Phage-based biocontrol strategies to reduce foodborne pathogens in foods

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There has been much recent interest in the use of phages as biocontrol agents of foodborne pathogens in animals used for food production, and in the food products themselves. This interest seems to be driven by consumers' request for more natural foods, as well as the fact that foodborne outbreaks continue to occur, globally, in many foods, some of which (such as fresh produce), lack adequate methods to control any pathogenic contamination present. Also, the many successes with respect to regulatory approval of phage based products destined for use in foods is leading to an increase in the number of phage products that are commercially available. At present, these products are directed against three main foodborne pathogens including *Escherichia coli* O157:H7, *Salmonella* spp and *Listeria monocytogenes*. In the future, it is likely that new phage products will be targeted against emerging foodborne pathogens. Here, we review the current literature and status of phage based strategies aimed at reducing the presence of foodborne pathogenic bacteria in food and the food production environment.

Introduction

Felix d'Herelle, largely considered to be the founding father of applied bacteriophage (phage) science, was the first to envision the use of phages as bacterial biocontrol agents, and an ever increasing number of peer-reviewed publications demonstrate the potential of phages to control pathogenic foodborne bacteria. While the results of these studies have been generally positive, obstacles nevertheless remain before widespread implementation of phage based interventions can be routinely achieved. For example, Hagens and Loessner[1] have listed several desirable properties of phages used as biocontrol agents in foods including: (1) Having a broad host range capable of infecting several strains of the target species and/or genus); (2) The phages should be strictly lytic (virulent); and (3) Should be propagated on nonpathogenic host; (4) The complete genome sequences of all phages used in a given product should be known; (5) There should be a lack of transduction of nonphage (i.e., bacterial) DNA; (6) There should be an absence of any genes encoding pathogenicity associated or

potentially allergenic proteins; (7) Oral feeding trials should show no adverse effects; (8) Phage based products should achieve GRAS (or some other appropriate regulatory approval, like a direct food additive—Goodridge LD) for use in foods; the phages should be sufficiently stable over long periods of storage and application; and (9) The phage product should be amendable to scale up for commercial production. Notwithstanding this comprehensive list, there is still considerable discussion over the exact properties that food grade phage products should have, and as yet, there is no industry standardized phage production procedures. These issues need to be addressed as the use of phages as biocontrol agents in foods becomes popular. Other challenges include the methods by which phage cocktails are produced (i.e., what is the basis for inclusion or exclusion of a phage from a cocktail), and whether or not to use cocktails or single phages in preparations. As the field of phage based biocontrol in food products continues to mature, there is no doubt that these issues will be addressed.

Pre-Harvest Control of Foodborne Pathogens in Food Producing Animals

Phage therapy has shown promise as an effective pre-harvest intervention by controlling foodborne pathogens in animals before they enter processing plants,[2-4] with several studies indicating that phage therapy is effective against a broad range of foodborne pathogens belonging to the genera *Salmonella*, *Campylobacter*, *Listeria* and *Escherichia*.[5-9] Phage therapy has been investigated for efficacy in red meat producing animals (cattle, sheep, swine) and white meat producing animals (poultry). Several studies have investigated the use of phages either alone, or in a cocktail, to control foodborne pathogens in sheep and cattle.[2-4,10] Generally, the use of only one phage in some experiments has led to resistance,[2,10] while the use of multiple phages in a cocktail has decreased the chance of developing resistance to a single phage.[4,7,11]

Ruminants. Phage based interventions have been aimed at controlling *Escherichia coli* (*E. coli*) serotype O157:H7 in cattle and other ruminants. This bacterial pathogen causes a myriad of foodborne disease manifestations, including diarrhea, hemorrhagic colitis, hemolytic uremic syndrome and thrombotic thrombocytopenic purpura.[12,13] Cattle and other ruminants are considered to be the principal reservoirs of *E. coli* O157:H7,[14] and the contents of the intestines and fecal material on the hide

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may contaminate meat during slaughter. Animals that shed high levels of this pathogen may pose an elevated risk of contaminating the food chain if presented to slaughter, and phage-based approaches to reduce fecal shedding of this pathogen have been designed to limit both the duration of shedding and concentration of *E. coli* O157 in the bovine gastrointestinal tract. For example, Callaway et al.[2] inoculated sheep with phages specific against *E. coli* O157:H7. This treatment caused a decrease in the concentration of *E. coli* O157:H7 throughout the gastrointestinal tract, but the differences were not statistically significant.[2] Bach et al.[10] evaluated the ability of a single phage (DC22) to eliminate *E. coli* O157:H7 in experimentally inoculated sheep. After introduction of phage DC22 to the sheep, there was no observed effect on the fecal shedding of *E. coli* O157:H7, probably due to nonspecific binding of the phage to food particles and other debris present in the rumen and gastrointestinal tract which may have ultimately limited their efficacy. The use of one phage in the study by Bach et al.[10] strengthens the idea that a mixture of phages might be more effective at controlling *E. coli* O157:H7 in livestock than a single phage.

