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## Prophage induction reduces Shiga toxin producing *Escherichia coli* (STEC) and *Salmonella enterica* on tomatoes and spinach: A model study

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## Prophage induction reduces Shiga toxin producing *Escherichia coli* (STEC) and *Salmonella enterica* on tomatoes and spinach: A model study

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### ABSTRACT

Fresh produce is increasingly implicated in foodborne outbreaks and most fresh produce is consumed raw, emphasizing the need to develop non-thermal methods to control foodborne pathogens. This study investigates bacterial cell lysis through induction of prophages as a novel approach to control foodborne bacterial pathogens on fresh produce. Shiga toxin producing *Escherichia coli* (STEC) and *Salmonella enterica* isolates were exposed to different prophage inducers (i.e. mitomycin C or streptonigrin) and growth of the cells was monitored by measuring the optical density (OD<sub>600</sub>) during incubation at 37 °C. Beginning at three hours after addition of the inducer, all concentrations (0.5, 1, 2 µg/mL) of mitomycin C, or 2 µg/mL streptonigrin significantly reduced the OD<sub>600</sub> in broth cultures, in a concentration dependent manner, relative to cultures where no inducer was added. PCR confirmed bacterial release of induced bacteriophages and demonstrated that a single compound could successfully induce multiple types of prophages. The ability of mitomycin C to induce prophages in STEC O157:H7 and in *S. enterica* (serovars Typhimurium and Newport) on fresh produce was evaluated by inoculating red greenhouse tomatoes or spinach leaves with  $5 \times 10^7$  and  $5 \times 10^8$  colony forming units, respectively. After allowing time for the inoculum to dry on the fresh produce samples, 6 µg/mL mitomycin C was sprayed onto each sample, while control samples were sprayed with water. Following overnight incubation at 4 °C, the bacterial cells were recovered and plate counts were performed. A 3 log reduction in STEC O157:H7 cells was observed on tomatoes sprayed with mitomycin C compared to those sprayed with water, while a 1 log reduction was obtained on spinach. Similarly, spraying mitomycin C on tomatoes and spinach inoculated with *S. enterica* isolates resulted in a 1–1.5 log and 2 log reduction, respectively. These findings serve as a proof of concept that prophage induction can effectively control bacterial foodborne pathogens on fresh produce.

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### 1. Introduction

In recent years, fresh produce has been implicated in an increasing number of foodborne outbreaks involving different bacterial pathogens, including Shiga toxin producing *Escherichia*

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*coli* (STEC) and *S. enterica* (Bennett, Littrell, Hill, Mahovic, & Behravesh, 2015; Callejon et al., 2015; CDC, 2011, 2013; Dechet et al., 2014; Herman, Hall, & Gould, 2015; McCollum et al., 2013). Contamination of fresh produce can occur on the farm, pre- or post-harvest, and all along the different steps of the food production chain because of inadequate hygiene, agricultural and/or manufacturing practices (EFSA, 2013, 2014). There are currently no effective means to completely remove foodborne pathogens from fresh produce during post-harvest processing steps, such as washing (Goodburn & Wallace, 2013; Hellstrom, Kervinen, Lyly, Ahvenainen-Rantala, & Korkeala, 2006). Furthermore, the majority of fresh produce is consumed raw, thereby eliminating the possibility of using heat to inactivate pathogens which may be present. Finally, a global trend towards eating more fruits and vegetables, as part of a healthy lifestyle, has been observed over the last thirty years (European Commission, 2007). Together, these factors contribute to an increased likelihood of contracting foodborne illnesses from eating fresh produce. Retrospective analyses of USA outbreak and epidemiological data for the period 1998–2008 indicated that 46% of illnesses were attributable to fresh produce (Painter et al., 2013) and *Salmonella* was the most common bacterial etiological agent (Gould et al., 2013). Analysis of Canadian outbreak data for 2001–2009 confirmed this trend and showed that *Salmonella* was responsible for 50% of incidents linked to fresh produce (Kozak, MacDonald, Landry, & Farber, 2013). Since 2008, there have been at least 18 salmonellosis outbreaks linked to fresh produce in the USA (CDC, 2017). A recent and large outbreak occurred in 2015 and involved contaminated cucumbers, which resulted in 907 cases and 6 deaths (CDC, 2017).

Some antimicrobial-based strategies to control pathogens on fresh produce have been developed with varying success. The majority of reported techniques include addition of organic acids, essential oils, bacteriocins, or a combination thereof, directly on foods to inhibit bacterial growth or destroy the pathogen (Azizkhani, Elizaquivel, Sanchez, Selma, & Aznar, 2013; Bari et al., 2005; Ganesh, Hettiarachchy, Griffis, Martin, & Rieke, 2012; Landry, Chang, McClements, & McLandsborough, 2014; Leverentz et al., 2003; Oliveira, Abadias, Colas-Meda, Usall, & Vinas, 2015; Park et al., 2011). Unfortunately, these methods generally have poor efficacy and/or alter the organoleptic qualities of the food, resulting in an undesirable product for the consumer. Also, while post-harvest washing, using chlorinated water, is often employed, it is not considered an intervention step, but is used instead to limit cross contamination (Gombas et al., 2017). During the past decade, bacteriophages (phages) have emerged as a new class of antimicrobials for the control of bacterial pathogens on foods. In this approach, cocktails of virulent phages are applied onto the food to control specific pathogens (Goodridge & Bisha, 2011; Leverentz et al., 2003; Magnone, Marek, Sulakvelidze, & Senecal, 2013; Oliveira et al., 2015; Sulakvelidze, 2013). This approach is dependent on the ability of a phage to successfully infect its bacterial host, which depends on a number of factors, including pH and temperature, which can limit the utility of the technology for specific foods and pathogens (J. W. Kim et al., 2012; Tsonos et al., 2014). The approach is also limited when trying to control a single diverse pathogenic species such as *S. enterica*, which contains more than 2500 serovars; as such, current commercial phage cocktails to control *Salmonella* are limited to only a few serovars that cause the majority of salmonellosis cases (Grant, Parveen, Schwarz, Hashem, & Vimini, 2017; K. H.; Kim, Lee, Jang, Kim, & Kim, 2013; Woolston et al., 2013). Also, current phage cocktails are designed to reduce a single pathogenic species or pathotype of bacteria, such as STEC, *S. enterica*, or *Listeria monocytogenes*. Given these factors, and that multiple bacterial foodborne pathogens can be associated with a single food commodity (Callejon et al., 2015; Gould et al., 2013),

there is a need to design a more robust and effective antimicrobial approach which will be able to target a broader spectrum of foodborne pathogens present on fresh produce and other foods.

