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Chapter 8

Phage Biocontrol Applications in Food Production and Processing

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Abstract

Bacteriophages, or phages, are one of the most – if not the most – ubiquitous organisms on Earth. Interest in various practical applications of bacteriophages has been gaining momentum recently, with perhaps the most attention (and most regulatory approvals) focused on their use to improve food safety. This approach, termed “phage biocontrol” or “bacteriophage biocontrol,” includes both pre- and post-harvest application of phages as well as decontamination of the food contact surfaces in food processing facilities. This chapter focuses on post-harvest applications of phage biocontrol, currently the most commonly used type of phage mediation. We also briefly describe various commercially available phage preparations and discuss the challenges still facing this novel yet promising approach.

Phages are Ancient and Abundant in Nature

Bacteriophages are the viruses that infect bacteria. They were discovered in 1917 by Félix d’Hérelle in 1917 (Salmond and Fineran, 2015; Sulakvelidze et al., 2001), who also coined the term “bacteriophage,” derived from "bacteria" and the Greek φαγεῖν (*phagein*) meaning “to eat” or "to devour" bacteria. Numerous studies, including recent metagenomic surveys, suggest that phages

are (i) arguably the oldest microorganisms on this planet that likely originated approximately 3 billion years ago (Brüssow, 2007), and (ii) likely the most ubiquitous organisms on Earth, abundant in all life-supporting environments including all natural untreated foods. To give a few examples: (1) There are an estimated 1.5×10^8 phage particles per gram of agricultural soil (Ashelford et al., 2003; Williamson et al., 2003); (2) There are an estimated 7 to 15×10^6 phages per mL in fresh water lakes (Mohiuddin and Schellhorn, 2015) and 10^6 to 10^9 particles per mL in sea water (Bergh et al., 1989); (3) Bacteriophages are likely present in 100% of fresh unprocessed foods and have been isolated from various food products such as beef, pork, chicken, fresh produce, dairy, and fermented foods (Aw et al., 2016; Park et al., 2011; Zhang et al., 2014); (4) Humans are constantly exposed to phages in their daily lives as highly diverse and abundant phage populations are present on various human organs such as the skin, vagina, etc. – with an estimated 10^{15} phages present in our gastrointestinal tract (Dalmasso et al., 2014; Hannigan et al., 2015; Shkoporov et al., 2019); (5) Similarly, animals carry a diverse and prolific phage population (Dalmasso et al., 2014; Delwart, 2012; Hannigan et al., 2015; Shkoporov et al., 2019). Altogether, phages are a significant part of the living biosphere and humans continuously interact with a multitude of phages, through food, water, and the general environment. The phage population is increasingly recognized as an active part of the mammalian microbiome and a contributor towards the health of their host (Cadwell, 2015).

Almost immediately after their discovery, the ability of phages to infect and kill bacteria led to the exploration of their therapeutic potential against bacterial pathogens, in a clinical approach known as “bacteriophage therapy” or “phage therapy” – with the first therapeutic use in humans described in 1919, just two years after their discovery by d’Herelle (Sulakvelidze and Kutter, 2005; Summers, 2001). Although the exact number of people treated therapeutically with phages is difficult to estimate, in all likelihood tens of thousands of people have been administered phages therapeutically since that initial clinical use, with no serious phage-related adverse effects ever reported (Kutter and Sulakvelidze, 2004). However, the discovery and increased use of antibiotics during the 1940s and 1950s, coupled with an incomplete understanding of

phage biology, as well as several other factors, led to a decline in the clinical use of phages in Western Europe and North America. In contrast, phage therapy continued to be utilized in the former Soviet Union and some Eastern European countries (e.g., Poland) where therapeutic phage preparations are still readily available in pharmacies today (Sulakvelidze et al., 2001; Sulakvelidze and Kutter, 2005; Summers, 2012). The situation has changed recently as phage therapy and other phage-related technologies – including phage biocontrol – have been gaining a renewed interest in the West.

Two major developments played a significant role in rekindling the interest in phages as antimicrobial agents. First, the emergence and widespread distribution of antimicrobial resistant (AMR) bacterial pathogens have limited our therapeutic options. The situation is further exacerbated by the limited number of new antimicrobial drugs entering the market; also, most new antibiotics approved in recent years are modifications of existing drugs and, therefore, are at higher risk of being rendered ineffective in a short period (Talbot et al., 2019). Secondly, there has been an increased appreciation of the damage broad-spectrum antibiotics can inflict upon the microbiome. In fact, antibiotic mediated microbiome perturbation is now believed to contribute to several non-communicable and chronic diseases (Dietert and Dietert, 2015). Also, due to the rapidly growing field of microbiome research, connections are increasingly being made and/or hypothesized between microbiome composition and various health-associated conditions, ranging from obesity to Alzheimer to certain forms of cancer. If these links are substantiated through further research, there would be a need for a modality(ies) capable of the targeted elimination of certain bacteria in our microbiome which would not deleteriously impact other, beneficial bacterial species (i.e., a modality to fine-tuning the microbiome). As a result, there has been an increased interest in targeted antimicrobials, such as phages, which are capable of killing disease-causing bacteria in the gastrointestinal tract without altering the normal microflora. Lytic phages demonstrate remarkable bactericidal activity against specific bacterial strains and can be employed to treat and manage AMR as well as non-AMR bacterial infections either alone or in conjunction with existing antimicrobials (Hesse and Adhya, 2019). Since lytic phages exert their bactericidal activity through very

different mechanisms than antibiotics, they demonstrate similar efficacy for both AMR and non-AMR bacterial pathogens (Hesse and Adhya, 2019; Kortright et al., 2019; Sulakvelidze and Morris, 2015). In addition, the mechanisms of bacterial resistance to phages differ from those for antibiotics and the strains that develop resistance to antibiotics typically remain susceptible and can be killed by bacteriophages (Chen et al., 2017). Therefore, phages may help to manage various bacterial infections, including those caused by pandrug-resistant bacterial strains (Sulakvelidze and Morris, 2015). The high specificity towards the target bacterial species provides an additional benefit, the preservation of microbial diversity and function (Cieplak et al., 2018). Thus, developing phage preparations for human clinical applications appears to be a logical fit for lytic bacteriophages. Yet, most commercial phage developments in the United States thus far have focused not on human therapeutic applications or microbiome modulations (although efforts in those areas are underway, including several clinical trials), but on using bacteriophages for improving the safety of our food products (Hesse and Adhya, 2019).