As such, other research groups have also evaluated the use of phage cocktails to decrease various bacterial pathogens in livestock during brief time periods. Callaway and coworkers[15] anaerobically isolated phage that targeted *E. coli* O157:H7 from fecal samples collected from commercial feedlot cattle in the central US. The host range of the phages was determined, and the phages were combined to form a cocktail of phage for in vivo studies. When a 21-phage cocktail was inoculated into sheep artificially contaminated with *E. coli* O157:H7, intestinal populations of *E. coli* O157:H7 were decreased (p < 0.05) in the cecum and rectum, a result that indicates that properly selected phages can be used to reduce *E. coli* O157:H7 in food animals. The authors concluded that phage therapy approaches could be an important part of an integrated foodborne pathogen reduction program. Raya et al.[16] isolated a bacteriophage, CEV1, from sheep that were resistant to *E. coli* O157:H7 colonization, and used this phage to reduce populations of *E. coli* O157 in sheep. In vitro, CEV1 efficiently infected *E. coli* O157:H7 grown both aerobically and anaerobically. Four sheep were treated once orally with 10^{11} PFU of phage CEV1, 3 d after challenge with *E. coli* O157. Sheep receiving a single oral dose of CEV1 showed a 2–3 log-unit reduction in cecal and rectal *E. coli* O157:H7 levels within 2 d compared with levels in the controls, although rumen concentrations remained unchanged.

This same research group later combined phage CEV1 with a newly isolated phage, CEV2, which had high specificity for *E. coli* O157:H7, and showed that cocktail was very effective at reducing *E. coli* O157:H7 by 3 log units compared with the untreated phage-free control.[17] The authors concluded the phage cocktails are more effective than individual phages at reducing *E. coli* O157:H7 populations in the ruminant gastrointestinal tract.[17]

These studies show that phage therapy may be useful to decrease *E. coli* O157:H7 counts in adult livestock, and may provide a viable method to reducing *E. coli* O157:H7 in live animals immediately before slaughter. Other studies have addressed the question of using phages to control shedding of pathogenic bacteria in younger animals.

For example, Waddell et al.[18] treated weaned 7- to 8-week old calves with a six phage cocktail up to 7 d before infection with *E. coli* O157:H7. The results indicated that most of the untreated calves shed *E. coli* O157:H7 in their feces for at least 12–16 d. In contrast, the treated calves stopped shedding *E. coli* O157:H7 after day 8, which corresponded with a dramatic increase in the concentration of phages that were shed in the feces of the animals. The increase in the number of phages that were excreted was due to phage replication in the calves, as determined by the fact that such a result was not observed in uninfected control calves that were only inoculated with the phage cocktail. Chase et al.[19] used a 37-phage cocktail in an attempt to reduce shedding in Black Angus calves ranging from 4–6 mo of age. The calves were orally inoculated with *E. coli* O157:H7 in two separate trials. The first trial evaluated ileal samples and the second trial evaluated fecal samples for the presence of *E. coli* O157:H7 and phages. In the first trial, a significant decline in the concentration of *E. coli* O157:H7 was observed at 8 h (p < 0.05). However, the concentration of *E. coli* O157:H7 increased back to the concentration of the control samples at 16 h. In the second trial, shedding of *E. coli* O157:H7 decreased significantly in the treated group (p < 0.05) at 24 h. As with the ileal samples, an increase in the concentration of *E. coli* O157:H7 was observed at 36 h. The increases in cell concentration were associated with a decrease in phage concentration. None of the *E. coli* O157:H7 cultured from the ileal or fecal samples showed resistance to the phage cocktail. These results highlight the ability of the 37-phage cocktail to transiently reduce *E. coli* O157:H7 in calves, without the formation of phage resistant mutants.

