log_{10}$ CFU/g on lettuce and sprouts, respectively. SalmoFresh™ was an effective biocontrol intervention to reduce *Salmonella* on lettuce and sprouts. On seeds, although a reduction was observed, *Salmonella* was able to grow exponentially during germination; therefore, the phage cocktail was not effective on mung bean seeds or sprouts obtained from adulterated seeds. The combination of hurdles, chlorination fallowed by the phage cocktail was the most effective treatment to reduce *Salmonella* on lettuce and sprouts.

1. Introduction

Contamination of fresh produce by pathogens is a serious health issue in all countries. In the US, 9.4 million cases of foodborne illnesses were caused by 31 common pathogens, with 11% of the hospitalizations and 28% of deaths being associated with non-typhoidal *Salmonella* (Scallan et al., 2011). The Food and Drug Administration (FDA) reported that \$10 - \$83 billion is spent every year for medications to relieve pain and suffering from foodborne illnesses (FDA, 2013). Consequently, efforts to develop effective antimicrobial strategies aimed at controlling foodborne pathogens have been increasing (Viazis et al., 2011). From 2007 to 2008, the majority of produce-generated foodborne illness outbreaks in North America were linked to *Salmonella* (Lynch et al., 2009).

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https://doi.org/10.1016/j.ijfoodmicro.2019.108250
Received 21 May 2018; Received in revised form 31 May 2019; Accepted 10 June 2019
Available online 12 June 2019

The consumption of fresh fruits and vegetables is widely acknowledged as crucial for a healthy human diet (Olaimat and Holley, 2012). Although consumption of produce rose by 9% from 1995 to 2006, it was associated with a 38.6% increase in foodborne illnesses linked to produce (CDC, 2016). *Salmonella* outbreaks linked to lettuce have been reported in North America, Australia, Finland, and England (Brandl and Amundson, 2008). Using data from outbreak-associated illness that occurred in the years 1998 to 2008, it was estimated that produce commodities accounted for 46% of the illnesses, where leafy vegetables responsible for almost half of the illnesses (22%) (Painter et al., 2013). Outbreaks can occur due to adulteration of produce at any stage of production from the growing stage through to packaging and storage. Lack of effective antimicrobial treatments during planting, harvesting and processing of produce can raise the risk of microbial contamination (Buck et al., 2003). Since fresh-cut produce offers better nutritional and sensory quality than preserved produce, it is often consumed raw and sometimes with few bacterial decontamination procedures. To control microbial growth and extend shelf-life, produce processing plants often process fresh produce at temperatures below 4 °C (Liu et al., 2013). However, washing, slicing, trimming, and shredding can damage plant cells and release intracellular nutrients that promote bacterial growth (Francis et al., 2012). Therefore, it is crucial to identify effective pathogen reduction strategies that can be applied to fresh-cut produce without altering its quality (Parish et al., 2003).

Antimicrobial treatments have been applied in produce processing industries to decrease the risk of pathogen contamination (Jahid and Ha, 2012). Among the conventional chemical sanitizers, chlorine is extensively used to disinfect fresh fruits and vegetables. However, chlorine gas released from treated water can cause discomfort to workers (Suslow, 2014) and if chlorine levels in water exceed 1%, produce can be discoloured and acquire undesirable odours (Baskaran et al., 2013). Chlorine can also react with organic materials and form carcinogenic organochlorine compounds such as chloramines (Jahid and Ha, 2012; Stopforth et al., 2008). Moreover, wastewater that contains chemical sanitizers also requires additional purification and treatment, significantly increasing produce production costs (Neal et al., 2012).

To satisfy the growing consumer demand for organic fruits and vegetables, bacteriophages, which have recently been used to control foodborne pathogens in fresh produce, offer a feasible alternative to kill pathogens (Sharma, 2013). Bacteriophages are abundant in nature and are naturally present in a variety of ready-to-eat foods such as cheese and sausages (Sharma et al., 2009). Bacteriophages replicate through a lytic cycle, which includes the stages of adsorption, infection, and propagation. After adsorption, phages inject nucleic acids into the bacterial host and DNA is translocated across the host cell wall. Subsequently, nucleic acid replication occurs which is followed by synthesis of the structural proteins which are assembled into new bacteriophage particles that are released upon lysis of the host cell (Campbell, 2003). A variety of commercial bacteriophage cocktails such as SalmoFresh™, Guard Listex, EcoShield™, and ListShield™ have been reported to be effective at controlling pathogens in food (Anany et al., 2014).

SalmoFresh™, a lytic *Salmonella* bacteriophage cocktail containing 6 *Salmonella* phages, has been granted GRAS (Generally Recognize As Safe) status by the FDA (FDA, 2013; Intralytix, 2015). Previous research has reported that SalmoFresh™ can effectively reduce pathogenic *Salmonella* on glass coverslips, stainless steel coupons (Woolston et al., 2013), fresh fruits (Magnone et al., 2013), whole and fresh-cut cucumbers (Sharma et al., 2017), chicken (Sukumaran et al., 2015) and turkey breast cutlets (Sharma et al., 2015). When SalmoFresh™ was combined with EcoShield™ and ShigActive™ in the washing solution, a > 4 log reduction of *Salmonella* on cantaloupe, broccoli and strawberries was achieved (Magnone et al., 2013). However, the effectiveness of *Salmonella* phages has not been evaluated on mung bean sprouts or seeds, nor has the effectiveness of phages on fresh produce storage at different temperatures. Therefore, this study was aimed to determine the effectiveness of SalmoFresh™ and chlorinated water against five common *Salmonella enterica* serovars (Typhimurium, Enteritidis, Heidelberg, Kentucky, and Newport) on lettuce, sprouts and mung bean seeds at 2, 10 and 25 °C over storage times of 1, 24, 48 and 72 h. *Salmonella* serovars were chosen based on their association with foodborne outbreaks in North America (CDC, 2015, 2018; Jackson et al., 2013). In addition, the impact of administering the phage through immersion or spraying a phage solution onto the surface of produce was examined.

Table 1
Salmonella strains used in tests.

Species	Serotype	Strain no.	Origin
Salmonella enterica	Newport	1194	Ground beef
Salmonella enterica	Braenderup	H9812 (ATCC BAA-664)	N/A[a]
Salmonella enterica	Typhimurium	ATCC 19585	N/A[a]
Salmonella enterica	Kentucky	F41-2	Chicken carcasses
Salmonella enterica	Heidelberg	32SusP	Chicken nuggets

[a] Information was not applicable.