Genomic analyses demonstrate that most bacterial genome sequences deposited in public databases contain prophage sequences (Canchaya, Proux, Fournous, Bruttin, & Brussow, 2003; Kang et al., 2017). This includes the presence of prophages integrated within the genomes of foodborne bacterial pathogens, such as *Salmonella* spp., *L. monocytogenes*, *E. coli*, *Shigella* spp., and *Vibrio* spp. (Allison & Verma, 2000; Hayashi et al., 2001; Herold, Karch, & Schmidt, 2004; Klumpp & Loessner, 2013; Moreno Switt et al., 2013; Waldor & Mekalanos, 1996). In fact, the ability of STEC to produce Shiga toxin and *Vibrio cholerae* to produce Cholera toxin is due to the integration of toxin encoding prophages (Brabban, Hite, & Callaway, 2005; Gamage, Patton, Hanson, & Weiss, 2004; Herold et al., 2004; Wagner & Waldor, 2002; Waldor & Mekalanos, 1996). Phages exhibit one of two lifestyles: a virulent lifestyle and a temperate lifestyle. In contrast to virulent phages, which can only grow lytically, temperate phages display lysogenic growth, meaning that once they infect their bacterial host, the phage DNA integrates into the bacterial chromosome (and becomes known as a prophage). Once integrated, prophages remain dormant until the cell experiences some form of stress, which will then induce the phages to activate their lytic cycle, replicate and lyse their host cell (Oppenheim, Kobiler, Stavans, Court, & Adhya, 2005). Different forms of stress that have been reported to induce prophages include hydrogen peroxide, ultraviolet light, and antibiotics, such as mitomycin C and streptonigrin (Cao et al., 2012; Gerner-Smidt, Rosdahl, & Frederiksen, 1993; Gervasi, Curto, Narbad, & Mayer, 2013; Horgan et al., 2010; Lan et al., 2009; Levine & Borthwick, 1963; Los, Los, Wegrzyn, & Wegrzyn, 2010; McDonnell, 2014; Mmolawa, Willmore, Thomas, & Heuzenroeder, 2002; Muschel & Schmoker, 1966; Pryshliak, Hammerl, Reetz, Strauch, & Hertwig, 2014; Wallin-Carlquist et al., 2010; Wormser & Pardee, 1957; Yee, De Grandis, & Gyles, 1993). In this study, cell lysis through induction of prophages was investigated as a novel approach to control bacterial pathogens on fresh produce.

## 2. Materials and methods

### 2.1. Bacterial strains and growth conditions

Strains used in this study are listed in Table 1. *E. coli* MC185 is a fluoroquinolone-resistant strain isolated from raccoon feces contaminating an agricultural production system, the STEC O157:H7 strain EC920333 was isolated from a bovine source, and the Shiga toxin negative *E. coli* O157:H7 strain was isolated from human feces. STEC O157:H7 and *S. enterica* serovars Typhimurium and Newport were chosen because they have been previously linked to outbreaks involving fresh produce (Callejon et al., 2015; CDC, 2012, 2017; Herman et al., 2015). Specifically, the *S. Newport* strain used in this study was involved in an international outbreak linked to sprouted chia seed powder (Harvey et al., 2017). All strains were grown on tryptic soy agar (TSA; Becton, Dickinson and Company, Sparks, Maryland, USA) from frozen stock, followed by subculture in tryptic soy broth (TSB; Oxoid Ltd, Basingstoke, Hampshire, England), unless indicated otherwise. Ciprofloxacin (2.5 µg/mL) was added to overnight cultures of *E. coli* MC185 to promote a selective growth environment. All strains were grown at 37 °C in an orbital shaker set at 225 rpm.

### 2.2. Induction of prophages

A 5 mL volume of TSB was inoculated with an overnight culture to a starting OD<sub>600</sub> equivalent to 0.1. The cells were grown to mid-

**Table 1**  
List of strains used in this study.

Taxon	Strain	Origin
<i>Escherichia coli</i>	MC185	USDA National Wildlife Research Centre
<i>Escherichia coli</i> O157:H7	EC920333	Health Canada
<i>Escherichia coli</i> O157:H7 – Shiga toxin negative	43888	American Type Culture Collection
<i>Salmonella</i> Typhimurium	LT2	American Type Culture Collection
<i>Salmonella</i> Newport	131174	Laboratoire de Santé Publique du Québec

logarithmic phase, at which point subinhibitory concentrations of the prophage inducers, mitomycin C (0.5–6 µg/mL) (Sigma, St Louis, Missouri, USA) or streptonigrin (0.25–2 µg/mL) (Sigma), were added to the culture. Following addition of the inducers, growth of the cells was monitored over time by measuring the OD<sub>600</sub> using a spectrophotometer (Ultrospec 100 Pro, Biochrom Ltd; Cambridge, England). To determine the number of viable cells after 20 h of treatment with 2 µg/mL mitomycin C, the cells were collected by centrifugation at 5,000×g for 10 min at room temperature, washed three times with phosphate-buffered saline (PBS), pH 7.5, diluted and plated onto TSA. The resulting number of colony forming units (CFU) were counted after incubating the plates at 37 °C for 20 h.

### 2.3. Tomato experiment

The stem scar of fresh Beefsteak greenhouse tomatoes was inoculated with  $5 \times 10^7$  CFU of an overnight culture of the respective bacteria. After allowing time for the inoculum to dry on the tomato samples, 5 mL of mitomycin C (2 or 6 µg/mL) was sprayed on the complete tomato surface, while control tomatoes were sprayed with an equal volume of water. Following overnight treatment at 4 °C, the tomatoes were immersed in 25 mL PBS and manually agitated for 2 min. The surviving bacterial cells were collected from the PBS by centrifugation (15,000×g for 2 min), washed three times with PBS to remove any residual inducer, and plate counts were performed. *E. coli* strain MC185 was plated onto TSA +2.5 µg/mL ciprofloxacin, STEC O157:H7 was plated onto Sorbitol MacConkey Agar (Oxoid Ltd), and the *Salmonella* spp. strains were plated on XLT4 Agar (Fluka Analytical – Sigma).