One concern that arises when phage therapy is applied to reduce pathogen concentration in ruminants is that fact that the viability of orally administered phage may be rapidly reduced under the acidic conditions of the abomasum[20] and in the presence of enzymes and other digestive compounds such as bile. To address this issue, Sheng and colleagues[21] evaluated the phage-based rectal treatment of ruminants based on a previous study[22] which showed the recto-anal junction to be the primary site of *E. coli* O157:H7 colonization in cattle. Two phages (designated KH1 and SH1) were tested, alone or in combination, for their ability to reduce intestinal *E. coli* O157:H7 in animals. To optimize bacterial carriage and phage delivery in cattle, *E. coli* O157:H7 was applied rectally to Holstein steers 7 d before the administration of 10^{10} PFU of SH1 and KH1. Phages were applied directly to the recto-anal junction mucosa at a multiplicity of infection (MOI) of 10^{2}. In addition, phages were maintained at a concentration of 10^{6} PFU/ml in the drinking water of the treatment group. Results showed that this approach reduced the average number of *E. coli* O157:H7 CFU among phage-treated steers compared with control steers (p < 0.05), but did not eliminate the bacteria from the majority of steers. In a similar study, Rozema et al.[23] compared oral and rectal administration of *E. coli* O157-specific phages for efficacy in reducing or eliminating the fecal shedding of *E. coli* O157:H7 by experimentally inoculated steers. Fecal shedding

was monitored over 83 d after oral, rectal, both oral and rectal, or no treatment with a four-strain O157-specific phage cocktail delivered in multiple doses. Orally treated steers produced the fewest *E. coli* O157:H7 culture-positive samples, but this number was not statistically significant when compared with control steers. Animals that received the phage cocktail rectally shed *E. coli* O157:H7 at a higher concentration than steers from the other treatment groups, though there was no statistical difference in the number of *E. coli* O157 positive samples among treatments. Phages were isolated from steers that did not receive the cocktail, showing that the phages could be acquired from the environment.

While these studies indicate the possibility of using phages to control *E. coli* O157:H7 colonization and shedding in ruminants, more studies need to be conducted to determine adequate phage dose, number of doses (a single dose vs. continuous dosing), standardized methods of phage delivery (water or feed delivery vs. rectal delivery), and the economics of phage therapy in food producing animals.

Swine. Few studies have been conducted to assess the efficacy of phage therapy to control foodborne pathogens in swine. Harris[24] developed and evaluated a cocktail of 26 phages for their ability to effect control of *Salmonella* in swine. Preliminary studies showed that the phage cocktail did not significantly reduce the concentration of *Salmonella* in pigs. Based on these observations, Lee and Harris[25] conducted another study in which a single *Salmonella*-specific lytic phage (Felix 01) was examined as a possible candidate to control Salmonella. Three week-old pigs received 10^{10} PFUs of Felix 01 phage, both orally and intramuscularly 3 h following challenge with *Salmonella enterica* serovar Typhimurium at a concentration of 10^8 CFUs. Nine hours post challenge, blood, liver, lung, spleen, ileocecal lymph node, tonsil and cecum content samples were obtained from sacrificed pigs, and analyzed for *S.* Typhimurium. The phage treatment significantly reduced the amount of *S.* Typhimurium in tonsil and cecum contents as compared with the control. It was concluded that the phage treatment could be considered as a short-term intervention strategy to reduce the rapid dissemination of *Salmonella* in swine.[25]

Poultry. The majority of phage therapy applications to control foodborne pathogens in live animals have been conducted in poultry. Poultry and egg products are important sources of the human pathogens *Salmonella* spp and *Campylobacter* which cause many cases of foodborne illness globally. The scientific literature indicates that phage therapy-based attempts at controlling *Salmonella* in poultry have reduced, but not eliminated the pathogen. Berchieri et al.[26] showed that newly hatched chicks challenged with Samonella *enterica* serovar Typhimurium followed by oral or feed-based introduction of a single phage for 7 d did not show decreased levels of *S.* Typhimurium in cecal contents. The researchers reported a high percentage of phage resistant *S.* Typhimurium isolates, which occurred in 34–82% of 50 bacterial isolates tested on each of days 2, 4, 7 and 10 post infection. However, when a second phage was used, significant reductions in mortality were observed over a 21-d period (from 56% to 20%). However, while the concentration of the challenge

strain was reduced by more than 2 log units within 3–6 h, these reductions were transient.