2. Material and methods

2.1. Bacterial cultures and bacterial cocktail preparation

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

The 5-strain *Salmonella* cocktail suspensions were prepared by transferring bacterial colonies of each individual strain from TSA plates (Criterion™, Hardy Diagnostics, Santa Maria, CA) into fresh TSB (Criterion™, Hardy Diagnostics, Santa Maria, CA) and then incubated for 16–18 h at 37 °C to achieve a population of 10^8 CFU/mL. TSB containing each *Salmonella* strain were streaked on XLT4 (Xylose-Lysine-Tergitol 4; BD Difco™) agar plates. A single colony was selected from the plates, transferred to 10 mL TSB and incubated overnight at 37 °C. The concentration of each individual culture was confirmed. One ml of each culture (10^8 CFU/mL) was transferred into bottles containing 20 mL of TSB and incubated overnight at 37 °C. After incubation, the bottles for each strain was centrifuged (Beckman-Coulter Allegra X-22R Centrifuge, Kansas City, MO) for 10 min at 4000 ×*g* and the supernatant was discarded. The bacterial pellet of each strain was mixed and suspended in 2 mL of TSB with 10% glycerol, and brought to 30 mL with fresh 10% TSB; two ml aliquots were prepared for future use. The concentration of the *Salmonella* cocktail stock culture was confirmed as 10^8 CFU/mL by enumeration on XLT4 agar plates.

2.2. SalmoFresh™

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

2.3. In vitro microplate virulence assay

Salmonella susceptibility to SalmoFresh™ was assessed using a microplate phage virulence assay based on Niu et al. (2009). *Salmonella* bacteriophage was serially diluted 10-fold by adding 20 µL of phage suspended in 0.1 M sodium chloride to 180 µL of mTSB (TSB with

Table 2
Genome size and composition of phage contained in SalmoFresh™.

Phage	#ATCC	GenBank Accession	GC%	Size (bp)	No. of opening reading frames	Undesirable genes	Family	Host Strain	Serotype	Biochemistry	Antibiotic[a] susceptibility
SBA-1781	PTA-5282	JX181814 - JX181821	39	88,124	741	None	*Myoviridae*	S.H178	Hadar	*Salmonella* spp.	Susceptible to all tested
SKML-39	PTA-12380	JX181829	50	159,624	1547	None	*Myoviridae*	S.K39	Kentucky	*Salmonella* spp.	Susceptible to all tested
SPT-1	PTA-5281	JX181822 - JX181823	39	87,248	725	None	*Myoviridae*	S.E378	Enteritidis	*Salmonella* spp.	Susceptible to all tested
SSE-121	PTA-5283	JX181824	45	147,745	1455	None	*Myoviridae*	S.A121	Agona	*Salmonella* spp.	Susceptible to all tested
STML-13-1	PTA-8365	JX181826 - JX181828	51	161,646	1650	None	*Myoviridae*	S.E236	Enteritidis	*Salmonella* spp.	Susceptible to all tested
STML-198	PTA-12381	JX181825	37	158,160	1350	None	*Myoviridae*	S.T198	Typhimurium	*Salmonella* spp.	Susceptible to all tested

Intralytix Inc. Source (FDA, 2013).
Intralytix Inc.
[a] The tested antibiotics included cefotaxime, ceftriaxone, ciprofloxacin, levofloxacin, and sulfamethoxazole/trimethoprim.

10 mM $MgSO_4$) in 96-well microplates (Nunc™, Roskilde, Denmark) in triplicate. Then, 20 μL of an overnight culture (10^8 CFU/mL) of each *Salmonella* strain was added to each well, and the plates were incubated at 2, 10 and 25 °C for 5 h. The negative controls contained only mTSB, while positive controls consisted of mTSB plus the *Salmonella* cocktail (10^8 CFU/mL). After incubation, the optical density (OD) of all wells was measured at 630 nm with a microplate reader (BioTek ELx800; BioTek Instruments Inc., Winooski, VT). Reduction of the *Salmonella* population in microplate wells was determined after 5 h. Treatment and control groups in microplates were serially diluted from 10^{-1} to 10^{-7} by adding 20 μL of each sample into 180 μL buffered peptone water (BPW) (Criterion™, Hardy Diagnostics). Diluted samples were spread-plated onto XLT4 agar and incubated at 37 °C overnight. The multiplicity of infection (MOI) was calculated as the initial number of phages (PFU/mL) in the highest dilution wells divided by the initial number of bacteria added (CFU/mL). The process was replicated three times generating 12 observations for each strain at each of the three temperatures.

To test *Salmonella* survival, 2 μL from control and treatment groups from the microplates were taken from each well and plated on XLT4 agar. The agar plates were incubated at 37 °C overnight and examined for the presence of *Salmonella* colonies.

2.4. Spot plate technique to assess Salmonella survival

The microplate virulence assay (Section 2.3.) will indicate if the bacteriophages are effectively reducing *Salmonella* populations based on optical density (OD) measurements. However, a clear well does not necessarily mean that the bacteria was killed, only indicates a reduction or a bacteriostatic effect; this is the case when testing phage effectiveness at low temperatures, like 2 and 10 °C. At 2 °C *Salmonella,* a mesophilic bacterium, is not able to grow or its growth rate will be very slow. Therefore, another technique such as spot plate inoculation is needed to test *Salmonella* survival. After the microplates were prepared and incubated as described in Section 2.3, 2 μL of sample from control and treatment groups was taken from each well and plated on XLT4 agar. Agar plates were incubated at 37 °C overnight and examined for the presence of *Salmonella* colonies.

2.5. Produce and mung bean preparation

Romaine lettuce (Dole™ pre-packed romaine hearts, California, U.S.A), mung bean sprouts (purchased from local supermarket's in bulk, no brand provided), and mung bean seeds (Mumm's Sprouting Seeds. Parkside, SK, Canada) were kindly donated by Mumm's. Romaine lettuce was prepared by removing the outer leaves and portions of the stems 3 cm from the root before washing. Whole sprouts with root, stem, and cotyledon were selected for the study. Mung bean seeds were examined under a stereomicroscope (Richter Optica U2B Binocular Lab Microscope, China) and only those seeds that exhibited no visible damage were selected for the study.