### 2.4. Spinach experiment

A sample of five pre-washed baby spinach leaves (cultivars C2-606, Escalade, and Stanton) were inoculated with a total of  $5 \times 10^8$  CFU of an overnight culture of the respective bacteria. After allowing time for the inoculum to dry on the spinach samples, 5 mL of mitomycin C (6 µg/mL) was sprayed onto the entire top surface of the spinach leaves, while an equal volume of water was sprayed onto control spinach leaves. The spinach leaves were exposed to the mitomycin C overnight at 4 °C. The next day, 25 mL PBS was added to the spinach and the sample was homogenized for 2 min in a stomacher (Stomacher Lab-Blender 400, Seward Laboratory System, London, England). The surviving bacterial cells were counted as described for the tomatoes.

### 2.5. DNA extraction

Bacterial DNA was extracted from an overnight culture using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) per the manufacturer's instructions. To extract phage DNA, the viral particles were collected from the lysates of bacterial cells exposed to the inducer for 20 h. The lysates were prepared as previously described (Y. Zhang & Lejeune, 2008). To remove any potentially contaminating bacterial DNA, lysates were treated with 1 µg/mL DNase I

(Roche Diagnostics, Indianapolis, Indiana, USA) for 30 min at 37 °C and the enzyme was then inactivated by incubating the lysate at 75 °C for 15 min. Previous studies indicated that this approach was efficient at degrading DNA (data not shown). Following the DNase treatment, viral particles were concentrated by adding a 1:100 (w/vol) ratio of Amberlite IRA-900 ion-exchange resin (Acros Organics, New Jersey, USA) to the treated lysate and incubating at room temperature for 60 min, while continuously mixing (Perez-Mendez, Chandler, Bisha, & Goodridge, 2014). Phage DNA was extracted from the particles bound to the resin beads by resuspending the beads in 200 µL of 0.85% saline. Following this step, the rest of the DNA extraction procedure was performed using the QIAamp MinElute Virus Spin kit (Qiagen) according to the manufacturer's instructions, where the resuspended beads were used instead of plasma or serum.

### 2.6. Amplification of phage-specific genes

PCR of prophage integrase genes was used to confirm the presence of prophages within bacterial genomes and the release of induced phages upon cell lysis. The integrase genes from phages with similarities to λ, SfiI, Fels2, and P2 were amplified as previously described (Balding, Bromley, Pickup, & Saunders, 2005) using a Peltier Thermal Cycler (PTC-100, Bio-Rad, Hercules, California, USA). After amplification, PCR products were separated using the QIAxcel automated capillary electrophoresis system with a DNA high resolution cartridge (Qiagen) following the manufacturer's instructions.

### 2.7. Whole genome sequencing and bioinformatics analysis

Whole genome sequencing was performed at the EcoGenomics Analysis Platform (IBIS, Université Laval, Québec, Canada). Initially, sequencing libraries were constructed using the KAPA Hyper Prep kit (Kapa Biosystems, Wilmington, MA, USA) per the manufacturer's instructions. Each 300-bp paired-end library was sequenced on an Illumina MiSeq instrument (Illumina technology, San Diego, CA, USA) with 30X coverage. The raw reads were assembled *de novo* using the A5 pipeline (Tritt, Eisen, Facciotti, & Darling, 2012) and annotation was performed using RAST (Overbeek et al., 2014). Whole genome sequences have been deposited at DDBJ/ENA/GenBank under accession numbers NPKK000000000 for *E. coli* MC185 and NPIW000000000 for *S. Newport*. The whole genome sequence of *S. Typhimurium* strain LT2 (NC\_003197.2) was retrieved from the National Center for Biotechnology Information database. Prophage regions within the bacterial genomes were identified using PHASTER (Arndt et al., 2016).

### 2.8. Statistical analysis

All experiments were independently performed in triplicate and the data are plotted as average values taken from repeat experiments ± the standard deviation. Statistical analysis was performed using GraphPad QuickCalcs (GraphPad Software, San Diego, CA, USA). An unpaired, 2-tailed Student *t*-test was used to determine

statistically significant differences ( $P < 0.05$ ) between the treated and control samples.