Fiorentin et al.[3] isolated a cocktail of phages from free-range chickens and used them to reduce the concentration of *Salmonella enterica* serovar Enteritidis phage type 4 (PT4) in the ceca of broilers. Five days post treatment, the concentration of *S.* Enteritidis PT4 per gram of cecal content in the phage-treated group was reduced by 3.5 logs, and samples collected up to 25 d after treatment revealed that the treated birds still had lower colony-forming units of *S.* Enteritidis PT4 per gram of cecal content compared with untreated broilers.

Both Andreatti-Filho et al.[27] and Sklar and Joerger[28] evaluated cocktails of phages for their ability to reduce *S.* Enteritidis in experimentally infected chicks and young chickens. The results of both studies showed that the cocktails significantly reduced the concentrations of *S.* Enteritidis recovered from the treated birds, but the reductions were not statistically significant.

A possible solution to the problem of transient bacterial reduction following phage therapy is the use of phage cocktails in combination with another biocontrol method such as competitive exclusion bacteria to sustain the ability of phages to control pathogens in the live animal. Toro et al.[4] used a mixture of three *Salmonella*-specific phages in combination with competitive exclusion bacteria to reduce *Salmonella* colonization in experimentally infected chickens. The phages were administered orally to the chickens several days prior and after *Salmonella* challenge. A competitive exclusion product consisting of a defined culture of seven different microbial species was used either alone or in combination with phage cocktail treatment, and was administered orally at hatch. *Salmonella* counts in the intestine, ceca and a pool of liver/spleen samples were evaluated in *Salmonella*-challenged chickens treated with the phage cocktail or with the cocktail and competitive exclusion bacteria. A reduction in *Salmonella* counts was detected in the cecum and ileum of phage treated, competitive exclusion treated, and phage treated/competitive exclusion treated chickens as compared with nontreated birds.

Atterbury and colleagues[29] individually administered three phages that had broad host ranges against *Salmonella enterica* serotypes Enteritidis, Hadar and Typhimurium to birds experimentally colonized with their specific *Salmonella* host strains. The first phage reduced *S.* Enteritidis cecal colonization by more than 4.2 log CFU within 24 h compared with controls. Administration of the second phage reduced *S.* Typhimurium by more than 2.19 log CFU within 24 h. The third phage was ineffective at reducing *S.* Hadar colonization. Phage resistance occurred at a frequency commensurate with the titer of phage being administered, with larger phage titers resulting in a greater proportion of resistant *Salmonella*e. The researchers concluded that the selection of appropriate phages and optimization of both the timing and method of phage delivery are key factors in the successful phage-mediated control of *Salmonella*e in broiler chickens. To investigate the effect that method of delivery has on the efficacy of phage therapy, Borie et al.[30] used three phages to reduce *S.* Enteritidis colonization in experimentally infected 10-d old chickens. The chickens were treated by coarse spray or

drinking water with a cocktail of the three phages 24 h before challenge with *S.* Enteritidis. Chickens were euthanized at 20 d of age and sampled for *S.* Enteritidis. The results showed that aerosol-spray delivery of the phage cocktail significantly reduced the incidence of *S.* Enteritidis infection when compared with the control group. Also, phage delivery by both coarse spray and drinking water significantly reduced the intestinal *S.* Enteritidis colonization. It was concluded that phage treatment, either by aerosol spray or drinking water, may be a plausible approach to reduce *Salmonella* infection in poultry.[30]

The collective research conducted to investigate the use of phages to control foodborne pathogens in ruminants and poultry indicates the successes, but also challenges, that are present when this biocontrol method is applied to live animals. A main challenge is the need for regulatory approval of phage based products prior to their large scale investigation in animals. Currently, there are no phage-based products approved for use to reduce pathogens within the live animal. The United States Department of Agriculture (USDA) issued two no objection letters for the use of *E. coli* O157:H7 and *Salmonella* targeted bacteriophages developed by Omnilytics™ (Salt Lake City, UT, USA) for use as hide sprays on cattle prior to slaughter.[31] The phages would aid in the reduction of *E. coli* O157:H7 and *Salmonella* spp on hides prior to further processing, to decrease transfer of these pathogens from the hide to meat. The hide sprays produced by Omnilytics™ are the only phage products currently approved for use in the animal industry.[32-34] Elanco (Greenfield, IN) in conjunction with Omnilytics™ has produced two products called Finalyse to reduce *E. coli* O157:H7 on cattle hides, and Armament for reduction of *Salmonella* on poultry. Further approval of phage based products may become easier to achieve with the European Food Safety Authority Biohazards Panel endorsement of the use of phages as a treatment for foods of animal origin including carcasses, meat and dairy products.[35]