2.6. Spot inoculation of Salmonella strains on lettuce and sprouts

To determine SalmoFresh™ effectiveness to reduce *Salmonella* on the surface of lettuce and mung bean sprouts, the first set of experiments was conducted using the spot-inoculation method. Spot-inoculation involves the placement of a drop of bacterial suspension onto the surface of produce allowing the control of the inoculation site, facilitating the application of phages directly on the bacteria inoculation site. The intention was to obtain data for proof of concept before embarking in scaled-up experiments. Before spot-inoculating *Salmonella* onto fresh produce, lettuce and sprouts were washed three times with tap water at 10 °C ($<$ 0.5 ppm free chlorine). Lettuce pieces were cut into 3×3 cm^2 with a sterile scalpel, and whole sprouts were selected and placed on square Petri dishes (10×10 cm^2 plastic, Fisherbrand®, Pittsburg, PA, US) using sterile tweezers. Lettuce and sprouts were sprayed with 70% ethanol and air-dried for up to 3 h to reduce the presence of background flora (Ferguson et al., 2013). Produce samples were spot-inoculated (2 μL/spot, 5 spots/lettuce piece, 3 spots/sprout) with the 10^5 CFU/mL diluted *Salmonella* cocktail. *Salmonella* inoculum concentration used in this paper (10^5 CFU/mL) was chosen following FDA recommendations and previously reported data (FDA, 2018; Sharma et al., 2017). The 5 spots for each lettuce piece were evenly inoculated over the leaf surface, and three spots for sprouts were inoculated onto the cotyledon, stem, and root. After spot inoculation, all samples were air-dried at 25 °C for 3 h. After air-drying, samples from treatment groups were treated with SalmoFresh™ (10^8 PFU/mL) using a commercial sprayer (HX50; Yuyao Dongxia Sprayer Plastic Industrial Co. Ltd., Ningbo, China) delivering 0.84 mL and 0.63 mL to each piece of lettuce and sprout, respectively. Samples from treatment and control groups were packed in sterile bags (Whirl-Pak™, Nasco, Fisher, US) and stored at 2, 10, and 25 °C for 1, 24, 48, and 72 h. Negative controls consisted of inoculated, untreated samples.

In this section, three repetitions by quadruplicates per treatment (n = 16 lettuce/sprouts pieces' x 3) were conducted; with a total of 48 pieces of produce per treatment.

To recover *Salmonella* from experimental lettuce and sprouts, after each storage, serial BPW (90 mL) was added to produce samples containing bags and homogenized for 1 min in a stomacher (Interscience, St. Nom, France). Samples (1 mL) were serially diluted (1:10) (10^{-1} to 10^{-7}) in sterile BPW and 100 μL were plated onto XLT4 agar. Each experiment was repeated three times by quadruplicates.

2.7. Scaled-up experiments

2.7.1. Lytic phage and chlorinated water treatments

A scaled-up experiment using a larger quantity of produce and mung bean seeds was conducted. Lettuce leaves (600 g), sprouts (300 g) and mung bean seeds (30 g) were washed three times with tap water at 10 °C. Excess water was removed using a sterile salad spinner (OXO Good Grips, Elmira, NY, US). Samples were adulterated by immersion in a 10^5 CFU/mL diluted *Salmonella* cocktail for 30 min to allow bacterial attachment. Inoculated samples were assigned to two different treatments. For Treatment 1 (Chlorination) (Tr1), the inoculated samples were washed with chlorinated water (200 ppm for lettuce and sprouts, 1500 ppm for seeds) with agitation for 3 min, followed by three rinses with tap water (0.02 ppm chlorine, 10 °C). For Treatment 2 (SalmoFresh™) (Tr2), the inoculated samples were immersed in 10^8 PFU/mL diluted SalmoFresh™ solution in sterile tap water for 15 min (lettuce and sprouts) or 1 h (mung bean seeds). For treatment 3 (Tr3), the inoculated samples were washed with chlorinated water as explained in treatment 1 and then treated with SalmoFresh™ as explained in treatment 2. Positive and negative controls were included. Positive controls consisted in samples inoculated with *Salmonella* cocktail immersed in the same volume of sterile, cold water. Negative controls consisted of a set of samples without treatment or inoculation. The chlorinated water used for Tr1 was prepared by mixing commercial bleach containing sodium hypochlorite as the main ingredient (Old Dutch®, Montreal, QC, Canada) in 10 °C deionized water to obtain two concentrations of either 150 or 1000 ppm. Concentrations of chlorine in the water were confirmed using Quantab test strips (Hach Co., Loveland, CO, US) before application. Lettuce and sprouts (10 g each) were randomly collected with sterile tweezers from treatment and control groups, packaged in sterile stomach bags (Whirl-Pak™, Nasco, Fisher, US) and stored at 2, 10, or 25 °C for 1, 24, 48, or 72 h. Adulterated seeds (10 g) were taken from treatment and control groups, dried at room temperature (22 °C) for up to 1 h, and stored in sterile bags (Whirl-Pak™, Nasco, Fisher, US) in the dark at 25 °C for 72 h. After 72 h, half of the treated seeds were germinated to sprouts by soaking the seeds in sterile water for 24 h and then arranged in sterile trays containing towel paper and sprayed with sterile water 2 times/day for 3 days at 37 °C.

At the end of storage and sprouting, each type of sample (lettuce, sprouts, MBS, and LG sprouts) were placed in individually labelled Whirl-Pak® bags with 90 mL BPW and homogenized using a stomacher for 2 min (Intersciences Inc., Markham, ON, Canada).

2.8. Chlorine effects on SalmoFresh™ effectiveness

2.8.1. Free chlorine concentration measurement on fresh produce

Free chlorine concentrations were measured in the water used to wash the produce, specifically on the water used to deliver the phage cocktail to the produce, the chlorinated water (200 and 1500 ppm) and the residual free chlorine in the produce. For this purpose, free chlorine strips (HACH Co., Loveland, CO, US) and a free chlorine kit (DR300, HACH Co., Loveland, CO, US) were used. Since the strips are less accurate, later in this paper we report the measurements obtained with DR300.

After produce was treated with chlorinated water (200 or 1500 ppm), the chlorinated water was removed and kept for free chlorine concentrations measurement. Later the produce was rinsed 3 times with tap water (0.02 ppm free chlorine) during a 3 min time interval. The water from each rinse was kept separate (R1, R2 and R3) and free-chlorine concentrations were measured.

2.8.2. Effect of free chlorine on phage cocktail

To test the effect of the chlorine on phages lytic activity, a microplate technique was used. For this purpose, 180 μL of chlorinated, deionized water at different concentrations (9.6 ppm, 6.4 ppm, 3.2 ppm, 1.6 ppm, 0.8 ppm, 0.4 ppm, 0.16 ppm) were added to different columns. Controls consisted in phage cocktail plus *Salmonella* cocktail only. Free chlorine concentrations were chosen based on the free chlorine obtained from the R1, R2 and R3. Then, 20 μL of SalmoFresh™ (10^8 PFU/mL) was transferred into all wells, followed by the addition of 20 μL of *Salmonella* cocktail dilution (10^5 CFU/mL). Blanks consisted on 200 μL of deionized water (0 ppm free chlorine). Microplates were incubated for 5 h. at 37 °C. After incubation, 10 μL of Dey-Engley neutralizing broth (Difco, BD) was added to each well. From each well, 2 μL of the mixture were spotted into XLT4.