Foodborne Pathogens – A Growing Challenge

Foodborne illnesses of microbial origin continue to be a serious food safety problem worldwide. In addition to being of significant public health importance, the economic impact of foodborne bacterial infections is considerable. For example, in the US alone, the average incidence of foodborne illness is estimated to cost ~$1500/person, with the total annual estimated cost of these foodborne diseases reaching over $75 billion (Scharff, 2012). In addition, substantive costs to the food industry are incurred due to product loss and brand-damaging negative publicity that is associated with the recall of products contaminated with pathogenic bacteria. Thus, there are both strong public health and economic incentives to develop novel approaches for managing contamination of a broad range of foods by specific foodborne bacterial pathogens.

Contamination prevention and the provision of safe food supply is one of the high priority areas to control and limit the foodborne pathogen outbreaks under the “One Health” approach (USDA, 2016). However, the challenge is not a

straightforward one to solve rapidly or easily. The epidemiology of foodborne pathogens is complex and involves multiple routes of transmission from food animals and agricultural produce to consumers. For example, animals are often asymptomatic carriers of Shiga-toxin producing *Escherichia coli* (STEC), *Salmonella*, *Campylobacter*, and *Listeria* and they can spread the pathogens to other food animals, crops, slaughter facilities, and, in some instances, directly to humans. Food processing facilities can also harbour foodborne pathogens as biofilms, which can potentially transfer to food products and reach consumers. According to estimates published by the Foodborne Disease Burden Epidemiology Reference Group (FERG) of the World Health Organization (WHO), pathogenic bacteria were responsible for almost 350 million illnesses and 187,000 deaths globally in 2010 (Havelaar et al., 2015). Four bacterial pathogens, *Escherichia coli* (including Enterotoxigenic, Enteropathogenic, and Shiga-toxin producing *E. coli*), *Campylobacter* spp., non-typhoidal *Salmonella enterica*, and *Shigella* spp. were responsible for 336 million (96%) of the illnesses. While these four pathogens cause diarrheal diseases that are generally self-limiting, there are still over 159,000 deaths, attributed to them yearly. Children under five years old are disproportionally impacted, due to under-developed immune systems, and a staggering 45% of those deaths were in children under 5 years of age (Havelaar et al., 2015).

Food processors routinely employ antimicrobial interventions to reduce contamination of the foods with foodborne pathogens. Also, in an effort to improve the safety of foods, FSIS regulations and the Food Safety and Modernization Act have mandated implementation of hazard analysis and critical control point systems (HACCP) to manage pathogens by all US establishments processing red meats, fresh fruit and vegetables, dairy, and other food products (Food and Drug Administration, 2015; Food Safety and Inspection Service, 2012; U. S. Department of Agriculture, 2012). Currently, the conventional pathogen decontamination protocols in food processing facilities focus primarily on using chemicals, physical disruption techniques, and irradiation to reduce the microbial burden from those facilities and from the foods produced in them (Gomez-Lopez, 2012; Hui, 2003; Maukonen et al., 2003). For example, various harsh chemical sanitizers, such as chlorine and

peracetic acid (PAA), are commonly utilized to reduce microbial contaminants of many fresh fruits and vegetables as well as Ready-To-Eat (RTE) food products (Beuchat and Ryu, 1997; Sohaib et al., 2016). Heat pasteurization is often used to reduce bacterial numbers, generally in liquids and dairy items, such as milk. High Pressure Processing (HPP) is also used to successfully reduce pathogens in liquid products, as well as pre-cooked, meant to be frozen meals (Bajovic et al., 2012; Wolbang et al., 2008). This technique exposes foods to high pressure to inactivate microbes. Ionizing radiation (i.e., irradiation) has been approved as a means for reducing the burden of pathogenic organisms in foods since 1997 (Food and Drug Administration, 1997). However, no single approach is 100% effective, and the above-mentioned approaches also have some significant drawbacks. For instance, many chemical sanitizers corrode and damage food processing equipment (Fatica and Schneider, 2009; Moye et al., 2018) and could have toxic chemical residues that may harm the environment (i.e., they are not environmentally friendly). Pasteurization and HPP are not suitable for fresh produce and meat products as they can adversely affect the organoleptic properties and/or the nutritional content of some foods (Bajovic et al., 2012; Wolbang et al., 2008). Irradiation, which can deleteriously affect the appearance of some foods, also has low customer acceptance, which is compounded by a labelling requirement for many food items treated with radiation (Suklim et al., 2014; Wheeler et al., 1999). As a result, the recent trend has been towards identifying alternative non-chemical, environmentally friendly (aka green) antimicrobial approaches. One such approach is phage biocontrol.