### 3. Results

#### 3.1. Mitomycin C induces prophages within *E. coli* resulting in cell death

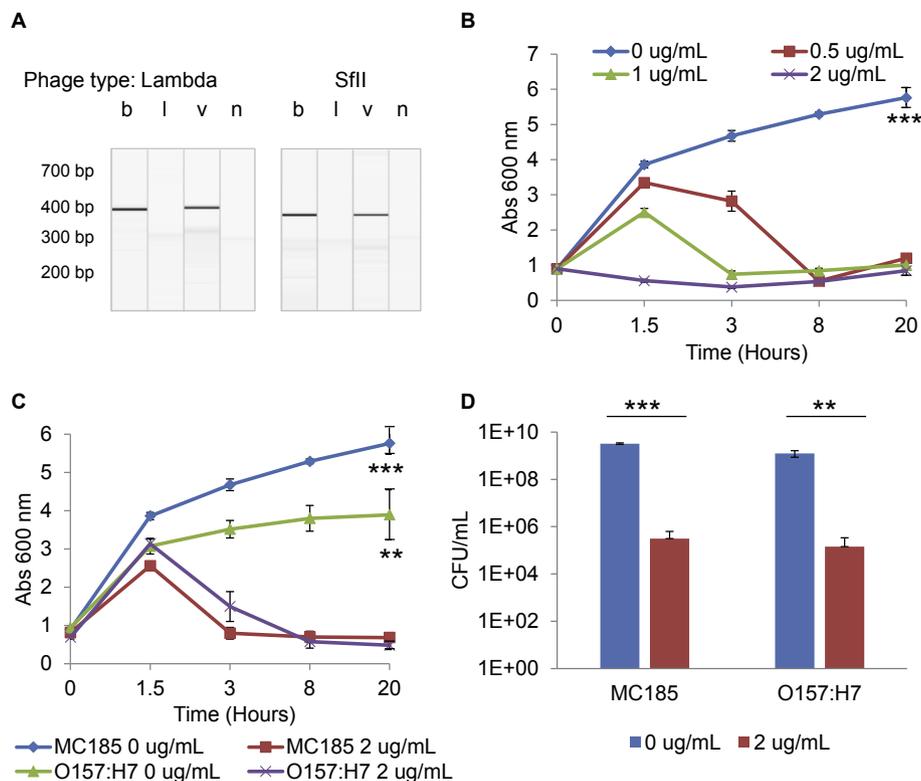
The presence of two prophages, with similarities to phages  $\lambda$  and SfiI, was detected within *E. coli* MC185 by amplifying phage-specific integrase genes from the bacterial DNA (Fig. 1A). Additionally, whole genome sequence analysis revealed the presence of phages  $\lambda$  and SfiI, as well as two additional prophages identified as Fels2 and phi4795, in the genome of *E. coli* MC185. To determine whether the prophages identified in *E. coli* MC185 could be induced, cells were exposed to different subinhibitory concentrations of mitomycin C, a potent prophage inducer (Mmolawa et al., 2002; Yee et al., 1993). After 1.5 h, a decrease in OD<sub>600</sub> was already observed at some concentrations of mitomycin C (i.e. 1–2  $\mu\text{g}/\text{mL}$ ), and this decrease was more significant over time and as the subinhibitory concentration of mitomycin C increased (Fig. 1B). These observations suggest successful prophage induction and potential lysis of the bacterial cell. To further support the hypothesis that cell lysis resulted from prophage induction, phage particles were isolated from the bacterial lysate after the cells had been exposed to mitomycin C for 20 h. Subsequently, the phage DNA was extracted and phage-specific integrases were amplified to confirm the presence of prophages in the bacterial lysate. Indeed, the integrases of phages  $\lambda$  and SfiI were identified in the purified lysate, indicating the release of these phages from the bacterial host

cell upon cell lysis (Fig. 1A).

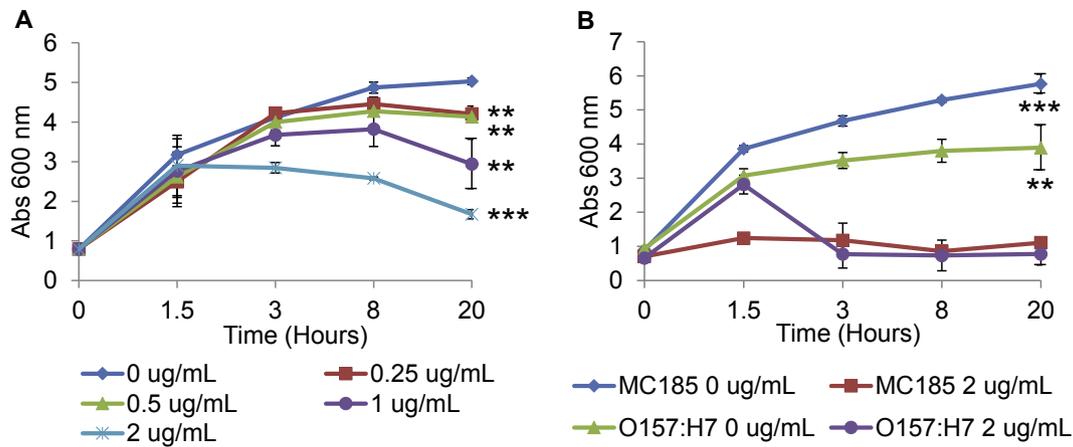
Next, the ability of mitomycin C to induce prophages in STEC O157:H7 was examined. Treatment of STEC O157:H7 with mitomycin C exhibited a decrease in OD<sub>600</sub>, which began at 3 h after addition of the inducing compound (Fig. 1C); a similar trend to that observed with *E. coli* strain MC185. The number of surviving cells after 20 h of exposure to mitomycin C was determined by measuring the CFU. Presence of mitomycin C resulted in a 4 log reduction in the number of cells for both *E. coli* strains compared to untreated cells, confirming that the observed decrease in OD<sub>600</sub> was due to phage-dependent cell death (Fig. 1D). Taken together, these results demonstrate that subinhibitory concentrations of mitomycin C effectively induced prophages within the different strains of *E. coli* used here, and that induction led to cell lysis and death.

#### 3.2. Streptonigrin also induces prophages within *E. coli*

The ability of different concentrations of streptonigrin, another prophage inducer (Levine & Borthwick, 1963; Muschel & Schmoker, 1966), to induce prophages in *E. coli* was evaluated. As with mitomycin C, treatment of *E. coli* strain MC185 with streptonigrin resulted in a decrease in OD<sub>600</sub> starting at 3 h, which became more significant over time and with higher concentrations of the inducer (Fig. 2A). Addition of streptonigrin to cultures of STEC O157:H7 also resulted in a significant decrease in OD<sub>600</sub> by 3 h (Fig. 2B). These findings indicate that subinhibitory concentrations of streptonigrin induce prophages within the *E. coli* strains used in this study, which results in a decrease in cell numbers.



**Fig. 1. Mitomycin C induces prophages within *E. coli* resulting in cell death.** A) Detection of bacteriophage-specific genes in bacterial DNA isolated from *E. coli* MC185 or bacteriophage DNA isolated from lysates of *E. coli* MC185 cells exposed to 2  $\mu\text{g}/\text{mL}$  mitomycin C. B) Growth of *E. coli* strain MC185 in TSB supplemented with different concentrations of mitomycin C. C) Growth of different strains of *E. coli* in TSB alone or supplemented with 2  $\mu\text{g}/\text{mL}$  mitomycin C. D) Number of surviving cells after exposure to 0  $\mu\text{g}/\text{mL}$  or 2  $\mu\text{g}/\text{mL}$  mitomycin C in TSB for 20 h: b: bacterial DNA, l: DNase-treated lysate, v: viral DNA, n: no DNA. Significant difference at  $P < 0.0001$  (\*\*\*),  $P < 0.005$  (\*\*).