Post-Harvest Control of Foodborne Pathogens in Meat, Fresh Produce and Processed Foods

Meat. The efficacy of phage based treatments is expected to be superior in foods than in the live animal, since phages in food based applications are not subjected to the dynamics observed in a living animal, such as interaction with the immune system, and constantly changing microenvironments. Still, the components of the food matrix, including fat, proteins and carbohydrates can all affect the ability of phages to find, and infect their target bacteria, as can intrinsic properties of the foods such as pH. Such characteristics of the food must be carefully considered during the development of food-based phage therapy approaches.

O'Flynn et al.[7] investigated the use of three phages (e11/2, e4/1c and PP01), individually, and combined as a cocktail, for their ability to lyse *E. coli* O157:H7 on meat. Eighteen pieces of beef were inoculated with 100 μl of a rifampin-resistant *E. coli* O157:H7 strain at 10^3 CFU/μl, and the three-phage cocktail (MOI of 10^6) was pipetted evenly onto nine of the pieces of beef. The remaining nine pieces of beef were inoculated with *E. coli* O157:H7, but not phage, and served as controls. Following

incubation for 1 h, the treated and control meat samples were enriched in BHI broth at 37°C for 2 h, followed by evaluation of the enriched samples by plate count. Seven of the nine phage-treated samples were devoid of *E. coli* O157:H7, while two of the samples had *E. coli* O157:H7 counts of less than 10 CFU/ml. In contrast, the control samples had *E. coli* O157:H7 concentrations of 10^5 CFU/ml.[7]

Others have assessed the phage-based control of *L. monocytogenes* in meat, with research studies demonstrating that the combination of a *L. monocytogenes* phage and nisin (a bacteriocin produced by lactic acid bacteria that is approved for use in ready to eat meats to control the presence of *L. monocytogenes*) provided an antimicrobial effect against *L. monocytogenes* in broth, but not in buffer or raw beef,[5] leading the researchers to conclude that the use of nisin and bacteriophages has potential to control *L. monocytogenes* in meats, but more research detailing the ecological aspects of complex systems like foods must be achieved before any practical use of these treatments can be realized.[5] Atterbury et al.[36] conducted experiments using phages to reduce *Campylobacter jejuni* on chicken skin. At 4°C, *Campylobacter* recovery from controls inoculated with 10^6 and 10^4 CFU remained constant through the entire course of the experiment, and in chicken samples inoculated with the lowest phage titer (10^3 PFU), no significant reduction in *C. jejuni* numbers was observed. In contrast, when the highest phage titer (10^7 PFU) was applied onto the chicken skin, there was a significant reduction of the pathogen at all sampling points. The efficacy of the phage treatment was more pronounced in frozen chicken samples, leading the authors to conclude that the use of phage therapy, when coupled with a freeze step, could be an effective treatment to reduce *C. jejuni* on poultry.[36] Goode and coworkers[6] artificially contaminated portions of chicken skin with *S.* Enteritidis and half of the contaminated samples were inoculated with *Salmonella* typing phage 12 at a MOI of 1. The samples were incubated at 4°C and samples were obtained prior to phage application and 24 and 48 h following addition of the phage. The results showed a statistically significant reduction in *Salmonella* concentration in samples treated with phage, when compared with nontreated controls.

Fresh produce. Fresh fruits and vegetables have increasingly become responsible for many cases of foodborne illness. For example, between 1990 and 2003, there were at least 554 foodborne outbreaks associated with vegetables, and these outbreaks resulted in approximately 28,000 illnesses and several deaths.[37] The recent outbreak of a pathogenic isolate of *E. coli* linked to bean sprouts produced on a German organic farm highlights the need to develop better interventions to control the presence of foodborne pathogens in foods that are consumed raw. Furthermore, organic farming practices dictate that only natural antimicrobials be used in production, which makes phages an excellent choice as a biocontrol approach in such establishments.

Pao and coworkers[38] conducted trials aimed at establishing whether phages could be used to control *Salmonella* in sprouting seeds. In this work, the researchers isolated and characterized two bacteriophages with different host ranges, and showed that

a mixture of both phages resulted in a 1.50 log reduction in the numbers of *Salmonella* in the soaking water of broccoli seeds.