The concept of phage biocontrol is to apply lytic bacteriophages, with strong lytic potency against one or more foodborne bacterial pathogens, onto the foods at high risk of contamination. The phages can lyse the targeted contaminating bacteria and significantly reduce (or eradicate) the foodborne pathogen(s), thus making the foods safe for consumption. Phage biocontrol is increasingly being accepted as a natural and green technology, effective at specifically targeting bacterial pathogens in various foods (Table 1) and the development and commercialization of bacteriophage products is now an emerging worldwide industry (Sulakvelidze, 2013) (Table 2). Phage biocontrol addresses many of the challenges facing the traditional chemical- or irradiation-based approaches

Table 1. A summary of studies of direct phage application onto a variety of foods†

Bacterium	Phages	Notes	Reference
Bacillus cereus	BCP1-1	*Bacillus cereus* counts were decreased after treatment with a single phage in fermented soya bean paste without affecting *Bacillus subtilis*, a critical component of the fermentation process.	(Bandara et al., 2012)
Campylobacter jejuni	Φ2	Counts of *Campylobacter* were reduced by ~1 log on the surface of chicken skin stored at 4°C after the application of a single phage.	(Atterbury et al., 2003)
Campylobacter jejuni; *Salmonella* spp.	*C. jejuni* typing phage 12673, P22, 29C; *Salmonella* typing phage 12	*C. jejuni* levels were decreased ~2 logs on experimentally-contaminated chicken skin after application of phage at an MOI of 100:1 or 1,000:1. *Salmonella* levels were reduced by ~2 logs on chicken skin treated with phage at an MOI of 100:1 or 1,000:1 and stored for 48 h, and bacterial counts were reduced below the limit of detection when lower levels of bacteria were used to contaminate the chicken.	(Goode et al., 2003)
Campylobacter jejuni; *Salmonella* spp.	Cj6; P7	*Campylobacter* levels were significantly decreased in beef after application of the phage Cj6 and decrease in bacterial levels were not significant at low levels of bacterial contamination (~100 CFU/cm^2). *Salmonella* counts were decreased ~2-3 logs at 5°C and >5.9 logs at 24°C in raw and cooked beef after P7 phage application. Surviving *Salmonella* colonies were still sensitive to P7. For both phages, the killing of bacteria was higher at an MOI of 10,000:1 and ~10,000 CFU/cm^2 of bacteria.	(Bigwood et al., 2008)
Cronobacter sakazakii	ESP 1-3, ESP 7321	In infant formula, *Cronobacter sakazakii* (formerly *Enterobacter sakazakii*) levels were decreased after phage addition. The reduction was dependent on the phage concentration, and the phages were more effective at 24°C than 37°C or 12°C.	(Kim et al., 2007)
Cronobacter sakazakii	Five phages	Growth of 36 of 40 test strains was inhibited by a phage cocktail tested in infant formula experimentally contaminated with *C. sakazakii*. Further, both high and low concentrations (10^6 and 10^2 CFU/mL) of bacteria were eliminated from the liquid culture medium treated with the individual phage (10^8 PFU/mL).	(Zuber et al., 2008)
Escherichia coli O157:H7	e11/2, pp01, e4/1c	After incubation at 37°C, a three-phage cocktail used to treat the surface of beef that was contaminated (10^3 CFU/g) with *E. coli* O157:H7 eliminated the bacterium from ~78% of the treated specimens.	(O'Flynn et al., 2004)
Escherichia coli O157:H7	EcoShield™ (formerly ECP-100)	*E. coli* 0157:H7 levels were decreased by ~1-3 logs or reduced below the limits of detection, on tomatoes, broccoli or spinach after treatment with a phage cocktail while *E. coli* O157:H7 levels were decreased by ~1 log when the phages were applied to ground beef.	(Abuladze et al., 2008)
Escherichia coli O157:H7	EcoShield™	A phage cocktail applied to experimentally contaminated lettuce and cut cantaloupe significantly reduced *E. coli* O157:H7 levels by up to 1.9 and 2.5 logs, respectively.	(Sharma et al., 2009)
Escherichia coli O157:H7	Cocktail BEC8	At various temperatures (4, 8, 23 and 37°C), the phage cocktail significantly reduced the level of *E. coli* O157:H7 on leafy green vegetables by ~2-4 logs. The inclusion of essential oil increased this effect.	(Viazis et al., 2011)

Escherichia coli O157:H7	EcoShield™	The levels of *E. coli* O157:H7 were reduced by ≥ 94% and ~87% on the surface of experimentally contaminated beef and lettuce respectively after addition of the phage cocktail; however, the single treatment did not protect foods after recontamination with the same bacteria (i.e., phage biocontrol had no continued technical effect on the foods).	(Carter et al., 2012)
Escherichia coli O157:H7	EcoShield™	After a 30 mins phage treatment at both 4 and 10°C, levels of *E. coli* O157:H7 were decreased by >2 logs on leafy greens under both ambient and modified atmosphere packaging storage.	(Boyacioglu et al., 2013)
Escherichia coli	FAHEc1	Contamination of raw and cooked beef was decreased by 2-4 logs at 5, 24 and 37°C in a concentration dependent manner after phage application. The *E. coli* displayed regrowth at higher temperatures.	(Hudson et al., 2013b)
Escherichia coli O157:H7	EcoShield™	A phage cocktail was applied to lettuce by spraying and dipping. A larger initial reduction (~0.8-1.3 logs) in *E. coli* O157:H7 counts was observed after spraying. Dipping required submerging the lettuce for as long as 2 mins, and the initial reductions were not significant. After 1 day of storage at 4°C, dipping in the highest concentration of the phage cocktail reduced *E. coli* by ~0.7 log.	(Ferguson et al., 2013)
Escherichia coli	EC6, EC9, EC11	Two *E. coli* strains were eradicated from raw and ultra-high temperature processed (UHT) milk after treatment with a three-phage cocktail at 5-9°C and 25°C. For a third *E. coli* strain, phage treatment eliminated the bacteria from UHT milk; however, after an initial reduction, regrowth occurred in the raw milk after 144 or 9 h at 5-9°C and 25°C storage, respectively.	(McLean et al., 2013)
Escherichia coli O157:H7	EcoShield PX™	Application, via spraying, of the phage cocktail reduced the *E. coli* O157:H7 levels by as much as 97% on various food products. In addition, the phage cocktail reduced the occurrence of *E. coli* O157:H7 on chuck roast beef by ≥80 %.	(Vikram et al., 2020)
Escherichia coli, *Salmonella*, *Shigella*	EcoShield™, SalmoFresh™, ShigActive™	Phage cocktails were as effective or more effective than chlorine wash at reducing targeted pathogenic bacteria from broccoli, cantaloupe and strawberries in samples containing a large amount of organic content. Combination of the phage cocktail and a produce wash produced a synergistic effect, i.e., higher reductions of bacteria.	(Magnone et al., 2013)
Listeria monocytogenes	ListShield™ (formerly LMP-102)	*Listeria* counts were decreased ~2 logs and ~0.4 logs after application of a phage cocktail on melon and apple slices respectively; a synergistic effect was observed when phage and nisin were used, decreasing levels of *Listeria* on the fruit ~5.7 logs and ~2.3 logs, respectively.	(Leverentz et al., 2003)
Listeria monocytogenes	ListShield™	Application of a phage cocktail 1, 0.5, or 0 h before honeydew melon tissue were contaminated with the bacterium was most effective at reducing *Listeria* counts. This effect was depended on the concentration of phage applied. *Listeria* counts decreased ~5-7 logs after 7 days when the phages were applied at the times described above.	(Leverentz et al., 2004)