**Fig. 2. Streptonigrin induces prophages within *E. coli*.** A) Growth of *E. coli* strain MC185 in TSB supplemented with different concentrations of streptonigrin. B) Growth of different strains of *E. coli* in TSB alone or supplemented with 2  $\mu\text{g}/\text{mL}$  streptonigrin. Significant difference at  $P < 0.0001$  (\*\*\*),  $P < 0.005$  (\*\*).

### 3.3. Mitomycin C reduces the number of *E. coli* cells on fresh produce

The next step was to determine if lysing bacterial pathogens by inducing the prophages within the cells could be used to efficiently control *E. coli* on tomatoes. Preliminary experiments demonstrated that 2  $\mu\text{g}/\text{mL}$  mitomycin C was not sufficient to allow detection of prophage induction in *E. coli* cells inoculated onto tomatoes, while 6  $\mu\text{g}/\text{mL}$  mitomycin C resulted in a detectable level of induction (Fig. 3A). Additional preliminary work, using a Shiga toxin negative *E. coli* O157:H7 isolate that did not contain inducible prophages, showed that 6  $\mu\text{g}/\text{mL}$  mitomycin C did not inhibit growth, demonstrating that this concentration was sub-inhibitory (Fig. 3B). Consequently, a subinhibitory concentration of 6  $\mu\text{g}/\text{mL}$  mitomycin C was used for the subsequent *in vivo* studies. Similarly to the experiments conducted in broth cultures, exposure to mitomycin C significantly reduced the number of *E. coli* cells on tomatoes compared to control tomatoes sprayed with water. A 3.5 log and 3 log reduction were obtained for *E. coli* MC185 and O157:H7, respectively (Fig. 3C).

A similar approach was used to determine whether mitomycin C could also reduce the number of *E. coli* on fresh spinach. The survival rate of *E. coli* on spinach sprayed with water was similar to that obtained on tomatoes (Fig. 3C–D). Additionally, exposure to mitomycin C successfully decreased the number of surviving *E. coli* cells on spinach by 1.5 log and 1 log for *E. coli* MC185 and O157:H7, respectively, compared to spinach sprayed with water (Fig. 3D). Together, these findings provide the proof of concept that prophage induction can effectively be used to control *E. coli* strains, including STEC O157:H7, on different types of fresh produce.

### 3.4. Mitomycin C induces prophages within *S. enterica*

The ability of mitomycin C to induce prophages within *Salmonella* spp was also examined. Amplification of phage-specific integrase genes from the bacterial DNA of the *Salmonella* strains identified two prophages with similarities to phages P2 and Fels2 in *S. Newport* and *S. Typhimurium*, respectively (Fig. 4A). Similarly, whole genome sequence analysis revealed the presence of Fels2 and Gifsy2 in *S. Typhimurium*, while Gifsy1 and Fels1 were present in the bacterial genomes of *S. Typhimurium* and *S. Newport*. Exposure of *S. Typhimurium* and *S. Newport* to mitomycin C resulted in a decrease in  $\text{OD}_{600}$ , at 1.5 h after addition of mitomycin C for cultures of *S. Newport* and 3 h for *S. Typhimurium*, compared

to untreated cells, and  $\text{OD}_{600}$  values remained low beyond 20 h, as was observed with strains of *E. coli* (Fig. 4B vs 1C). Furthermore, prophage induction resulted in bacterial concentrations that were more than 3 logs lower for both *S. Typhimurium* and *S. Newport* when the cells were exposed to mitomycin C for 20 h, compared to untreated cells (Fig. 4C).

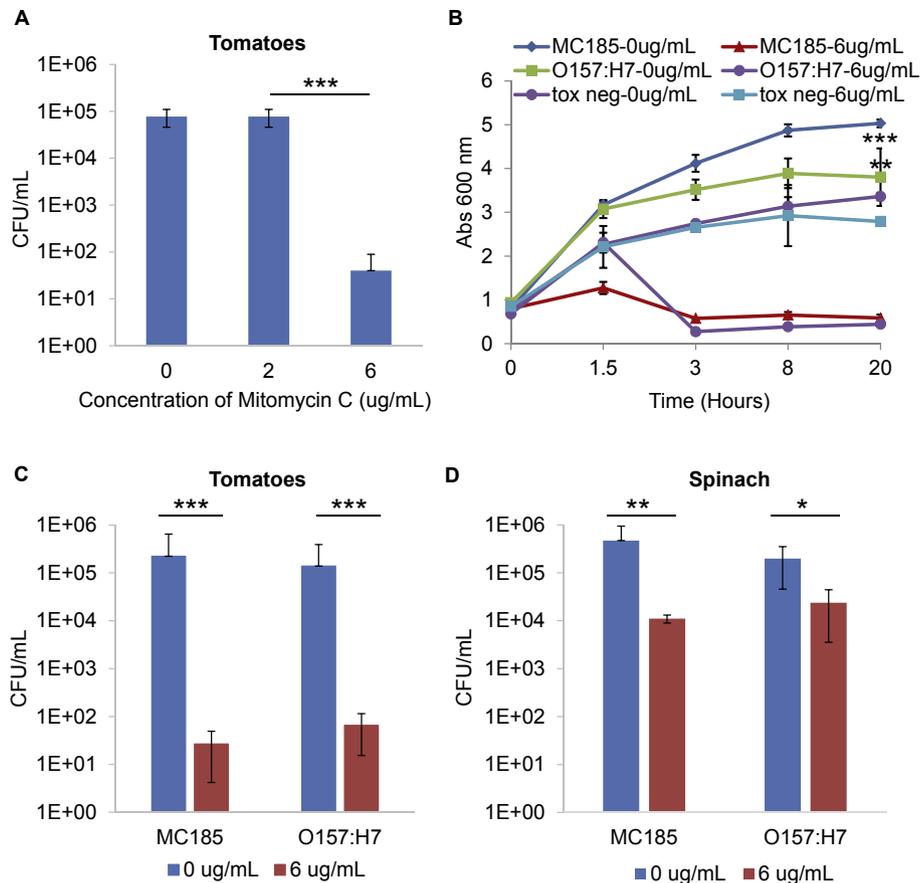
The presence of integrase genes of phages with similarities to P2 and Fels2 were identified in phages recovered from the *S. Newport* and *S. Typhimurium* lysates, respectively (Fig. 4A), suggesting that prophages were induced and lysed the bacterial host cells upon release of the phage. Together, these data confirm that subinhibitory concentrations of mitomycin C successfully induced prophages found within the two different serovars of *Salmonella*, which lead to cell lysis and resulted in a decrease in the number of surviving cells.