2.9. Lysis-from-without (LO)

To explore if LO could be present in the *Salmonella* phage cocktail a microplate assay was applied as explained in Section 2.3. with some modifications. The microplates incubation time were 5, 10 and 15 min. and the temperatures at which the phages (10^8 PUF/mL) were tested were 2 and 25 °C. The culture media were added to the microplates 24 h before inoculation to ensure a uniform temperature (2 °C). *Salmonella* strains and phages were also chill before added to the microplate when tested at 2 °C. Each *Salmonella* strain was tested individually (10^5 CFU/mL) (Abedon, 2011).

2.10. Salmonella detection

After storage, lettuce and sprouts were homogenized in 90 mL of BPW in a stomacher (Interscience) for 1 min; while mung bean seeds were sonicated for 30 s. (ultrasonic bath, Fisherbrand®, UK). Serial dilutions (1:10) (10^{-1} to 10^{-7}) were prepared in sterile BPW and 100 μL aliquots were spread-plated on XLT4 agar plates. For Tr1, D/E neutralizing broth (Difco) was used to neutralize chlorine and prevent downstream inhibition of *Salmonella.*

For instances where *Salmonella* colonies were not recovered by direct plating, samples were subject to immunomagnetic separation (IMS). The IMS method is based on the use of beads coated with antibodies against *Salmonella* allowing *Salmonella* concentration in a small volume by exposure to a magnetic field (Cudjoe et al., 1995); therefore, this method is recommended when the target pathogen is suspected to be present in low numbers. Briefly, 10 g of sample was enriched in 90 mL of modified tryptic soy broth [TSB (Oxoid, Nepean, ON, Canada) containing 10 mg/L of novobiocin (Alfa Aesar Co. Inc., Ward Hill, MA) for 24 h at 37 °C. After incubation, IMS was conducted using Dynabeads® (Dynal, Lake Success, NY) and a bead retriever tube rack (Dynal Lake Success, NY, US) as per the manufacturer's instructions. Briefly, 1 mL of the enriched sample was mixed with 10 μL of Dynal anti-*Salmonella* beads in a 1.5 microcentrifuge tube for 15 min. Beads were washed three times with 1 mL phosphate buffered saline (PBS)-Tween 20 (TEKnova, Hollister, CA, US), before 100 μL of the bead-bacteria mixture was plated onto XLT4 agar using a sterilized cotton swab. Plates were incubated overnight at 37 °C and *Salmonella* colonies were confirmed by latex agglutination (Oxoid, Basingstoke, United Kingdom).

2.11. Bacteriophage-resistance test

Up to 10 surviving *Salmonella* colonies from each SalmoFresh™ treatment group by repetition were isolated by streaking onto XLT4 agar. Isolates were grown to of 10^8 CFU/mL in 200 μL TSB + 15% glycerol at 37 °C for 24 h and stored at −80 °C. Susceptibility of these isolates to SalmoFresh™ was subsequently tested using the *in vitro* microplate virulence assay as described in Section 2.3.

2.12. Statistical analysis

Data were subject to analysis of variance using the ANOVA procedure of SAS (SAS version 9.2, SAS Institute Inc., Cary, NC, US). A complete random design (CRD) was used with the MIXED procedure of SAS. The fixed effects of treatment, time and temperature along with associated interactions were included in the model. Where interactions were not significant, only main effect variables were tested. Responses were considered significant at $P < 0.05$.

3. Results

3.1. In vitro microplate virulence of SalmoFresh™

The phage cocktail exhibited strong lytic activity against individual *Salmonella* strains when used at high concentrations (10^8 PFU/mL) with an overall reduction of 5.34 log_{10} CFU/mL of the 5 strain mixture of *Salmonella* (Fig. 1). As Table 4 shows, after 5 h, there was virtually no growth of *Salmonella* at 2 and 10 °C as showed by optical density measurements when compared to controls. At 25 °C, microplate wells showed lysis; however, the spot-plate method indicated that all *Salmonella* isolates survived in microplates after bacteriophage treatment. After 5 h at 2 and 10 °C, SalmoFresh™ killed all 5 strains at 10^8 PFU/mL, but when the phage concentration was diluted to 10^7 PFU/mL, some *Salmonella* survived. All positive controls showed the existence of *Salmonella*.

The overall MOI values were higher at 2 and 10 °C (MOI = 90) than

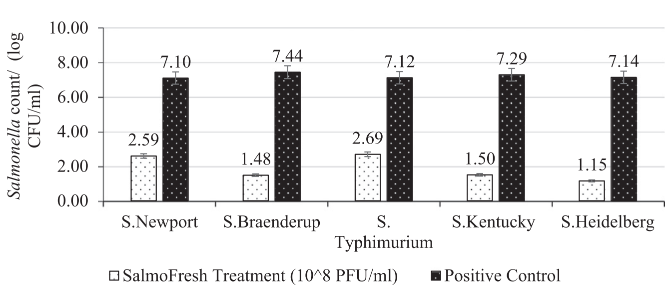

Fig. 1. *Salmonella* count in the *in vitro* microplate virulence assay for control cultures (10^8 CFU/mL) or cultures (10^8/mL) treated with 10^8 PFU/mL of bacteriophage (SalmoFresh™) at 25 °C for 5 h.

Table 3
Effect of temperature on the multiplicity of infection of (MOI) of SalmoFresh™ required for lysis of five *Salmonella* strains.

Salmonella strains	Temperature		
	2 °C	10 °C	25 °C
S. Newport	145^A	145^A	0.1^B
S. Braenderup	96^A	956^A	0.006^B
S. Typhimurium	65^A	65^A	0.06^B
S. Kentucky	49^A	49^A	0.03^B
S. Heidelberg	100^A	100^A	0.1^B

A, B:: different letters within a row indicate differences at $P < 0.05$.
MOI = Plaque forming units (PFU) of phages used for infection divided by the number of cells.

at 25 °C (MOI = 0.05) ($P < 0.001$), indicating that more bacteriophage particles were required to lyse 10^8 CFU/mL of *Salmonella* at lower temperatures (Table 3.).

