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

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**A B S T R A C T**

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 mg/mL) of mitomycin C, or 2 mg/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 × 10<sup>5</sup> and 5 × 10<sup>6</sup> 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 coli* (STEC) and *Salmonella enterica*. Prophage induction offers a promising non-thermal method to control these pathogens on fresh produce.
control speciﬁc approach, cocktails of virulent phages are applied onto the food to combat bacterial pathogens on foods. In this manner, which can limit the utility of the technology for speciﬁc 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 & Loesser, 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 prophage to activate their lytic cycle, replicate and lyse their host cell (Oppenheim, Kobler, 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 streptomycin (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, Wegryn, & Wegryn, 2010; McDowell, 2014; Mmolawa, Willmore, Thomas, & Heuzenroeder, 2002; Muschel & Schmoker, 1966; Pryshliak, Hammerl, Reetz, Strauch, & Hertwig, 2014; Wallin-Carquist 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 isolated 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 OD600 equivalent to 0.1. The cells were grown to mid-
logarithmic phase, at which point subinhibitory concentrations of the prophage inducers, mitomycin C (0.5–6 μg/mL) (Sigma, St Louis, Missouri, USA) or streptomycin (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 OD₅₆₀ using a spectrophotometer (Ultraspéc 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 × 10⁶ 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) (Sigma) 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 × 10⁶ 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/v) 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, SFI, 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, Quebec, 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 NPKK00000000 for E. coli MC185 and NPIW00000000 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$_{600}$ was already observed at some concentrations of mitomycin C (i.e. 1–2 $\mu$g/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$_{600}$, 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$_{600}$ 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$_{600}$ 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$_{600}$ 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.

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**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 $\mu$g/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 $\mu$g/mL mitomycin C. D) Number of surviving cells after exposure to 0 $\mu$g/mL or 2 $\mu$g/mL mitomycin C in TSB for 20 h: bacterial DNA, l: DNAase-treated lysate, v: viral DNA, n: no DNA. 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 μg/mL mitomycin C was not sufficient to allow detection of prophage induction in E. coli cells inoculated onto tomatoes, while 6 μg/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 μg/mL mitomycin C did not inhibit growth, demonstrating that this concentration was sub-inhibitory (Fig. 3B).

Consequently, a subinhibitory concentration of 6 μg/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 inte-grase genes from the bacterial DNA of the Salmonella strains identified two prophages with similarities to F2 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 OD600 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 OD600 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 &
In this study, subinhibitory concentrations of mitomycin C and streptonigrin were used as inducers and were shown to effectively induce multiple prophages within \textit{E. coli}, \textit{S. Typhimurium} and \textit{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 \textit{E. coli}, \textit{S. enterica} serovar Typhimurium, \textit{L. monocytogenes}, \textit{Vibrio vulnificus}, \textit{Vibrio parahaemolyticus}, \textit{Clostridium perfringens}, \textit{Clostridium difficile}, and \textit{Staphylococcus aureus} among others (Cao et al., 2012; Gerner-Smidt 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, \textit{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 (Azizkhan 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 \textit{Salmonella 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 \textit{Salmonella O157:H7}.
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, & Ptasnine, 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 antibacterials, 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 (carboxadox 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 multidrug 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 intraintestinal 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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