### 3.5. Mitomycin C reduces the concentration of *S. enterica* on fresh produce

Finally, the use of mitomycin C was evaluated to control different serovars of *S. enterica*, *S. Typhimurium* or *S. Newport*, on fresh tomatoes and spinach. In tomatoes, treatment with mitomycin C resulted in a 1 log reduction for *S. Typhimurium* and a 1.5 log reduction for *S. Newport*, when compared to control tomatoes sprayed with water (Fig. 4D). A 2 log reduction was observed for both *S. enterica* serotypes on spinach treated with mitomycin C compared to those sprayed with water (Fig. 4E). Overall, these results demonstrate the feasibility of using prophage inducers to control *Salmonella* spp. on fresh produce and that this approach can target multiple foodborne pathogens with a single inducing compound.

## 4. Discussion

The current study demonstrates the feasibility of using prophage inducers as a novel approach to efficiently control bacterial pathogens on fresh produce. The success of this antimicrobial approach is dependent on the presence of prophage(s) within target bacteria. Studies of more than 11,000 bacterial genomes demonstrated that most bacterial species contain prophages, and identified the presence of multiple prophages within these genomes (Canchaya et al., 2003; Kang et al., 2017). Studies have also identified prophages within the genome of foodborne pathogens (Allison & Verma, 2000; Hayashi et al., 2001; Herold et al., 2004; Klumpp & Loessner, 2013; Moreno Switt et al., 2013; Waldor &



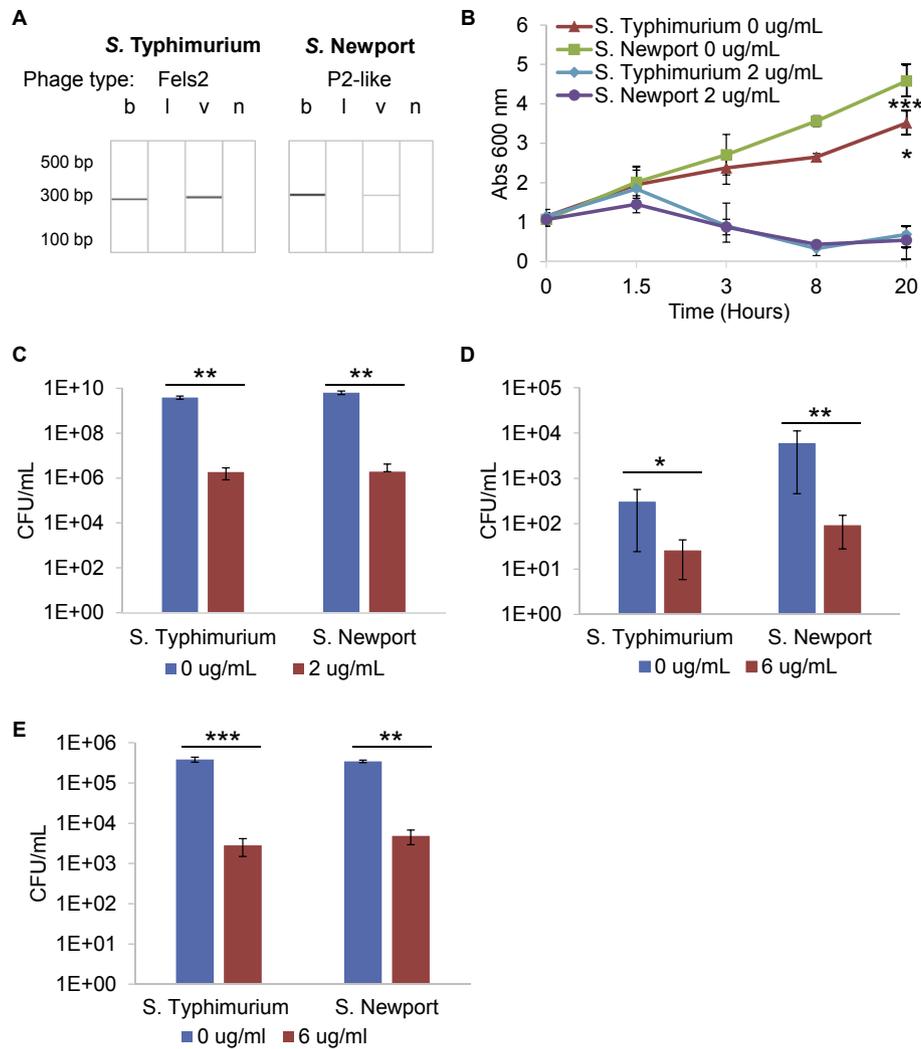
**Fig. 3. Mitomycin C reduces the number of *E. coli* cells on fresh produce.** A) Number of surviving cells recovered from tomatoes inoculated with *E. coli* strain MC185 and sprayed with water (0 µg/mL) or different concentrations of mitomycin C. B) Growth of *E. coli* strains in TSB alone or TSB supplemented with 6 µg/mL of mitomycin C. C-D) Number of surviving cells recovered from tomatoes (C) or spinach (D) inoculated with *E. coli* strains and sprayed with water (0 µg/mL) or mitomycin C (6 µg/mL). Six tomatoes or four spinach samples were used per treatment and per strain. O157:H7: STEC O157:H7, tox neg: Shiga toxin negative *E. coli* O157:H7. Significant difference at  $P < 0.0001$  (\*\*\*),  $P < 0.005$  (\*\*),  $P < 0.05$  (\*).

Mekalanos, 1996). Perhaps the most striking example of this is the STEC O157:H7 Sakai strain, which contains 18 prophage genomic elements, representing 16% of the total bacterial genomic content (Canchaya et al., 2003). Additionally, we recently analyzed 1,355 *Salmonella* genomes from the *Salmonella* Food Syst-OMICs (SalFoS) database (<https://salfos.ibis.ulaval.ca/>), and determined that at least 90% of these genomes contained prophages (data not shown). Collectively, these studies support the potential of prophage induction in controlling bacterial pathogens in food.

