Lytic bacteriophages
Potential interventions against enteric bacterial pathogens on produce

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Foodborne illnesses resulting from the consumption of produce commodities contaminated with enteric pathogens continue to be a significant public health issue. Lytic bacteriophages may provide an effective and natural intervention to reduce bacterial pathogens on fresh and fresh-cut produce commodities. The use of multi-phage cocktails specific for a single pathogen has been most frequently assessed on produce commodities to minimize the development of bacteriophage insensitive mutants (BIM) in target pathogen populations. Regulatory approval for the use of several lytic phage products specific for bacterial pathogens such as *Escherichia coli* O157:H7, *Salmonella* spp. and *Listeria monocytogenes* in foods and on food processing surfaces has been granted by various agencies in the US and other countries, possibly allowing for the more widespread use of bacteriophages in the decontamination of fresh and minimally processed produce. Research studies have shown lytic bacteriophages specific for *E. coli* O157:H7, *Salmonella* spp. and *Listeria monocytogenes* have been effective in reducing pathogen populations on leafy greens, sprouts and tomatoes.

Foodborne illness from the consumption of contaminated produce is an increasing problem in the US and around the world. Overall, foodborne infections from the consumption of contaminated produce cost $38 billion annually in the US[1] The number of outbreaks of foodborne illness arising from the consumption of fresh and fresh-cut produce has increased dramatically over the last two decades.[2] From 1990–2005, fresh produce was associated with 768 outbreaks, resulting in 35,060 cases of illness.[3] In addition, the average number of illnesses per produce outbreak was significantly higher than those from other foods.[3] While the reasons behind this increase are somewhat unclear, several factors may play an important role. First, the per capita consumption of fresh produce in the US has increased

significantly. From 1982–1997, US consumption of raw fruits and vegetables increased by 18% and 29%, respectively.[4] Second, the produce industry has become increasingly global, with large volumes of produce being imported into the United States, making adherence to Good Agricultural Practices (GAPs) and Good Manufacturing Practices (GMPs) during harvesting and packaging difficult to evaluate to determine if measures are being taken to reduce foodborne illness. Third, convenience foods such as fresh-cut fruits and bagged salads have also grown exponentially, yet are potentially more conducive to microbial growth and spoilage than the whole produce from which they are derived.[5] However, the increased consumption of leafy greens does not fully explain the increased incidence of outbreaks associated with these commodities. The incidence of foodborne outbreaks associated with leafy greens increased by 39% between 1996–2005, while leafy green consumption increased by only 9%.[6,7] This indicates that other factors may also be responsible for the increased number of these outbreaks.

Until recently, it was thought that enteric pathogens such as *Escherichia coli* O157:H7 and *Salmonella* survived poorly in the harsh environment encountered on plant surfaces, where microorganisms must survive sunlight, desiccation, nutrient limitation and drastic temperature fluctuations. Recent research has shown this not to be the case.[8,9] Enteric pathogens have been demonstrated to persist in a variety of agricultural settings including water, soils, manure, the plant rhizosphere and even on exposed (foliar) plant surfaces.[8,9]

As bacteriophages and their derived products are increasingly approved by regulatory agencies in several countries, they may provide a safe, natural, effective intervention against pathogenic bacterial contamination of fresh and fresh cut produce items.

In addition to the lytic cycle which bacteriophages can use to kill cells using lysis, phages may also employ "lysis from without" (LO) to be effective in decontaminating or disinfesting produce surfaces. The concept of LO is critical to understand when discussing the application of bacteriophages to produce. LO is the lysis of bacterial cells through adsorption of bacteriophages to cell surfaces without completing the infection cycle.[10] LO requires that a sufficient number of phage particles be adsorbed to the cell, resulting in cell wall damage and subsequent cell lysis, due to stress placed on structural weak points in the cell envelope.[10] LO

is an applicable concept when decontaminating produce commodities because of the low storage temperature (4°C) used for many fresh fruits and vegetables. At 4°C, most pathogenic bacteria will not be metabolically active and the cycle of phage infection cannot be completed. However, the initial adsorption and lysis can occur at a low temperature, rendering LO an important mechanism to kill pathogenic bacteria on fresh and minimally processed produce.