Listeria monocytogenes	PhageGuard Listex™ (formerly Listex™; P100)	Levels of *L. monocytogenes* were reduced at least 3.5 logs after a single phage was administered to the surface of ripened red-smear soft cheese. The surviving *L. monocytogenes* colonies isolated from the cheese after phage treatment were not resistant to the phage.	(Carlton et al., 2005)
Listeria monocytogenes	A511, PhageGuard Listex™	Levels of *L. monocytogenes* in experimentally contaminated chocolate milk and mozzarella cheese brine were eradicated after phage treatment at 6°C. When the phage cocktail was applied to various solid foods, including sliced cabbage, iceberg lettuce leaves, smoked salmon, mixed seafood, hot dogs, and sliced turkey meat, a reduction of *Listeria* of up to 5 logs was observed.	(Guenther et al., 2009)
Listeria monocytogenes	PhageGuard Listex™	*L. monocytogenes* counts were decreased between 1.8-3.5 logs after application of a single phage at ~10^8 PFU/g to the surface of raw salmon fillets that were stored at 4°C or 22°C.	(Soni and Nannapaneni, 2010)
Listeria monocytogenes	PhageGuard Listex™	Levels of *L. monocytogenes* were decreased 1.4-2.0 logs CFU/g at 4°C, 1.7-2.1 logs CFU/g at 10°C, and 1.6-2.3 logs CFU/g at room temperature (22°C) after application a single phage to the surface of raw catfish fillets. Regrowth was not observed after ten days of storage at either 4°C or 10°C.	(Soni et al., 2010)
Listeria monocytogenes	A511	The natural microbial community on soft cheese was maintained after the addition of the phage. Levels of *Listeria* on experimentally contaminated cheese were decreased 2 logs and additional phage administrations did not improve the reduction of *Listeria*.	(Guenther and Loessner, 2011)
Listeria monocytogenes	FWLLm1	*Listeria* levels were decreased 1-2 logs on the surface of experimentally contaminated chicken stored in vacuum packages at 4°C or 30°C. Subsequent regrowth of *Listeria* was observed at 30°C but not at 4°C.	(Bigot et al., 2011)
Listeria monocytogenes	PhageGuard Listex™	Counts of *Listeria* decreased ~3 logs in experimentally contaminated queso fresco cheese after the addition of a single phage; however, subsequent growth was observed. Regrowth was prevented, and a similar log reduction was observed when PL + SD were included with the phage. Reduction of *Listeria* was lower, and regrowth occurred when LAE was included with phage.	(Soni et al., 2012)
Listeria monocytogenes	PhageGuard Listex™	Compared to PL or PL + SD, a single phage was most effective at decreasing *Listeria* levels on RTE roast beef and turkey after storage at 4°C or 10°C, and subsequent bacterial growth was observed at both temperatures. Similar log reductions occurred when PL or PL + SD were used in conjunction with the phage, and regrowth was prevented or diminished at both 4°C and 10°C.	(Chibeu et al., 2013)
Listeria monocytogenes	PhageGuard Listex™	Counts of *Listeria* were decreased by ~1.5 logs on experimentally contaminated melon and pear slices but not apple slices after two days at 10°C. Additionally, treatment with phage did not impact *Listeria* levels in apple juice but decreased bacterial contamination by ~4 and ~2.5 logs in melon and pear juice respectively.	(Oliveira et al., 2014)