In this study, subinhibitory concentrations of mitomycin C and streptonigrin were used as inducers and were shown to effectively induce multiple prophages within *E. coli*, *S. Typhimurium* and *S. Newport*, and consequently led to cell lysis of the bacterial host. Mitomycin C, an antibiotic that inhibits DNA synthesis via intercalation and adduct formation (Iyer & Szybalski, 1963, 1964; Tomasz & Palom, 1997), has been reported to be a potent prophage inducer capable of inducing a wide range of phages in various foodborne bacterial pathogens including *E. coli*, *S. enterica* serovar Typhimurium, *L. monocytogenes*, *Vibrio vulnificus*, *Vibrio parahaemolyticus*, *Clostridium perfringens*, *Clostridium difficile*, and *Staphylococcus aureus* among others (Cao et al., 2012; Gerner-Smith et al., 1993; Gervasi et al., 2013; Horgan et al., 2010; Lan et al., 2009; Mmolawa et al., 2002; Pryshliak et al., 2014; Wallin-Carlquist et al., 2010; Yee et al., 1993). Streptonigrin is another antibiotic shown to induce prophages in bacteria (Levine & Borthwick, 1963; Muschel & Schmoker, 1966). In this case, the antibiotic mechanism involves

causing irreversible cleavage of nucleic acids (Cohen, Shaw, & Craig, 1963; Miller, Laszlo, McCarty, Guild, & Hochstein, 1967). *In vitro* experiments were carried out with mid-exponential phase cells to evaluate whether prophage induction would lead to growth cessation of the bacterial host, which would not have been possible if stationary phase cells were used. However, stationary phase cells were used in experiments involving fresh produce, because, *Salmonella* does not actively grow on fresh produce. Therefore stationary phase cells are more representative of the growth state of bacterial cells that would be naturally found on fresh produce, although lag phase cells could also be present.

We have established that spraying mitomycin C on tomatoes can lead to as much as a 3.5 log reduction of the targeted bacterial population, while a reduction of up to 2 logs was observed on spinach. Previous studies on the ability of other antimicrobial strategies to control pathogens on food have exhibited varying success (Azizkhani et al., 2013; Bari et al., 2005; Ganesh et al., 2012; Landry et al., 2014; Leverentz et al., 2003; Magnone et al., 2013; Oliveira et al., 2015; Park et al., 2011). For example, organic and inorganic acids, including malic, tartaric, lactic and phosphoric acids, sprayed electrostatically on spinach previously inoculated with STEC O157:H7 (7.0 log CFU/mL) yielded a 1.1–4.0 log CFU/g reduction (Ganesh et al., 2012). Emulsions of essential oils derived from various plants (e.g. oregano, clove, thyme) have also been studied as potential antimicrobials. Carvacrol, the essential oil found in oregano, resulted in a 2–3 log reduction of STEC O157:H7



**Fig. 4. Mitomycin C reduces the number of *S. enterica* serovars Typhimurium and Newport in broth and on fresh produce.** A) Detection of bacteriophage-specific genes in bacterial DNA isolated from *Salmonella* spp. or bacteriophage DNA isolated from lysates of *Salmonella* spp. cells exposed to 2  $\mu\text{g/mL}$  mitomycin C. B) Growth of *Salmonella* spp. in TSB in presence of 0 or 2  $\mu\text{g/mL}$  mitomycin C. C) Number of surviving cells after exposure to 0  $\mu\text{g/mL}$  or 2  $\mu\text{g/mL}$  mitomycin C in TSB for 20 h. D-E) Tomatoes (D) and spinach leaves (E) inoculated with *Salmonella* spp. cells and sprayed with water (0  $\mu\text{g/mL}$ ) or mitomycin C (6  $\mu\text{g/mL}$ ). Three tomatoes or three spinach samples were used per treatment and per strain. b: bacterial DNA, l: DNase-treated lysate, v: viral DNA, n: no DNA. Significant difference at  $P < 0.0001$  (\*\*\*),  $P < 0.005$  (\*\*),  $P < 0.05$  (\*).

and *S. Enteritidis* on sprout seeds incubated at ambient temperature and a 0.5 log reduction of STEC O157:H7 on baby leaf salads stored at 7 °C (Azizkhani et al., 2013; Landry et al., 2014). Factors such as the type of antimicrobial approach used, the organism(s) targeted, the food matrix, the pH, the temperature, the exposure time, and the concentration of the antimicrobial were all shown to influence the outcome of the challenge studies performed. The use of phages to control bacterial foodborne pathogens has previously been demonstrated by spraying virulent phage cocktails on food, including fresh produce. For example, a recent study, in which phages were used to control STEC O157:H7 on fresh produce at 4 °C and 25 °C, reported a 2.4–3.0 log CFU/g reduction on cut green peppers and a 3.4–3.5 log CFU/g reduction on spinach leaves (Snyder, Perry, & Yousef, 2016). A different group examined the effectiveness of phage cocktails introduced in packaging materials to control *L. monocytogenes* on cantaloupes or *E. coli* O104:H4 on alfalfa sprouts (Lone et al., 2016). A 1–2 log reduction in the number of *L. monocytogenes* on cantaloupes and a 1 log reduction of *E. coli* on germinated sprouts was observed. However, the efficacy of this type of phage-based antimicrobial method relies on successful

infection of the bacterial host by the phage, which can be limited by the host range of the phage, as well as composition of the food matrix, pH, and temperature (J. W. Kim et al., 2012; Tsonos et al., 2014).

The prophage induction approach described here offers several advantages over traditional phage therapy approaches. In contrast to traditional phage-based antimicrobial approaches, the compounds used in this study, mitomycin C and streptonigrin, are not reliant on phage-host interactions, and instead, cause DNA damage and initiate the SOS response in bacteria (Campoy et al., 2006). The bacterial SOS system consists of several genes aimed at guaranteeing cell survival in the presence of extensive DNA damage (Walker, 1984), and is induced by the activation of RecA after it binds to single-stranded DNA fragments (ssDNA) (Sassanfar & Roberts, 1990). Activated RecA promotes the autocatalytic cleavage of the LexA repressor, resulting in prevention of LexA from binding to its specific recognition motif in the promoter region of SOS genes, thereby allowing the transcription of all the genes required in the SOS response (Campoy et al., 2006). In addition to the genes directly regulated by LexA, the induction of the SOS

response, via ssDNA activation of RecA, promotes cleavage of other repressors, including lytic cycle repressors of temperate phages (Roberts & Roberts, 1975; Sauer, Ross, & Ptashne, 1982).