Use of Bacteriophages on Produce Commodities

Bacteriophages specific for foodborne pathogens *(Escherichia coli* O157:H7, *Salmonella* spp. and *Listeria monocytogenes)* have effectively reduced pathogens on a variety of fresh and fresh-cut produce. Most studies described below employ a mixture (or cocktail) of bacteriophages specific for a single bacterial foodborne pathogen in order to minimize the opportunity for the development of bacteriophage insensitive mutants (BIM), which is more likely to occur if only a single bacteriophage is employed. The development of resistance to lytic infection from multiple phages simultaneously in bacteria in foods is unlikely because the conditions (food matrices, storage temperature of foods, temperatures in processing plants) are not permissive for bacterial replication, an essential prelude to the development of bacterial resistance to lytic phage infection. In a well-designed cocktail of bacteriophages specific for a pathogen, it is unlikely that bacteria will develop resistance to multiple lytic bacteriophages simultaneously because these phages should utilize different receptor molecules during the adsorption process at the outset of the lytic infection cycle. The multiplicity of infection (MOI), the average number of bacteriophages available to infect a single bacterial cell, is also important to address when using bacteriophages on produce. For targeted food interventions, using phages that can infect bacterial cells at a low MOI is beneficial because less bacteriophages are needed to achieve population reductions. For a summary of studies examining effectiveness of lytic bacteriophages on produce commodities, see **Table 1**.

Lytic phages and leafy greens

A combination of three *E. coli* O157:H7-specific lytic bacteriophages (termed ECP-100), were sprayed on to fresh cut lettuce at a level of 5.9 log PFU (plaque forming units)/cm² , which had been inoculated with *E. coli* O157:H7 at a level of 2.6 log CFU (colony forming units)/cm² , and stored at 4°C for up to 2 d.[11] *E. coli* O157:H7 populations on the fresh cut lettuce treated with control (phosphate buffered saline) were significantly (p < 0.05) higher than those on lettuce receiving ECP-100 spray.[11] On days 0, 1 and 2, cut lettuce receiving control treatment had *E. coli* O157:H7 populations of 2.64, 1.79 and 2.22 log CFU/cm² while lettuce receiving ECP-100 treatment had O157-populations of 0.72, < 0.58 and 0.58 log CFU/cm², respectively. Furthermore, the bactericidal effect due to the application of ECP-100 occurred very quickly after spraying: the initial application of ECP-100 reduced *E. coli* O157:H7 counts by 1.92 logs CFU/cm² on day 0 within 30 min. The bacteriophage cocktail did reduce *E. coli* O157:H7 populations when sprayed on to fresh cut lettuce at an MOI of apporximately 1000. Spraying the same ECP-100

bacteriophage mixture at 9×10^7 PFU/ml on spinach inoculated with ca. 4 log CFU *E. coli* O157:H7 /g resulted in reductions of 100% after 24 h and 120 h, and 99% by 168 h when stored at 10°C.[12] In another study, a mixture of eight lytic bacteriophages (BEC 8) specific for *E. coli* O157:H7 were deposited at a titer of 10^6 PFU/leaf on fresh cut baby Romaine lettuce or baby spinach leaves and inoculated with either 10^4, 10^5 or 10^6 CFU *E. coli* O157:H7/leaf, and stored at either 4, 8, 23 or 37°C.[13] The results from these experiments also showed that as the multiplicity of infection (MOI) and incubation temperature increased, the greater the inactivation of the pathogen on the leafy green surface. Increasing the treatment time from 10 min to 1 h to 24 h also increased the reduction of *E. coli* O157:H7 populations on spinach and lettuce leaves.

Lytic bacteriophages have also been evaluated under minimal processing and commercial storage conditions used for leafy greens. Immersion of fresh-cut lettuce in solutions of bacteriophages to simulate wash water used for fresh-cut leafy greens did provide protection against cross-contamination with *E. coli* O157:H7. Fresh-cut lettuce was immersed in solutions of *E. coli* O157-specific bacteriophages at 9.8 log PFU/mL for 2 min, inoculated with *E. coli* O157:H7, and stored at 4°C for up to 7 d.[14] Unlike with the spray application of bacteriophages described previously, statistically significant reductions were not observed immediately after phage treatment but only after 24 h. After immersion treatment, populations were reduced to below the detectable limits after 3 d of storage at 4°C. The lack of immediate reduction by immersion is most likely due to the lower number of phages distributed to the surface of lettuce by immersion compared with spraying. Bacteriophages specific for *E. coli* O157:H7 were also effective on spinach packaged under modified atmosphere packaging (MAP) and stored at 4°C.[15] MAP is commonly used to preserve the quality of fresh-cut leafy greens sold at retail. Bacteriophage treatment significantly reduced populations by 2.18 log, 3.50 log and 3.13 log CFU/cm² after 24 h on spinach, green leaf and romaine lettuce, respectively, compared with untreated controls under MAP conditions.[15] Finally, the treatment of fresh-cut lettuce with a combination of *E. coli* O157-specific bacteriophage (spray treatment) and 50 ppm sodium hypochlorite (immersion treatment) reduced *E. coli* O157:H7 populations on fresh-cut iceberg lettuce more than either bacteriophage or hypochlorite treatment alone.[14] All of these findings describing the effectiveness of lytic bacteriophages on leafy greens after immersion, under modified atmosphere packaging and on fresh cut products shows that phages can be integrated into current leafy green processing and packaging conditions.