Leverentz and colleagues[39] investigated the ability of two phage cocktails to reduce concentrations of *L. monocytogenes* on fresh cut apples and honey dew melons. The researchers determined that the phage cocktails reduced *L. monocytogenes* concentrations on honey dew melons by 2.0 to 4.6 logs as compared with the control. On the fresh cut apples, the phage cocktail reduced the *L. monocytogenes* concentration by less than 0.4 log units. One possible reason for the reduced efficacy of the phage cocktail on the apple slices may be the low pH on the cut surface of the apples. For example, on the apple slices the pH was measured at 4.4, and the phage concentration was reduced to undetectable levels within 30 min of application.[39] Other studies have confirmed that low pH can reduce or eliminate *Salmonella* phage populations.[40,41]

As with the Dykes and Moorhead[5] study, Leverentz' group showed that the efficacy of phage reduction was shown to increase when phage were employed in combination with the nisin. In a follow up study with honey dew melons, the authors showed that a cocktail of six phages resulted in a larger reduction in the *L. monocytogenes* concentration, when the phages were applied at higher concentrations,[42] highlighting once again the importance of phage dosage.

More recently, Sharma et al.[43] investigated the ability of a cocktail of three *E. coli* O157:H7 specific bacteriophages to reduce the presence of this pathogen on artificially contaminated fresh-cut iceburg lettuce and cantaloupe. Samples of both produce were inoculated with *E. coli* O157:H7; cantaloupe samples were spot-inoculated with ECP-100, while lettuce samples were sprayed with the same phage cocktail, followed by storage for up to 7 d at 4°C or 20°C . After 2 d of storage, the lettuce samples were tested for the presence of the bacteria, and results showed statistically significant reductions in the levels of *E. coli* O157:H7 on phage sprayed samples, as compared with the control. The cantaloupes were sampled for up to 7 d, and as with the lettuce, the results showed much lower concentrations of *E. coli* O157:H7 on the phage treated samples than the controls. Finally, as part of a large study using many ready to eat foods, Guenther and coworkers[44] demonstrated the efficacy of two wide host range phages (A511 and P100) for control of *L. monocytogenes* in leaves of lettuce and cabbage. The vegetables were spiked with bacteria (103 CFU/g), followed by addition of each phage to separate vegetable samples at concentrations of (3 x 106 to 3 x 108 PFU/g), and storage at 6°C for 6 d. The results indicated that both phages were able to reduce the concentrations of the *L. monocytogenes* strains by more than 2 logs in both the lettuce and cabbage, when compared with controls.[44]

Others have combined the use of phage cocktails and other antimicrobials in an attempt to increase the efficacy of the phage treatment. Viazis et al.[45] produced a phage cocktail (BEC8) of eight previously isolated phages that were shown to individually infect and lyse *E. coli* O157 and *E. coli* O26 strains with high efficiency. To evaluate the ability of the cocktail to infect target strains, the BEC8 cocktail was tested, individually, in liquid culture against four *E. coli* O157:H7 strains at a concentration of

approximately 10^6 CFU/ml. Different tubes containing the individual bacterial strains were inoculated with the BEC8 cocktail at three different MOIs including 1, 10 and 100. The tubes were incubated at room temperature and 37°C for 5 h, and reductions in the bacterial concentration were determined by plate count. Significant reductions in bacterial counts were observed at both temperatures, with the greatest reductions occurring at 37°C. At this temperature and a MOI of 100, the phage cocktail reduced the *E. coli* O157:H7 concentrations by greater than 5 logs.[45] Following up on this work, the same research group investigated the ability of the BEC8 cocktail alone, and in combination with the essential oil trans-cinnamaldehyde (TC), to reduce the presence of a mixture of *E. coli* O157:H7 strains on whole baby romaine lettuce and baby spinach leaves.[46] The bacterial strains were spot inoculated onto the leaves at low (10^4), medium (10^5), and high concentrations (10^6 CFU/ml). After the leaves were dried, the phage cocktail was applied at a concentration of approximately 10^6 PFU/leaf, either alone, or in combination with TC (0.5% v/v). The leaves were incubated at 4, 8, 23 and 37°C for 10 min, 1 h and 24 h. Bacterial reductions were determined by plate count. At the low bacterial concentration, no bacteria were recovered following treatment with the phage cocktail or TC individually after incubation at 23 or 37°C for 24 h. The efficacy of both the phage cocktail and TC decreased at higher bacterial concentrations, and shorter incubation times. Still, when the treatments were combined, no bacteria were recovered after 10 min at all temperatures and inoculum levels.[46] In contrast to the previous studies, bacterial numbers were not reduced at low temperatures. This result may be due to the relatively short time of incubation (24 h in this study, as compared with up to 7 d) at refrigeration temperatures, and not the efficacy of the phage cocktail at low temperatures.