3.2. Effectiveness of SalmoFresh™ in reducing Salmonella on fresh produce and mung bean seeds

3.2.1. Spot-inoculation lettuce and sprouts with SalmoFresh™ delivered by spraying

For spot-inoculated lettuce and sprouts sprayed with SalmoFresh™, the overall reduction regardless of temperature or storage time was 0.76 log_{10} CFU/g for lettuce and 0.83 log_{10} CFU/g for sprouts. The maximum reduction observed after 72 h on lettuce was about 1 log_{10} at 2 and 10 °C with this same reduction being achieved after 24 h at 25 °C ($P < 0.001$) (Fig. 2). Similarly, other researchers showed a 1–2 log CFU/cm^2 reduction of *E. coli* O157:H7 on fresh-cut spot-inoculated lettuce surfaces stored at 4 °C when sprayed with a mixture of lytic bacteriophages (Sharma et al., 2009). For sprouts, reductions ($P = 0.001$) were about 0.9 log_{10} CFU/g at 2 °C after 48 h, and ~1 log_{10} CFU/g at 10 and 25 °C after 24 h of storage (Fig. 2).

3.2.2. Scaled-up immersion inoculation and immersion treatment with bacteriophage

3.2.2.1. Scaled-up lettuce study. Immersion in a bacteriophage solution resulted in a 2.43 log10 CFU/g reduction ($P < 0.001$) of *Salmonella* on lettuce (Fig. 3.), higher than that obtained by spraying the bacteriophages onto lettuce. Washing with chlorinated water at 200 ppm achieved 2.41 log_{10} CFU/g reduction ($P < 0.001$). When both treatments were combined (Tr3), the overall reduction was 3.8 log_{10} CFU/g. Treatment efficacy was affected by temperature ($P < 0.001$), where all of the treatments were more effective at 2 and 10 °C while less effective at 25 °C. Among the three treatments, the combination of chlorinated water fallowed by SalmoFresh™ at 2 and 10 °C, showed to be the most effective treatment.

3.2.2.2. Scaled-up sprouts. The reduction in *Salmonella* on sprouts (2.26 log_{10} CFU/mL) as a result of bacteriophage treatment (Fig. 4) was lower ($P < 0.001$) than that achieved with lettuce. Washing with 200 ppm chlorinated water reduced ($P = 0.0075$) *Salmonella* (1.83 log_{10} CFU/g) on sprouts as compared to SalmoFresh™ (2.16 log_{10} CFU/g) at all three temperatures. The combination of treatments showed a reduction of 2.7 Log_{10} CFU/g. When comparing immersion with spray as a method to deliver the phages, similarly to what was observed in lettuce.

3.2.2.3. Scaled-up Mung bean seeds and germinated seeds. Mung bean seeds were adulterated with a 10^5 CFU/mL *Salmonella* cocktail; however, only 2.33 log CFU/g of *Salmonella* attached to the seeds. After the seeds were treated with the phage cocktail and chlorinated water (1000 ppm) *Salmonella* was reduced by 2.28 and 0.04 log^{10} CFU/g respectively. Using both methods, the reduction was 1.28 log_{10} CFU/

Table 4
Result for survival (spot plate assay) and lysis (microplate assay) of *Salmonella* strains at 2 °C, 10 °C and 25 °C after being treated with SalmoFresh™ at various concentrations.

Temp.	SalmoFresh™ Conc. (PFU/mL)	*S.* Newport		*S.* Braenderup		*S.* Typhimurium		*S.* Kentucky		*S.* Heidelberg	
		Lysis	Survival	Lysis	Survival	Lysis	Survival	Lysis	Survival	Lysis	Survival
2 °C[a]	10^8	+	–	+	–	+	–	+	–	+	–
	10^7	+	+	+	+	+	+	+	+	+	+
10 °C[a]	10^8	+	–	+	–	+	–	+	–	+	–
	10^7	+	+	+	+	+	+	+	+	+	+
25 °C	10^8	+	+	+	+	+	+	+	+	+	+
	10^7	+	+	+	+	+	+	+	+	+	+

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

g. However, after seed germination, *Salmonella* numbers recovered to reach a population of 7.74 and 8.04 logs CFU/mL on sprouts originating from seeds treated with the phage cocktail and chlorinated water respectively (Fig. 5). The combination chlorinated water and SalmoFresh™ did not further reduce the numbers or prevent the growth of *Salmonella* on contaminated seeds during sprouting (Fig. 5).

To test if *Salmonella* developed phage resistance, surviving isolates from produce, were exposed to the phage cocktail, and all the tested colonies exhibited sensitivity to the phage cocktail.

3.3. Chlorine effects on phage cocktail

Results for free chlorine concentrations in scaled-up trials were as follows for lettuce, the chlorinated water (CW) concentration was 225 ppm, and the recovered chlorinated water concentration was 200 ppm. Lettuce rinses were as follow: R1 (3.6 ppm), R2 (0.01 ppm), R3 (0.0 ppm). Sprouts were as follows: CW (225 ppm), recovered chlorinated water (13.5 ppm). Sprout rinses were R1 (0.55 ppm), R2 (0.01 ppm), R3 (0.05). or Mung beans seeds were CW (1505 ppm), recovered chlorinated water (1495 ppm), seed rinses R1 (66.5 ppm), R2: 0.85 ppm), R3 (0.01 ppm). Results showed that for all produce and seeds, after the third rinse, there was no free chlorine remaining.

3.4. Chlorine effects on phage cocktail

Microplate results from the different chlorinated water concentrations on the phage cocktail showed that concentrations of 9.6 to 0.16 ppm did not significantly affect phage lytic activity when compare with controls without chlorine (Table 5).

3.5. Lysis from without

At 2 and 25 °C optical density measurements showed no differences with controls at 5, 10 and 15 min. At both temperatures, all wells were turbid indicating no lysis. Spot-plate results did not show any differences with positive controls. Among strains, no differences were observed (data not shown). Results collected from the microplates indicated that the *Salmonella* phage cocktail did not possess LO (Abedon, 2011).

4. Discussion

The process of ensuring food safety is complicated because microbial contamination can occur at any of the pre-harvest and post-harvest steps in the farm-to-consumer chain. Among the various antimicrobial strategies, bacteriophages are considered a novel tool for post-harvest biocontrol of foodborne pathogens in the food processing industry, and