Lytic phages and tomatoes

Reports in the literature have described varied results with the application of lytic bacteriophages on tomatoes. ECP-100 applied at 9×10^6 PFU/g reduced initial *E. coli* O157:H7 populations of 2.81 log CFU/g on cut sliced tomatoes by 99%, 94% and 96% after 24, 120 and 168 h, respectively after spray application.[12] A cocktail of five lytic bacteriophages specific for *Salmonella* spp. were applied to blossoms of tomato plants at a level of 6 log PFU/ml, and also treated with 6 log CFU/ml of *Salmonella*

Table 1. Summary of studies cited examining effectiveness of lytic bacteriophages on produce commodities

Commodity	Pathogen	MOI	Method of application of phages to produce commodity	Pathogen reduction	Ref.
Leafy greens					
Lettuce (green leaf)	*E. coli* O157:H7	100	Spray	3.5 log CFU/cm²	Boyacioglu et al.[15]
Lettuce (Iceberg)	*E. coli* O157:H7	1000	Spray	1.9 log CFU/cm²	Sharma et al.[11]
Lettuce (Iceberg)	*E. coli* O157:H7	10000	Immersion in solution before bacterial inoculation	1.9 log CFU/cm² (after 72 h)	Ferguson et al.[14]
Lettuce (Romaine)	*E. coli* O157:H7	1	Spot	0.7–3.7 log CFU/leaf	Viazis et al.[13]
		10	Spot	1.8–3.2 log CFU/leaf	
		100	Spot	2.0–3.6 log CFU/leaf	
Lettuce (Romaine)	*E. coli* O157:H7	100	Spray	3.5 log CFU/cm²	Boyacioglu et al.[15]
Spinach	*E. coli* O157:H7	1000	Spray	99–100%	Abuladze et al.[12]
Spinach	*E. coli* O157:H7	1	Spot	0.4–2.9 log CFU/leaf	Viazis et al.[13]
		10	Spot	1.7–3.0 log CFU/leaf	
		100	Spot	1.5–3.1 log CFU/leaf	
Spinach	*E. coli* O157:H7	100	Spray	2.2 log CFU/cm²	Boyacioglu et al.[15]
Tomatoes					
Tomato (fresh cut)	*E. coli* O157:H7	10000	Spray	94–99%	Abuladze et al.[12]
Tomato (whole)	*Salmonella* Javiana	ND1	Dip in combination with E. absuriae	2.26 log CFU/ml (internalized populations)	Ye et al.[16]
Sprouts					
Brocoli sprouts (seeds)	*Salmonella* spp.	< 1	Dip (soak)	1.5 log CFU/ml	Pao et al.[17]
Mung bean sprouts	*Salmonella* spp.	1	Dip (soak)	3.4 log CFU/g	Yet et al.[1]
Melons					
Cantaloupe	*E. coli* O157:H7	100	Spot	2.5 log CFU/ml	Sharma et al.[11]
Honeydew	*Salmonella* Enteritidis	< 100	Spot	3.5 log CFU	Leverentz et al.[19]
Honeydew	*Listeria monocytogenes*		Spray	3.9 log CFU	Leverentz et al.[19]

Javiana.[16] Resulting tomato fruits had a significantly lower incidence of internalized *S.* Javiana compared with fruits which were inoculated with *S.* Javiana and no bacteriophage. However, when flowers of tomato plants were treated with a combination of *Enterobacter absuriae* and *Salmonella*-specific phages, the resulting *S.* Javiana incidence on tomatoes was not significantly different from fruits from plants where the blossom was only treated with *E. absuriae*. These results indicate that *E. absuriae* contributed more to reducing *S.* Javiana counts than this particular bacteriophage mixture when inoculated on tomatoes in this instance.[16]