These results demonstrate the effectiveness of using phages to control pathogens on fresh produce. In addition to the bacterial reductions, the studies are noteworthy because they show that phage treatments can control foodborne pathogens at refrigeration temperatures. It should be noted that phage based products to reduce the spread of bacteria on fresh vegetables were the first to ever receive regulatory approval. In 2002, the Environmental Protection Agency (EPA) approved the use of a pesticide for control of bacterial spot (rot) of tomatoes and peppers, which consists of two bacteriophages that infect the plant pathogens Xanthomonas campestris subsp vesicatoria and Pseudomonas syringae (EPA 2007). The pesticide was developed by Omnilytics™, and consists of a mixture of the two phages which constitute the active ingredient. The Xanthomonas phage controls bacterial spot on tomatoes and peppers and the Pseudomonas phage controls bacterial speck on tomatoes. The pesticide is approved for various uses including direct application to plants and the surrounding soil. The early regulatory approval of such products paves the way for the development of phage biocontrol products to reduce the presence of foodborne pathogens on fresh produce.

Processed foods. Processed foods are a significant source of foodborne outbreaks. For example, outbreaks of listeriosis, salmonellosis, and hemorrhagic colitis and hemolytic uremic syndrome

caused by *E. coli* O157:H7 have been linked to foods including sausages, deli meats, cheeses, and powdered milk.[47-50] The control of foodborne pathogens in these foods represents another use of phage as biocontrol agents, highlighting the diversity of foods that can be treated with phages, and the flexibility of phages as antimicrobial agents.

Carlton et al.[8] characterized the virulent listeriophage P100 which infects and kills a majority of *L. monocytogenes* strains, and showed its use as an antimicrobial by producing surface ripened red-smear soft cheese, and contaminating the cheese with low concentrations of *L. monocytogenes* at the beginning of the ripening period. Phage P100 was applied to the surface during the rind washings. Depending on the time points, frequency, and the dose of phage applications, the researchers observed a significant reduction ranging from 3.5 logs to complete elimination of the *L. monocytogenes* bacteria. The authors did not observe the presence of any *L. monocytogenes* resistant cells that were recovered from the samples. This study indicated the possibility of using bacteriophages to control *L. monocytogenes* surface contamination of soft cheeses, which are the cause of many cheese-borne outbreaks of listeriosis.[51-54] Modi et al.[55] evaluated the effects of phages on the survival of *S.* Enteritidis during the manufacture and storage of cheddar cheese. The authors concluded that the addition of phages to raw and pasteurized milk significantly reduced the *S.* Enteritidis concentration in cheddar cheeses produced from these milks.

Recently, Guenther and Loessner[56] evaluated phage A511 for its ability to control *L. monocytogenes* on Camembert and Limberger type cheeses. The surface of unripened cheeses were inoculated with two strains of *L. monocytogenes* at levels of 10^1 to 10^3 CFU/cm^2, followed by application of the A511 phage either in a single dose or repeated doses. Regardless of the number of phage doses, and the type of cheese, counts in all *L. monocytogenes* inoculated cheeses were reduced by greater than 2.5 logs at the end of the ripening period (21 d). Repeated doses of A511 did not lead to greater inhibition of *L. monocytogenes* on Camembert cheese, but did lead to delayed regrowth of *L. monocytogenes* on Limburger type cheese.[56] This same research group had previously conducted a comprehensive study using phage A511 and another *L. monocytogenes* specific phage, P100, on a large variety of ready-to-eat foods including hot dogs, turkey deli meat, smoked salmon, pasteurized chocolate milk (3.5% fat), mozzarella cheese brine, iceberg lettuce and cabbage.[57] The authors observed the need to optimize phage applications for the different food types, and reported reductions of greater than 2 logs of the *L. monocytogenes* concentrations in most foods except for the turkey deli meat and smoked salmon samples.[57] Phage P100 is the active component of LISTEX™ P100, and in 2007 the FDA and USDA announced that they had approved LISTEX™ P100 as a natural phage product against *Listeria*, (and produced by EBI Food Safety, Wageningen, The Netherlands), as GRAS (generally recognized as safe), for all food products.[58] GRAS status exempts the additive in question from a formal pre-market safety review.[59] The USDA has recently amended the GRAS status of LISTEX™ such that the product is now recognized as a processing aid (de Meester D, personal communication).