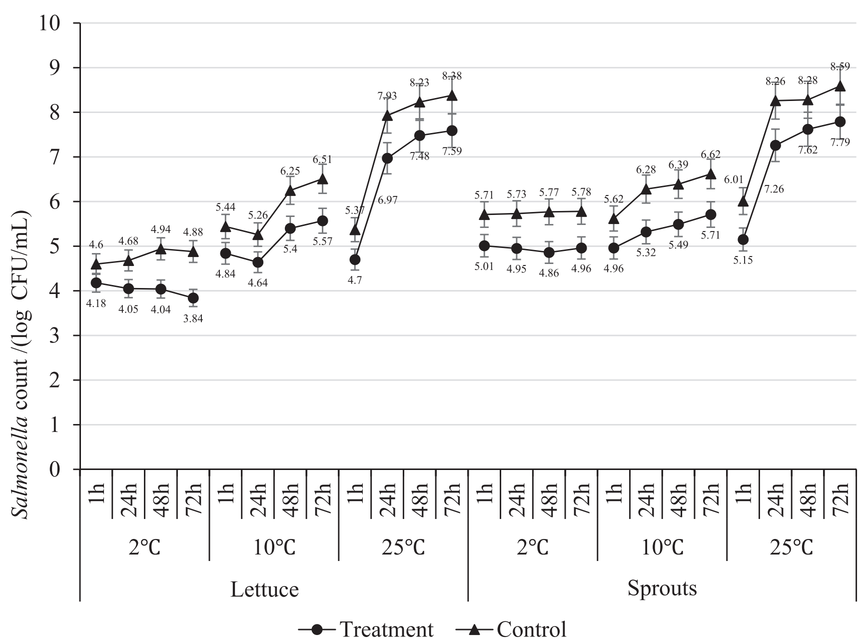

Fig. 2. *Salmonella* count in a mixture of 5 *Salmonella* strains spot-inoculated (CFU/g) onto a) lettuce and b) sprouts after spraying with a mixture of bacteriophage (SalmoFresh™) relative to positive controls at 2, 10 and 25 °C and stored for 1, 24, 48 and 72 h.

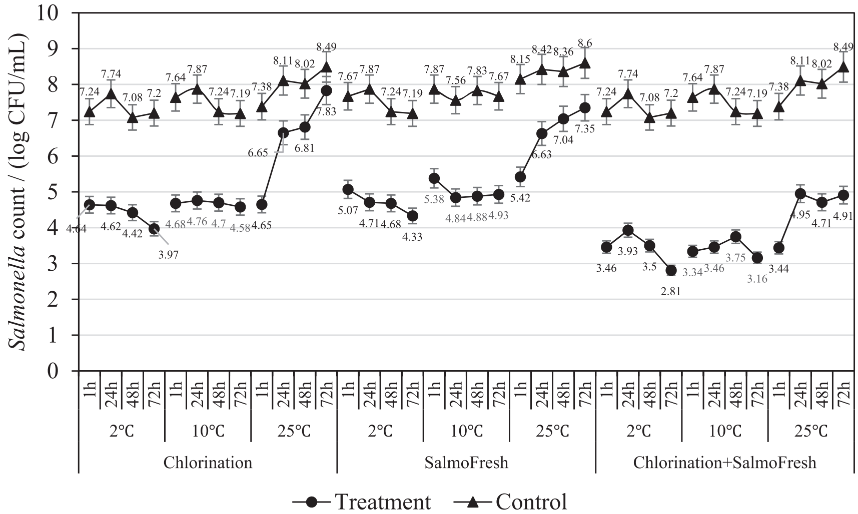

Fig. 3. *Salmonella* count for scaled-up lettuce treated by a) 150 ppm chlorinated water b) 10^8 PFU/mL SalmoFresh™ delivered through immersion c) SalmoFresh (10^8 PFU/mL + chlorinated water 150 ppm related to positive control. Each group at 2, 10 and 25 °C was treated for 1, 24, 48 and 72 h.

they have been increasingly used for this purpose in recent years (Sharma, 2013). Since the consumption of fresh fruits and vegetables are widely acknowledged as a crucial component of a healthy diet for humans, using bacteriophage to mitigate food-borne pathogens in fresh produce is of increasing interest.

In this study, a 5.34 $\log_{10}$ CFU/mL reduction in *Salmonella* was achieved by phages with MOI of 0.05 at 25 °C in broth culture. However, when phage was applied to produce at 2 and 10 °C, the MOI required to kill *Salmonella* was 1800 times higher than that needed at 25 °C. At 25 °C, bacteriophages were efficient at infecting *Salmonella*, which upon bacterial cell lyses released additional phage progeny which infected additional host cells and enhanced the disinfection process. In contrast, at 2 and 10 °C bacteria are metabolically active but at a slow rate, and as a result, the lytic cycle of bacteriophages is inhibited (Sharma, 2013). At refrigeration temperatures, the process of 'lysis from without (LO)' has been proposed to be responsible for the ability of bacteriophage to lyse bacterial cells (Ferguson et al., 2013). The proposed mechanism of LO action is that the adsorption of large numbers of bacteriophages to the bacterial cell envelope can result in the formation of weak points within its molecular structure which leads to host lysis (Abedon, 2011). When the number of bacteriophages attaching to the cell wall exceeds a threshold level (MOI > 50), the contents of cells are liberated due to distension and the rupture of the cell wall (Abedon, 2011). In this way, bacteriophages can still effectively kill pathogens at lower temperatures even though the lytic cycle of bacteriophage reproduction is not active. Data from the LO test showed that the tested *Salmonella* cocktail did not exhibit LO. Nevertheless, the cocktail is composed by 6 phages. Ideally each phage should be tested separately. This was not possible since the host for propagation was not disclosed by the company neither individual phages were provided. However, individual strains were tested which show the specific phage performance. Results from this research indicated that none of the tested *Salmonella* strains were lyse after being exposed to the phage cocktail during 5, 10 or 15 min. and no differences were observed by temperature; therefore, is not likely that this phage cocktail has LO properties. More research is needed to determine mechanistic behind phage effectiveness on mesophilic foodborne pathogens under refrigerated storage.

Another concern related to using bacteriophages as a biocontrol method is the emergence of bacteriophage-resistant bacteria (Demerec and Fano, 1945; Leon and Bastias, 2015). In an attempt to resist bacteriophage infection, bacteria can alter or not produce cell surface

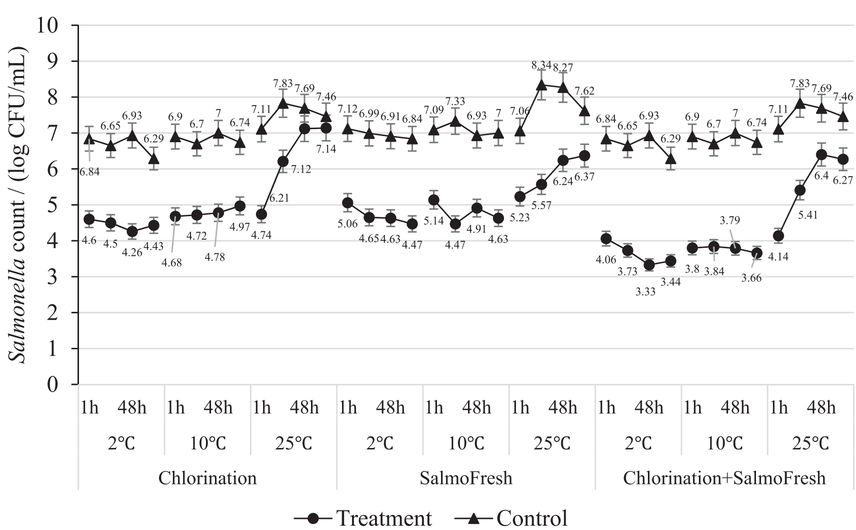

Fig. 4. *Salmonella* count for scaled-up sprouts treated by a) 150 ppm chlorinated water b) 10^8 PFU/mL SalmoFresh™ delivered through immersion c) 10^8 PFU/mL SalmoFresh + 150 ppm chlorinated water related to positive control. Each group at 2, 10 and 25 °C was treated for 1, 24, 48 and 72 h.