Lytic phages and sprouts

Bacteriophages specific for *Salmonella* serotypes have also been used on various seeds and sprouts to limit contamination with the pathogen.[17] A combination of two bacteriophages, one with specific lytic ability against *S.* Montevideo, and one with specificity against both *S.* Typhimurium and *S.* Enteritidis, applied at a level of 6.7 log PFU/ml, reduced *Salmonella*

populations by 1.50 log CFU/ml from an initial population of ca. 7 log CFU/ml in soak-water of broccoli seeds compared with non-bacteriophage-treated seeds. A cocktail of six lytic bacteriophages specific for *Salmonella* spp. applied a concentration of 6 log PFU/ml in combination with *E. absuriae*, reduced *Salmonella* spp. populations on inoculated mung bean sprouts by ca. 6 log CFU/ml compared with sprouts which did not receive any antimicrobial treatments.[18] Interestingly, the combination of *Salmonella*-specific lytic phages and *E. absuriae* reduced *Salmonella* spp. populations on mung bean sprouts more than either bacteriophages or *E. absuriae* alone. Although different phage cocktails and *Salmonella* serovars were used on mung bean sprouts, these results differ from those observed with tomatoes which showed the bacteriophage mixture contributing a minimal antimicrobial effect in combination with *E. absuriae*. These contrasting results with the same bacteriophage mixture indicate that lytic phages may be more effective on some types of produce commodities compared with others.

Lytic phages and melons

Lytic bacteriophages have been effective in reducing pathogenic bacterial populations on several types of melons. A cocktail of four lytic bacteriophages (SCPLX-1), specific for *Salmonella* Enteritidis, was applied to inoculated fresh-cut honey dew melons through a spot treatment (5×10^6 PFU/spot) and reduced *Salmonella* by approximately 3.5 log CFU when stored at 5 and 10°C after 3 d when compared with untreated controls. Inoculated melons treated with SCPLX-1 but stored at 20°C contained higher populations of *S.* Enteritidis than those treated with SCPLX-1 and stored at 5 and 10°C.[19] Moreover, this work showed that the activity of bacteriophages occurs almost instantaneously once applied to inoculated melons, and that bacteriophage activity can be enhanced at lower temperatures, which limit growth and potential regrowth of the pathogen. Bacteriophages specific for *Listeria monocytogenes* were also applied to honeydew melons to determine their effectiveness in reducing levels of the pathogen.[20] A combination of six (LMP-102) or 14 lytic bacteriophages (LMP-103) lytic bacteriophages specific for *L. monocytogenes* were applied to cut honeydew melons by either spot or spray application. Squares (30 mm²) of fresh cut honeydew melons were sprayed with either water, nisin (a bacteriocin effective against *L. monocytogenes*) or a combination of nisin and LMP103/LMP102 or LMP103/LMP102 alone to deliver 5×10^5 PFU/mm² over 25 mm² of area. Treatments of LMP103/LMP102 were significantly more effective ($p < 0.05$) in reducing *L. monocytogenes* on honeydew squares when squares of the flesh (mesocarp) were stored at 10°C for up to 7 d compared with treatments with water or with nisin alone. Furthermore, a combination of nisin and LMP cocktails was more effective in reducing *L. monocytogenes* than treatment with nisin alone under the same conditions.[20]

The spot application of ECP-100 at a concentration of 5×10^5 PFU/spot, to fresh cut cantaloupes inoculated with *E. coli* O157:H7 and stored at 4°C for 7 d reduced the pathogen by 2.5 log CFU / ml compared with untreated controls.[11] After 5 d of storage at 20°C, treatment of cantaloupes with ECP-100 reduced counts by 1.24 CFU/ml compared with untreated controls.[11]

All three of these studies indicated that bacteriophages can be effective in reducing pathogenic bacterial counts in or fresh cut melon tissues when combined with temperatures less than 10°C. The lytic activity of bacteriophages against bacterial foodborne pathogens on fresh cut melons is enhanced at low storage temperatures, which prevents the growth of bacteria.