Listeria monocytogenes	PhageGuard Listex™	*Listeria* levels on soft cheese were decreased ~2-3 logs after 30 mins and ~0.8-1 log after storage for 7 days at 10°C.	(Silva et al., 2014)
Listeria monocytogenes	ListShield™	Counts of *L. monocytogenes* were decreased 0.7 and 1.1 logs on experimentally contaminated cheese and lettuce respectively after a 5 min treatment with phage and decreased the bacteria 1.1 logs on the surface of apple slices after 24 h when combined with an anti-browning solution. The phage cocktail also eliminated *L. monocytogenes* from experimentally contaminated frozen entrees that were frozen and thawed after treatment. It was also effective in eliminating environmental contamination by *L. monocytogenes* in a smoked salmon processing plant.	(Perera et al., 2015)
Listeria monocytogenes	PhageGuard Listex™	When applied to the surface of experimentally contaminated sliced pork ham, the phage reduced *Listeria* counts below the limit of detection after 72 h and performed better than nisin, sodium lactate or combinations of these antibacterial measures.	(Figueiredo and Almeida, 2017)
Mycobacterium smegmatis	Six phages	*M. smegmatis* counts were reduced below the limit of detection in milk treated with a six-phage cocktail or each component phage. Subsequent bacterial growth occurred when the component phages were used, but no growth was observed after 96 h at 37°C when the cocktail was applied.	(Endersen et al., 2013)
Salmonella spp.	SJ2	*Salmonella* levels were reduced by 1-2 logs in raw and pasteurized cheeses created using milk that was treated with phage, while cheese made from milk without phage saw *Salmonella* counts rise ~ 1 log.	(Modi et al., 2001)
Salmonella spp.	SCPLX-1 (an early version of SalmoFresh™)	Counts of *Salmonella* were decreased by ~3.5 logs at 5 and 10°C and ~2.5 logs at 20°C on melon slices after application of a four-phage cocktail; treatment of apple slices with phage showed no reduction of bacteria.	(Leverentz et al., 2001)
Salmonella spp.	Felix-O1	*Salmonella* counts were decreased by 1.8-2.1 logs after phage application to chicken frankfurters.	(Whichard et al., 2003)
Salmonella spp.	PHL4	The levels of *Salmonella* recovered from experimentally contaminated broiler and naturally contaminated turkey carcasses were reduced by as high as 100% or 60% respectively after phage administration.	(Higgins et al., 2005)
Salmonella spp.		Levels of *Salmonella* were decreased ~3 logs after application of a phage cocktail to sprouts; addition of an antagonistic bacteria to the phage cocktail increased this reduction to ~6 logs.	(Ye et al., 2010)
Salmonella spp.	FO1-E2	In chocolate milk and mixed seafood, *Salmonella* levels were reduced to undetectable after phage treatment and storage for 24 h at 8°C and remained below the limit of detection. When foods were treated with phage and stored at 15°C, *Salmonella* counts were reduced to undetectable levels within 24-48 h for hot dogs, sliced turkey breast, and chocolate milk, but regrowth occurred after 5 days. *Salmonella* levels were initially inhibited ~0.5-2 logs and ~1-3 logs in egg yolk and mixed seafood respectively after phage addition; but bacterial recovery matched controls in egg yolks after two days, while the log reduction was maintained in seafood.	(Guenther et al., 2012)

Salmonella spp.	UAB_Phi 20, UAB_Phi78, UAB_Phi87	*Salmonella* counts were decreased by ~1 log on the shells of fresh eggs and 2-4 logs on lettuce 60 mins after application of the phage. After an initial reduction of 1-2 logs when chicken breasts were dipped in a phage cocktail, no further decrease in the bacterial counts was observed over the next seven days at 4°C. The levels of *Salmonella* were reduced 2-4 logs on pig skin after phage application and storage for 6 h at 33°C.	(Spricigo et al., 2013)
Salmonella spp.	wksl3	*Salmonella* counts were decreased by ~3 logs on chicken skin after application of a single phage, and no further decrease in bacterial levels was observed over the next seven days at 8°C. Phage cocktail was also administered to mice to test for safety. Mice received a single dose of phage orally and displayed no adverse effects.	(Kang et al., 2013)
Salmonella spp.	Five phages	The levels of *Salmonella* were decreased by ~1 log on chicken skin after application of a five-phage cocktail comprised of closely related phages. The reduction was comparable to treatment with 200 ppm dichloroisocyanurate, 10 ppm peroxyacetic acid, and 2 % lactic acid.	(Hungaro et al., 2013)
Salmonella spp.	P22	After the administration of a single temperate phage and storage at 4°C, levels of *Salmonella* decreased by 0.5-2 logs on chicken samples; below the limits of detection in whole and skimmed milk; by ~3 logs in apple juice; by ~2 logs in liquid egg; and by ~2 logs in an energy drink.	(Zinno et al., 2014)
Salmonella Enteritidis	Five phages	The levels of *S.* Enteritidis were reduced by as much as 3.2 logs and 2.8 logs after 10 days storage at 18°C and 4°C, respectively in raw salmon fillets. The phage treatment of smoked salmon fillets resulted in 1.9 logs and 1.2 logs reduction in Salmonella after 10 days of storage at 18°C and 4°C, respectively.	(Galarce et al., 2014)
Salmonella spp.	SalmoFresh™	The stability of a *Salmonella*-specific phage preparation was determined in various chemical antimicrobials. Treatment of chicken breast fillets with a combination of phage and chemical antimicrobials did not produce a synergistic effect on the reduction of *Salmonella*; however, application of chlorine or PAA followed by spraying with phage significantly reduced *Salmonella* from chicken skin by up to 2.5 logs, compared to use of chlorine, low levels of PAA, or phage alone (0.5-1.5 logs).	(Sukumaran et al., 2015)
Salmonella spp.	SalmoFresh™	Treatment of chicken breast fillets by dipping or surface application of a *Salmonella*-specific bacteriophage preparation and storage at 4°C significantly reduced *Salmonella* contamination by up to 0.9 logs; further, storing the meat in modified atmospheric packaging after surface application produced a higher reduction in bacterial counts (up to 1.2 logs).	(Sukumaran et al., 2016)
Salmonella spp.	SalmoLyse®	A phage cocktail was sprayed onto experimentally contaminated raw pet food ingredients, including chicken, tuna, turkey, cantaloupe, and lettuce, and reduced the levels of the targeted bacteria by 0.4-1.1 logs.	(Soffer et al., 2016)