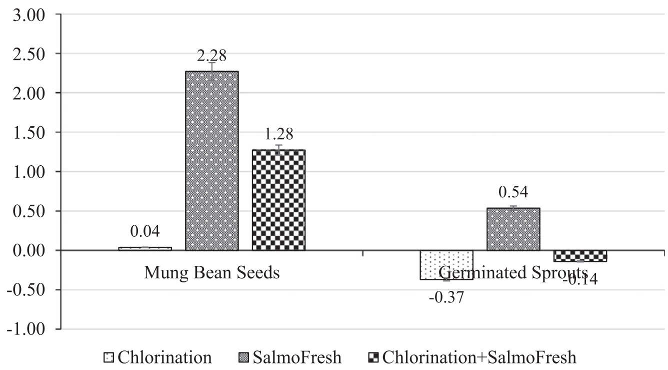

Fig. 5. *Salmonella* reduction for scaled-up seeds and germinated sprouts treated by a) 1000 ppm chlorinated water b) 10^8 PFU/mL SalmoFresh™ delivered through immersion c) 10^8 PFU/mL SalmoFresh + 1000 ppm chlorinated water related to positive control. Mung-bean seeds were adulterated with a 10^5 CFU/mL *Salmonella* mixture; control seeds showed an average *Salmonella* load of 2.3 log CFU/g.

Table 5
Spot plate results from microplate assay for *Salmonella* cocktail (10^5 CFU/mL) after exposure to phage cocktail and different chlorine concentrations.

Chlorine (ppm)	*Salmonella* cocktail control	*Salmonella* phage cocktail
9.60	+	–
6.40	+	+/−
3.20	+	+/−
1.60	+	+/−
0.80	+	+/−
0.40	+	+/−
0.16	+	+/−
0.00	+	–

+: > 10 colonies observed (> 10).
+/−: < 3 colonies observed (≦ 3).
–: no colony observed (0).

proteins (OmpA, OmpC, and OmpF) or lipopolysaccharides that serve as specific receptors for bacteriophage (Kudva et al., 1999; Tanji et al., 2004). Because bacteria are likely to develop resistance to a single bacteriophage, simultaneous application of multiple bacteriophages within a cocktail is often used as a method to thwart resistance (Fischer et al., 2013). SalmoFresh™, consists of a 6-bacteriophage cocktail probably assemble that way as a means of avoiding resistance.

The ability of bacteriophage to reduce *Salmonella* was lower when it was applied to produce by spraying as compared to immersion. A higher reduction in *Salmonella* on lettuce (2.43 log10 CFU/g) was observed when bacteriophages were applied by immersion rather than by spraying (0.76 $\log_{10}$ CFU/g). One possible explanation for this discrepancy could be that the previous study sprayed larger volumes of bacteriophages (0.04 mL/cm^2) (Ferguson et al., 2013) which would more closely duplicate the conditions associated with immersion in a bacteriophage cocktail. Immersion more closely emulates industry practices as produce is most commonly immersed in chlorinated water (Gil et al., 2015) followed by at least three rinses with chilled fresh water to remove chlorine. In this study, lettuce was inoculated with bacteria by either spot inoculation or immersion. With immersion, the possibility of increased capillary effects may result in *Salmonella* cells entering the interior of lettuce leaves as opposed to just being associated with the outer surfaces. *Salmonella* in the interior of the lettuce leaves may be less susceptible (limited collision) to bacteriophage attack than those that lie on the leaf surface.

When SalmoFresh™ was applied to inoculated produce, the reduction of *Salmonella* was higher at 2 and 10 °C than at 25 °C. At 25 °C, the large population of *Salmonella* cells due to the greater replication at optimum growing temperature provide the bacteriophage more targets to start the lytic cycle. However, maintaining produce at refrigeration temperatures is the primary means of inhibiting the growth of bacteria at retail and in households. At 25 °C, the population of *Salmonella* on both lettuce and sprouts increased by at least 3 logs CFU/g after 72 h. In contrast, over this same storage period at 10 °C, the *Salmonella* population increased by only 1 log CFU/g, and growth was negligible at 2 °C. These results agree with Leverentz et al. (2001), emphasizing that maintaining produce at 2 °C is an important strategy for restricting the growth of foodborne pathogens. Therefore, when bacteriophages were applied to produce, the reduction of *Salmonella* was more significant at 2 and 10 °C compared to 25 °C likely due to the growth restriction of *Salmonella* at low temperatures. SalmoFresh™ effectiveness to reduce *Salmonella* on produce has been evaluated by others. A research investigating SalmoFresh™ ability to reduce *Salmonella* Newport (5 log CFU) on fresh cucumbers when the SalmoFresh™ was applied by spraying, and under refrigerated storage reported that *Salmonella* populations on phage-treated cucumbers stored at 10 °C were 1.72 ± 0.77 and 1.56 ± 0.46, significantly lower than those on control-treated cucumbers (3.20 ± 0.48 and 2.33 ± 0.25) on days 1 and 4. These results are similar to the ones observed here when using spraying to deliver the phages (Sharma et al., 2017).