Lytic phages and apples

A cocktail of *Salmonella*-specific (SCPLX-1) bacteriophages was unable to reduce *S.* Enteritidis populations on apple slices stored at 10°C for 7 d.[19] In this same paper, the authors showed that the titer of the *Salmonella*-specifc phages at pH 4.4 were 4 times lower than at pH 5.8. This indicates that pH of the apple slices (4.2) may have inhibited the lytic activity and the viability of SCPLX-1 against *S.* Enteritidis. However, lytic bacteriophages specific for *L. monocytogenes* (LMP103/LMP 102) reduced bacterial populations of the pathogen on apple slices compared with slices which did not receive phage treatment.[19] Lytic phages specific for *L. monocytogenes* were not as affected by the low pH on apple slices as lytic phages specific for *S.* Enteritidis.

Regulatory Status of Bacteriophages on Foods

Several US federal agencies have issued various degrees of approval for the use of lytic bacteriophages for specific and distinct purposes but hardly any are specific to produce commodities. The U.S. Dept. of Agriculture (USDA) issued two no objection letters for the use of bacteriophages targeting *E. coli* O157:H7 (Finalyse) and *Salmonella* spp.(Armament) developed by Omnilytics™, for use as hide sprays on cattle prior to slaughter.[21,22] The U.S. Food and Drug Administration (FDA), and subsequently the U.S. Department of Agriculture's Food Safety and Inspection Service (FSIS), approved the use of a mixture of six bacteriophages (*ListShield*™, manufactured by Intralytix Inc.) specifically for the bacterial foodborne pathogen *L. monocytogenes* on ready-to-eat meat and poultry products.[23] This product was approved as a direct food additive, similar to chemical ingredients as the FDA made its own determination that *ListShield*™ does not currently pose a human health risk when used in the manner described in the regulations. Another lytic bacteriophage product, *Listex P100* (Micreos Food Safety), is a single bacteriophage which the FDA declared as GRAS (Generally Recognized as Safe), suggesting that it has no current scientific objection to the use of the product (Anonymous, 2006b). Specifically, it was to be used to inhibit the growth of *L. monocytogenes* in Brie, cheddar and Swiss cheeses.[24] Its GRAS status was expanded for use in other ready-to-eat foods as well.[25]

EcoShield™ (Intralytix Inc.), formerly known as ECP-100, has also been cleared by the FDA through a "Food Contact Notice" to be used, without labeling, on red meat parts and trim intended to be ground.[26] The same approval has been granted by FSIS.[21] Furthermore it has also received a temporary exemption for two years from the U.S. Environmental Protection Agency, stating that its use does not require the establishment of a tolerance of lytic bacteriophages when used on food contact surfaces in food processing plants.[27] Outside the United States, the aforementioned *Listex P100* has been approved for use as a "processing

aid" for meat, seafood, cheese and ready-to-eat foods by Food Standards Australia and New Zealand.[28] Most recently, FDA approved *SalmoFresh*—a *Salmonella*-specific cocktail of bacteriophages—as GRAS, direct applications onto poultry, fish, shellfish, and fresh and processed fruits and vegetables.[29] These regulatory approvals by federal agencies indicate that the use of lytic bacteriophages in foods pose a minimal risk to human health. Although they are not anticipated to affect the sensory, nutritional or phytochemical status of foods to which they are applied, more studies are needed to evaluate the effect of bacteriophages on compositional and sensory aspects of produce commodities. It should be noted that bacteriophage treatments which do not receive regulatory approval will not be a commercially viable antimicrobial treatment to growers, handlers or retailers of fresh or minimally processed fruits and vegetables.

produce commodity chosen for application. In instances where bacteriophages have been effective, the maximum reduction on pathogenic bacterial populations has occurred on the same day as application of the bacteriophage to the comodity. This could either be due to lysis from without or the initial cycle of infection which kills bacterial cells without giving the cells the opportunity for regrowth. Because lytic bacteriophages for specific pathogens can be composed of phages from different families which may have different adsorption properties to bacterial cells and different attachment properties to produce surfaces, each cocktail must be evaluated individually on each specific produce commodity. The studies described above emphasize the need to evaluate and select bacteriophages that are effective in reducing pathogenic populations in a rapid manner. Bacteriophages used for produce safety applications should be evaluated carefully and systematically in vitro and in carefully defined experimental conditions before being applied to commodities intended for human consumption.

Conclusions

The reduction of bacterial pathogens on produce commodities is dependent on multiple factors, but the most important may be the lytic ability of the bacteriophage on the specific

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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