Salmonella spp.	SJ2	Application of the phage SJ2 significantly reduced *Salmonella* in experimentally contaminated ground pork and eggs with a larger reduction observed at room temperature compared to 4°C. After treatment, *Salmonella* colonies were screened for phage resistance, and more phage-resistant *Salmonella* isolates were recovered from eggs compared with ground pork.	(Hong et al., 2016)
Salmonella spp.	PhageGuard S™ (formerly Salmonelex™)	Boneless chicken thighs and legs were experimentally contaminated with *Salmonella* and treated with phage solution. A larger reduction of *Salmonella* was achieved when the bacteriophage preparation was diluted in tap water compared to filtered water prior to application, and the phage cocktail was more effective against *Salmonella* isolated from other sources compared to those from ground chicken.	(Grant et al., 2017)
Salmonella spp.	PhageGuard S™	Treatment with a bacteriophage cocktail or irradiation significantly reduced (~1 log) the level of *Salmonella* on experimentally contaminated ground beef trim, and a combination of these methods was even more effective and decreased bacterial contamination by ~2 logs.	(Yeh et al., 2018)
Salmonella spp.	PhageGuard S™	Bacteriophage application reduced the *Salmonella* levels >1 log on skinless and skin-on poultry products.	(Hagens et al., 2018)
Salmonella spp.	BSPM4, BSP101, BSP22A	The phage cocktail treatment achieved a reduction of 4.7-5.8 logs of *Salmonella* on lettuce and cucumber.	(Bai et al., 2019)
Salmonella spp.	LPSTLL, LPST94, LPST153	Application of phage cocktail reduced 3.0 log *Salmonella* inoculum to below detectable limits on chicken breast and in milk. Phage cocktail was effective against *Salmonella* biofilm grown for 72 h on microtiter plates and steel chips, resulting in >5.23 log reduction in *Salmonella* viable cells.	(Islam et al., 2019)
Salmonella spp.	SalmoFresh™	Phage biocontrol reduced *Salmonella* by 2-3 log on lettuce and sprouts. The pairing of the phage cocktail with chlorinated water resulted in 2.7-3.8 log reduction in viable *Salmonella* counts.	(Zhang et al., 2019)
Shigella spp.	SD-11, SF-A2, SS-92	*Shigella* counts were reduced by ~1-4 logs in spiced chicken after application of a phage cocktail or each of the component phages and storage at 4°C.	(Zhang et al., 2013)
Shigella spp.	ShigaShield™	Application of a five phage *Shigella*-specific cocktail to various RTE foods (including lettuce, melon, smoked salmon, corned beef, and pre-cooked chicken) reduced the levels of *Shigella* ~1.0-1.4 logs compared to control.	(Soffer et al., 2017)
Staphylococcus aureus	Φ88, Φ35	*S. aureus* levels were decreased below the limit of detection in experimentally contaminated whole milk after treatment with a two-phage cocktail and storage at 37°C. After phage treatment, *S. aureus* was not recovered from the acid curd after storage for 4 h at 25°C and was eliminated from the renneted curd after 1 h at 30°C.	(Garcia et al., 2007)
Staphylococcus aureus	vB_SauS-phi-IPLA35, vB_SauS-phi-SauS-IPLA88	Counts of *S. aureus* were significantly decreased in cheese made using milk treated with phage compared to milk made without the addition of phage. The microbiota of the cheese was not impacted by the addition of the phage.	(Bueno et al., 2012)

† Modified from (Moye et al., 2018)

Table 2. Phage products approved for food safety applications†

Company	Phage Product	Target Organism(s)	Regulatory	Certifications	References
FINK TEC GmbH	Secure Shield E1	*E. coli*	FDA, GRN 724; USDA, FSIS Directive 7120.1		
Intralytix, Inc.	Ecolicide® (Ecolicide PX™)	*E. coli* O157:H7	USDA, FSIS Directive 7120.1		
	EcoShield PX™	*E. coli* O157:H7 and other STECs	FDA, GRN 834; USDA FSIS Directive 7120.1; FCN No. 1018		(Vikram et al., 2020)
	EcoShield™	*E. coli* O157:H7	FDA, FCN 1018; Israel Ministry of Health; Health Canada	Kosher; Halal	(Abuladze et al., 2008; Boyacioglu et al., 2013; Carter et al., 2012; Ferguson et al., 2013; Magnone et al., 2013; Sharma et al., 2009)
	ListShield™	*L. monocytogenes*	FDA, 21 CFR 172.785; FDA, GRN 528; EPA Reg. No. 74234-1; National Food Service of Israel approved as a food processing aid for the treatment of ready-to-eat meat and poultry products (Ref: 70275202); Health Canada (iLONO)	Kosher; Halal; OMRI	(Leverentz et al., 2003; Leverentz et al., 2004; Perera et al., 2015)
	SalmoFresh™	*Salmonella* spp.	FDA, GRN 435; USDA, FSIS Directive 7120.1; Israel Ministry of Health; Health Canada	Kosher; Halal; OMRI	(Sukumaran et al., 2015, 2016)
	ShigaShield™ (ShigActive™)	*Shigella* spp.	FDA, GRN 672		(Mai et al., 2015; Soffer et al., 2017)
Micreos Food Safety	PhageGuard Listex™	*L. monocytogenes*	FDA, GRN 198/218; FSANZ; EFSA; Swiss BAG; Israel Ministry of Health; Health Canada	Kosher; Halal; OMRI; SKAL	(Carlton et al., 2005; Chibeu et al., 2013; Figueiredo and Almeida, 2017; Guenther et al., 2009; Oliveira et al., 2014; Silva et al., 2014; Soni et al., 2012; Soni and Nannapaneni, 2010; Soni et al., 2010)
	PhageGuard S™	*Salmonella* spp.	FDA, GRN 468; USDA, FSIS Directive 7120.1; FSANZ; Swiss BAG; Israel Ministry of Health; Health Canada	Kosher; Halal; OMRI; SKAL	(Grant et al., 2017; Yeh et al., 2018)
		E. coli O157:H7	FDA, GRN 757		
Passport Food Safety Solutions	Finalyse®	*E. coli* O157:H7	USDA, FSIS Directive 7120.1		