SalmoFresh™ was more effective than chlorinated water alone, and the combination of both treatments at reducing *Salmonella* on mung bean seeds. However, when the seeds were germinated, up to 10^8 CFU/g *Salmonella* were recovered from the sprouted seeds. Mung bean seeds need a warm environment and moisture to germinate. During soaking and germination, seeds release macromolecules such as proteins and other nutrients, and these conditions provide the perfect environment for bacterial growth (Yang et al., 2013). Organic material can also inactivate free chlorine, therefore reducing it antimicrobial effects. In this work neither phages nor chlorinated water were able to kill *Salmonella* on adulterated mung bean seeds completely. Therefore, the remaining *Salmonella* cells had the ideal conditions for recovery on the laboratory sprouted seeds, which explained the high *Salmonella* population recovered. Kocharunchitt et al. (2009) reported similar results, as application of lytic bacteriophage (bacteriophage SSP5 and SSP6) resulted in a 1 log reduction of *Salmonella* on inoculated alfalfa seeds after 3 h, but bacteriophages failed to inhibit the growth of *Salmonella* during germination. Warriner et al. (2003) reported that once *Salmonella* came in contact with mung bean seeds, they could be isolated from both the surface as well as from interior tissues after germination. Since internalized *Salmonella* are hard to kill by post-harvest biocidal methods, care should be taken to ensure that mung bean seeds and other seeds used for the preparation of sprouts are not contaminated during harvest and processing. The internalization of *Salmonella* through the use of contaminated irrigation water during germination likely accounts for the negligible *Salmonella* reduction on germinated sprouts (Ye et al., 2010). Bacteria may enter the plant following physical/biological damage, by natural openings on the tissue surface (*i.e.*, stomata, lenticels, sites of lateral root emergence) or through capillary action promoting the flow of bacteria into internal tissues (Deering et al., 2012). Others have also documented the internalization of *E. coli* O157:H7 in lettuce during washing (Erickson et al., 2010; Li et al., 2008). Moreover, *Salmonella* has been reported to have the advantage of more effectively attaching to sprouted seeds as compared to *E. coli* O157:H7, potentially explaining why sprout-related outbreaks have been more common for *Salmonella* (Barak et al., 2002). One potential reason for this difference is that *Salmonella* has a better ability to produce aggregative or curli fimbriae in low temperature and low osmolarity environments and *E. coli* O157:H7 (Barak et al., 2002). Regarding pathogen internalization, Jones et al. (2007) reported that bacteriophage was absorbed into the plant through the roots after 3 h of immersion in a bacteriophage solution, and was still detectable in roots and stems two weeks later. In this way, the lysis of pathogens by bacteriophages might occur inside the plant tissue if both were y internalized.

The observation that bacteriophage, the chlorinated water or a combination of both interventions were not able to completely eliminate *Salmonella* on lettuce, sprouts and seeds, complicates the use of bacteriophage as a biocontrol intervention. However, the reduction achieved by the combination of treatments (Tr3) at 2 and 10 °C on lettuce is promising. The reductions in this case were 4.30 and 4.03 at 2 and 10 °C respectively, which are better than reduction reported by others, mentioned in previous sections, using only SalmoFresh™. However, this combination of treatment did not work as well for sprouts and seeds. It is important to mention that the free chlorine remaining in the third rinse was negligible, indicating that the free chlorine is not affecting phage lytic activity, therefore, likely allowing higher *Salmonella* reduction. Enhanced reduction might be due to a synergistic effect, where *Salmonella* numbers were first reduced by chlorine and later further reduced by the phage cocktail. Researchers have reported that treatment of bacteriophage combined with other interventions (*i.e.*, organic acid) can reduce *Salmonella* to a safe level when contamination is < 2 log CFU/mL (Hara-Kudo and Takatori, 2011; Viazis et al., 2011). In this research chlorinated water effectiveness to reduce *Salmonella* was similar to the phages effectiveness or less, the combination of both treatments was effective to reduce *Salmonella* on lettuce, but was not as effective for sprouts and seeds. This reduced effectiveness on sprouts and seeds is probably due to the larger organic material that can be detached from sprouts and seeds, which can inactivate free chlorine. In this research, lettuce and sprouts were treated with 225 ppm of free chlorine. After exposure the free chlorine was reduced to 200 ppm still remained in lettuce, but only 13.5 free chlorine remained on sprouts, showing that when chlorine is exposed to sprouts, the free chlorine can be inactive to a greater degree than lettuce. Regarding seeds, the inactivation of free chlorine was lower, from 1505 ppm to 1495 ppm. A potential problem with the seeds is that *Salmonella* strains could be able to survive sheltering within the seed's crevices and cracks. Thus, it is important to consider the complex interaction between bacteriophage and host cells as well as the type of food plus other potential combinatory antimicrobial hurdles. Factors like the minimum host threshold, host resistance, and host specificity, are important for the successful application of bacteriophage as a biocontrol intervention on produce (Hudson et al., 2005). There are 15 *Salmonella* host serotypes that can be used by the bacteriophage in SalmoFresh™: Typhimurium, Enteritidis, Heidelberg, Newport, Hadar, Kentucky, Thompson, Georgia, Agona, Grampian, Senftenberg, Alachua, Infantis, Reading, and Schwarzengrund (Intralytix, 2015) serotypes often related to human infection. This means SalmoFresh™ is unlikely to be able to target all > 2700 *Salmonella* serotypes. To reduce the chance of commercial produce contamination by *Salmonella* serotypes insensitive to SalmoFresh™, it may be necessary to develop a cocktail with a wider range of bacteriophage types that target those *Salmonella* serotypes that most commonly associated with human illness.

In this study, no bacteriophage resistant strains were observed. Bacteria may become resistant to bacteriophage through several mechanisms including preventing bacteriophage adsorption, blocking the injection of bacteriophage nucleic acids and restricting resistance modification systems (Kim and Kathariou, 2009). It is of interest that Kim and Kathariou (2009) reported that the expression of the bacterial genes involved in bacteriophage resistance can be temperature-dependent. The low metabolic rate of bacteria at 2 and 10 °C might potentially reduce the ability of bacterial cells to initiate resistance mechanisms.

5. Conclusions

When SalmoFresh™ was applied to lettuce and sprouts by immersion, it was observed that the phages were more effective at reducing *Salmonella* than when they were applied by spraying. Overall, SalmoFresh™ resulted in similar or even greater reduction in *Salmonella* than chlorinated water, however when both interventions were combined a synergistic effect was observed for lettuce only at 2 and 25 °C. The susceptibility of surviving *Salmonella* isolates to bacteriophages was assessed and there was no evidence of emergence of bacteriophage resistance. However, for mung bean seeds, neither bacteriophage nor chlorinated water or a combination of both was able to significantly reduce *Salmonella* as this pathogen was recovered at levels of up to 4 $\log_{10}$ CFU/g after germination.

Although the sole use of SalmoFresh™ is not sufficient to completely eliminate *Salmonella* on fresh produce, it could be promising as part of a hurdle intervention approach to reduce this pathogen on lettuce and sprouts.

The phage cocktail was not effective at eliminating *Salmonella* from the seeds, and so far no other intervention has been reported to be effective at eliminating 100% pathogen contamination from seeds. Therefore, it is clear that additional precautions need to be taken to ensure that mung bean seeds are not contaminated with pathogens prior to germination.

Acknowledgement

Special thanks is given to the Ontario Ministry of Agriculture and Rural Affairs (OMAFRA), Canada (Grant # FS2014-2294) for funding this project, and to Mumm's for the kindly donation of the seeds.

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