In addition, multiple studies have demonstrated the formation of bacteriophage insensitive mutants (BIMs) due to mutations in bacterial cell surface appendages used as phage receptors (Labrie, Samson, & Moineau, 2010; O'Flynn, Ross, Fitzgerald, & Coffey, 2004). These mutations could significantly limit the effectiveness of traditional phage-based approaches. As the SOS response is germane to bacterial survival in the presence of DNA damaging compounds and stresses (Baharoglu & Mazel, 2014), the prophage induction approach described here would seem to target an essential cellular response, making it difficult for bacterial cells to develop resistance to this approach.

In this study, single inducing compounds, including mitomycin C and streptonigrin, were shown to induce at least five different prophages in *E. coli* and *Salmonella*, meaning that bacteria would potentially have to develop non-SOS based mechanisms to disrupt induction of multiple types of prophages to overcome this approach. Furthermore, as these, and related prophages, have been observed within the genomes of bacterial isolates from many bacterial species (Kang et al., 2017), a single compound could be used to simultaneously induce prophages (and therefore destroy bacteria) from multiple bacterial species. This represents an important advantage over traditional phage-based antimicrobials, which target only a single bacterial species. Further studies will be required to determine whether other inducers will effectively induce a broad range of phages. A combination of different inducers could also be used to ensure that many prophages from different bacterial species are induced. Thus, the prophage induction approach could simultaneously control multiple pathogens on foods, as well as extend the shelf life of foods by targeting several bacterial species involved in food spoilage.

As the mitomycin C and streptonigrin compounds used in this study are antibiotics, and could not be used in foods due to concerns over antibiotic resistance, the results presented here represent a proof of concept that prophage induction can be an effective approach to control foodborne pathogens. Future studies will focus on the identification of natural, non-antibiotic prophage inducers. For example, hydrogen peroxide is already used as an antimicrobial to control the presence of bacterial pathogens in foods (McDonnell, 2014), and has been shown to cause DNA damage mediated prophage induction (Los et al., 2010).

One potential concern regarding the use of prophage induction as a method to reduce bacterial pathogens in foods is the possibility of horizontal transfer of virulence and antimicrobial resistance (AMR) genes from the induced prophages to other bacteria. While horizontal gene transfer due to prophage induction has received much discussion in the scientific literature, at least one recent study has called into question the frequency of temperate phage-based horizontal gene transfer (Enault et al., 2017). According to their observations, the authors concluded that the presence of AMR genes in temperate phages tends to be vastly overestimated. Recent metagenomic studies demonstrating the presence of AMR genes in temperate phages have not demonstrated transfer of these genes to other bacteria (Quiros et al., 2014). In addition, most of this work has been conducted *in vitro*, with few studies being conducted on prophage transduction of AMR and virulence genes *in vivo*. Where such studies have been conducted, temperate phage-based horizontal gene transfer generally did not occur. For example, one recent study directly examined prophage induction and horizontal gene transfer in animals. In that work, the authors used metagenomics to evaluate the effect of two antibiotics in feed (carbadox and ASP250 [chlortetracycline, sulfamethazine, and penicillin]) on swine intestinal phage metagenomes (Allen et al., 2011). They also

monitored the bacterial communities using 16S rRNA gene sequencing. The authors observed that AMR genes, such as multi-drug resistance efflux pumps, were identified in the phage metagenomes, but in-feed antibiotics caused no significant changes in their abundance. The abundance of phage integrase-encoding genes was significantly increased in the phage metagenomes of medicated swine over that of non-medicated swine, demonstrating the induction of prophages with antibiotic treatment. This means that while prophages were induced in the swine gut, this did not result in horizontal transfer of AMR genes from the prophages to bacteria. In another study, Cornick, Helgerson, Mai, Ritchie, and Acheson (2006) evaluated the ability of a kanamycin-marked Shiga toxin encoding phage to move into a commensal, ovine *E. coli* strain in the ruminant gastrointestinal tract. While transduction was detected in 19/24 samples, subtherapeutic doses of the quinolone antibiotic, enrofloxacin, did not increase the rate of transduction.

Several *in vivo* studies conducted in mice have demonstrated temperate phage transduction of virulence genes to bacteria. For example, subtherapeutic doses of ciprofloxacin given to streptomycin-treated mice increased the concentration of intra-intestinal Shiga toxin and mortality compared to control mice, even though the viable number of STEC O157:H7 decreased by three orders of magnitude (X. Zhang et al., 2000). In another study, an increase in phage transduction also occurred in mice inoculated with an *E. coli* K-12 strain carrying a kanamycin-marked Shiga toxin encoding phage and treated with subtherapeutic doses of ciprofloxacin, when compared to the transduction rate in control mice (Cornick et al., 2006). However, the pre-treatment of the mouse intestine with antibiotics, such as streptomycin, which removes a majority of the natural facultative intestinal flora, may facilitate donor-recipient cell interaction within the intestine.

These studies, when taken collectively, and combined with the fact that prophage induction occurs regularly in the animal gut (De Paepe, Leclerc, Tinsley, & Petit, 2014), suggests that concerns regarding temperate phage-based horizontal transfer of virulence and AMR genes may be exaggerated. Additional *in vivo* studies will need to be conducted before definitive conclusions regarding prophage induction and horizontal gene transfer can be made.

## 5. Conclusion

The present study serves as a clear proof of concept that the prophage induction approach described here has the potential to work as a practical and effective antimicrobial technique. Such an intervention could be used in the food industry to eliminate contaminating bacterial pathogens resulting in safer fruits and vegetables for human consumption. Future studies will be required to determine if this method can be used to inactivate other foodborne bacterial pathogens which can be found in fresh produce. Moreover, the use of this novel method needs to be evaluated for its ability to control foodborne pathogens on other potentially hazardous foods, such as meat, poultry, eggs, and dairy products.

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