Phagelux	SalmoPro®	*Salmonella* spp.	FDA, GRN 603; USDA, FSIS Directive 7120.1		
		Salmonella spp.	FDA, GRN 752; USDA, FSIS Directive 7120.1		

† Modified from (Moye et al., 2018)

briefly described above, as well as many of the concerns voiced by consumers. For example, the traditional decontamination methods are broad-spectrum, killing not only the pathogen of concern but also the natural microflora of the foods, which are often beneficial. In contrast, phage biocontrol, due to the specificity of bacteriophages, enables targeted elimination of the foodborne bacteria in the foods, while maintaining the natural microbial population and preserving the nutritional composition/value of those foods (Moye et al., 2020).

As bacteriophages are the natural enemy of the pathogenic bacteria, phage biocontrol could be considered the most natural, environment-friendly antimicrobial intervention available today. The application rates for typical phage biocontrol interventions are low and are expected to have little if any, environmental impact. For instance, the US meat and poultry industry, the largest US agricultural sector, produced 52 billion pounds of meat and 48 billion pounds of poultry in 2017 (NAMI, 2020). If phages were applied at the maximum approved amount (10^9 PFU/g for one phage product, all other current approvals are for up to 10^7-10^8 PFU/g) to all 100 billion pounds of meat and poultry produced in one year, then the total phages applied would be ~4.5 x 10^{22} PFU, which is just 0.000005% of the minimum estimate of 1 x 10^{30} PFU present in the world. This calculation is a gross overestimate, as it assumes the maximum approved amount of phage is applied, especially considering that most GRAS approvals only permit an application of up to 10^8 PFU/g food (reducing the phage estimate by a factor of 10). Moreover, this estimate assumes bacteriophage biocontrol is universally used by all relevant food industries in the United States. If both assumptions were true, theoretical sales for the phage industry would exceed $650 billion per year. Additionally, our estimate envisions

a worst-case scenario, wherein all the phages applied to the foods eventually end up in the environment. Again improbable, as they will likely be inactivated prior to reaching the environment, either through cooking, consumption (and subsequent inactivation in the GI tract) or due to other environmental factors. In short, the number of phages added to the environment as a result of phage biocontrol is less than negligible, especially when compared to naturally present phage populations.

There are several other characteristics of commercial phage biocontrol preparations that make them consumer friendly. For example, many of them do not contain any additives or preservatives, and several are certified Kosher, Halal, and “Organic” – that is to say, suitable for use in organic foods (e.g., OMRI-listed in the USA; SKAL in EU) (Table 2). Phage biocontrol may also provide some cost benefits. For example, the costs for some of the currently implemented non-phage interventions (e.g., irradiation and HPP) range from 10-30 cents per pound of treated food (Viator et al., 2017). In contrast, phage biocontrol costs range between 1-4 cents per pound, similar to the cost range of harsher chemical sanitizers. Therefore, phage biocontrol provides a ‘green’ and safe alternate method to control foodborne pathogens, which may demonstrate superior biocontrol compared to other interventions while preserving the environment and encouraging sustainability.

While the biological properties of lytic bacteriophages provide advantages for improving food safety, these properties also lead to some of the limitations and drawbacks to phage biocontrol. As mentioned previously, bacteriophages are highly specific and as such, they are only effective against the pathogen of interest. Still, if foods are contaminated with multiple pathogens, a combination of phage biocontrol products could be used to target more than one pathogen. Since bacteriophages themselves are also microorganisms, commonly used disinfectants or chemicals could inactivate them, so their use needs to be carefully coordinated within the processing line. In addition, the currently marketed phage preparations require refrigerated storage (typically 28°C). Thus, a good understanding of the biological properties of bacteriophages and designing optimal application regimens that consider those properties is

essential for the best possible efficacy of phage biocontrol intervention. The main *Pros* and *Cons* of phage biocontrol are summarized in Table 3 and are also discussed in more detail later in this chapter.

Table 3. Pros and Cons of phage biocontrol

Pros	***Cons***
Phages are a natural product	Not all phages make a good biocontrol agent, e.g. temperate phages. These *pros* refer to wild-type lytic phages.
Specific, only targets problem foodborne bacterial pathogens	May not ensure full safety of foods if the foods are contaminated by a different foodborne pathogen (e.g. one not targeted by the phage biocontrol)
Effective in killing targeted bacteria	Narrow host range limits their usage in theory; this shortcoming can be overcome by using a “cocktail” or a combination of phages
Single dose application	Residual activity has not been observed on foods despite the ability of lytic phages to infect new hosts; possibly due to physical inaccessibility on food surfaces
Narrow potential for resistance and lack of cross resistance with antibiotics	Phage resistant bacterial strains can emerge, but using a cocktail is shown to reduce the phage resistance
Rapid discovery and relative ease for formulation and application	
Low inherent toxicity and no adverse environmental impact	
Does not impact organoleptic, nutritional, and rheological properties of the food	

For food safety applications, lytic bacteriophages can be used for both pre- and post-harvest interventions. When applied to live animals, phages can be administered via animal feed or spray-applied to hides or feathers prior to slaughter. For post-harvest applications, the phage preparations are generally applied directly to food surfaces, either via direct spraying, through the packaging materials, or by some other means (Lone et al., 2016; Sulakvelidze,

2013). Phage biocontrol could also be utilized as a disinfectant for surfaces within the food processing facility (Abuladze et al., 2008; Woolston et al., 2013). Several earlier reviews of those approaches are available (Endersen et al., 2014; Greer, 2005; Sulakvelidze, 2013; Woolston and Sulakvelidze, 2015). Other phage-related methods such as the use of phage endolysins or using bacteriophages to manage food spoilage have also been discussed in various earlier reviews (Greer, 2005; Schmelcher and Loessner, 2016). This chapter provides an updated overview of studies describing phage biocontrol predominantly in post-harvest applications, the segment that currently appears to be gaining the most momentum (Table 1).