Another comprehensive study was conducted by Abuladze et al.[60] to evaluate an *E. coli* O157:H7 phage cocktail in several foods. A three phage cocktail (called ECP-100) was utilized to reduce experimental contamination of hard surfaces found in food production facilities (glass and gypsum), tomato, spinach, broccoli and ground beef with a three strain *E. coli* O157 mixture. Following bacterial contamination, the hard surfaces and foods were treated with ECP-100 (test samples) or sterile phosphate-buffered saline buffer (control samples), and the efficacy of phage treatment was evaluated by comparing the number of viable organisms recovered from the test and control samples. When treated for 5 min with ECP-100 at three different phage concentrations (10^{10}, 10^9 and 10^8 PFU/ml), the treated samples had statistically significant reductions of less than a log to greater than 4 logs in the number of *E. coli* O157:H7 organisms recovered from the glass and gypsum board surfaces. With respect to the food samples, the observed reductions ranged from 94% in tomato samples (120 ± 4 h post-treatment of tomato samples) to 100% (at 24 ± 4 h post-treatment of spinach samples).[60] This data confirms the other reports that phages may be useful for reducing contamination of various foods, but is also useful in that it demonstrates the ability of phages to be used to decontaminate hard surfaces. In the food industry, contamination of food contact surfaces is a primary issue due to concerns of cross-contamination occurring between contaminated and noncontaminated foods. Phages may represent a new method to eliminate foodborne pathogens from these surfaces. Recently, Intralytix, Inc. the makers of ECP-100 (trade name: EcoShield™) received regulatory clearance in the form of a "Food Contact Notification" (FCN) from the Food and Drug Administration (FDA) for its phage-based EcoShield™ food safety product, effective against *E. coli* O157:H7. The FCN will allow the use of EcoShield™ on red meat parts and trim intended to be ground. Previously, the FDA approved a phage cocktail produced by Intralytix, called ListShield™, which contains six individual phages for use on ready-to-eat (RTE) meat and poultry products as an antimicrobial agent against *L. monocytogenes*.[61]

Others have investigated the use of phages to control pathogens in skim milk and sausages. Kim and colleagues[62] used phages that were newly isolated against *Enterobacter* (now *Chronobacter*) *sakazakii* to effect reductions of this bacterium in reconstituted infant formula milk, and showed that the treatment was able to suppress the growth of *C. sakazakii* in prepared infant formula, both at 24 and 37°C. Whichard et al.[63] tested the broad host range *Salmonella* phage Felix-O1 for ability to control *S.* Typhimurium on sausages, and reported a 99% reduction of viable cells of phage treated samples.

Conclusion

The scientific literature demonstrates the possibility of using phage therapy to effectively reduce the presence of foodborne pathogens in food producing animals and in fresh and processed foods. To date, the literature indicates more progress made in using phages to control foodborne pathogens in foods than in or on live animals. Thus, for this latter application in particular,

there are many questions that remain to be answered through the collection of scientific data from rigorously designed experiments. Such studies should be designed to solve issues associated with phage-bacteria interactions and ecology, phage efficacy under different environmental conditions and physiological conditions of food producing animals, and continued work should be conducted to more fully address the issue of phage resistance. It is possible that in the live animal, phage therapy will come to be viewed in a similar way to other biocontrol strategies such as competitive exclusion. If that is case, then future research should also be accomplished to develop methods to integrate phage

therapy approaches with other technologies, including competitive exclusion and vaccines, as well as other antimicrobials such as bacteriocins. In that way, the development of a hurdle approach for foodborne pathogen control in live animals, much like the approach developed for control of pathogens in foods, is a possibility. Finally, the cost of production will become a major consideration. Regardless of the remaining work to be accomplished, the usefulness of phage treatments to reduce bacterial pathogens in foods has been clearly demonstrated, and it is likely that more phage products will be developed and used to reduce contamination during food production.

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