Phage Biocontrol of Foodborne Pathogenic Bacteria

Listeria monocytogenes

Listeriosis, an infection caused by *Listeria monocytogenes*, results in a range of clinical symptoms including mild febrile gastroenteritis to more severe sepsis, meningitis, rhombencephalitis, perinatal infections, and abortions (Allerberger and Wagner, 2010). *L. monocytogenes* is generally transmitted when food is handled in environments contaminated with the pathogen and is usually contracted by consuming improperly processed or cooked meat and milk. *L. monocytogenes* can proliferate at the lower temperatures generally used to refrigerate (2-8°C) foods in households and transportation, making it a serious food safety threat. Therefore, the detection and control of *L. monocytogenes* is critically important for ensuring the safety of the food chain, especially in RTE foods. *L. monocytogenes* infections appear to have a very high mortality rate. For instance, Havelaar et. al. estimated that of the 14,000 *L. monocytogenes* global infections, recorded in 2010, approximately 22% of infections resulted in death (Havelaar et al., 2015). According to the CDC, *L. monocytogenes* infections result in approximately 1,600 illnesses and 320 mortalities each year in the US alone.

L. monocytogenes was the first foodborne pathogen for which a commercial phage biocontrol preparation was developed. That preparation, called ListShield™ (originally LMP-102) (Figure 1), was developed and is currently marketed by Intralytix, Inc. (Columbia, MD, USA) as either a Food Additive or

GRAS (Generally Recognized as Safe) food processing aid (Moye et al., 2018). Several studies have reported that the ListShield™ is highly efficacious in significantly reducing or eliminating *L. monocytogenes* contamination in a variety of foods, including RTE foods (Table 1) such as fruits and vegetables, cheese, and smoked fish (Perera et al., 2015). In addition, ListShield™ has been shown to reduce *L. monocytogenes* by approximately 2.2 logs in prepackaged frozen foods, such as those usually served in-flight (Perera et al., 2015). Similar efficacy has been reported for Listex™, another commercial monophage preparation. Listex™ reduced *L. monocytogenes* on pork ham by ca. 2.8 logs to undetectable levels; in comparison, nisin (a polycyclic antibacterial peptide) and sodium lactate resulted in 2 logs and <0.5 log reduction in *L. monocytogenes* levels, respectively (Figueiredo and Almeida, 2017). Only the phage mediated reductions were sustained during storage of the ham for 72 h at 6-8°C, suggesting phage biocontrol was more effective than antimicrobial treatments (Figueiredo and Almeida, 2017). The same monophage preparation was also shown to reduce *L. monocytogenes* on the surface of other deli meats (cooked sliced turkey and roast beef) stored at 4°C and 10°C (Chibeu et al., 2013). Additionally, Listex™ demonstrated synergistic activity against *L. monocytogenes*, with other antimicrobials such as sodium diacetate or potassium lactate (Chibeu et al., 2013).

Figure 1. *ListShield*™, the first phage-based product ever to be cleared by the FDA for food safety applications

Salmonella spp.

Non-typhoidal *Salmonella enterica* was estimated to account for 95.1 million cases of enterocolitis and 50,771 fatalities in 2017, worldwide (Stanaway et al., 2019). *Salmonella* infections are often self-limiting, with symptoms typically including stomach cramps, fever, nausea, and diarrhoea, but life-threatening instances can occur in cases of dehydration and when the bacteria invade the internal organs. According to the CDC estimates, non-typhoidal *Salmonella* causes about 1.35 million illnesses, 26,500 hospitalizations, and 420 deaths yearly in the US (Centers for Disease Control and Prevention, 2019c). While *Salmonellae* are typically associated with poultry products, the past several years have seen outbreaks caused by a variety of foods, including fruits and vegetables, nuts, and fish. In 2019, the CDC reported three fruit-related outbreaks, which sickened over 300 people in at least 11 states (Centers for Disease Control and Prevention, 2019b, 2020).

Phage biocontrol of *Salmonella* has been evaluated by several investigators (Table 1). In the US, two phage preparations are currently available commercially (Table 2). In general, both commercial and non-commercial phage-preparations have been shown to be an efficacious approach to control *Salmonella* contamination of various foods. For example, a recent study demonstrated an average of ca. 5 log reduction on lettuce and 0.8 log reduction on sprouts in *Salmonella* levels following application of the commercial six-phage cocktail SalmoFresh™ (Zhang et al., 2019). Another study showed ca. 2 log reduction in *Salmonella* population by the same phage cocktail on whole cucumbers (Sharma et al., 2017). The same phage cocktail was also reported to be effective in reducing *Salmonella* concentration on poultry products, with reductions up to 1.2 logs and 1.3 logs recorded in chicken breast and turkey breast, respectively (Sharma et al., 2015; Sukumaran et al., 2016). Finally, reports from our laboratory have shown that SalmoFresh™ can also be effective in reducing *Salmonella* contamination of dry pet food as well as raw pet food-ingredients (Soffer et al., 2016) and glass and stainless steel surfaces (Woolston et al., 2013). Raw pet foods present a high risk to not only the pets but also to the unsuspecting owners (Soffer et al., 2016).

Other *Salmonella*-specific phages with good efficacy were also reported for controlling *Salmonella* in various food applications. For example, Salmonelex™, a cocktail of the two phages Fo1a and S16, reduced a mixture of three *Salmonella* serovars by 0.4 logs and 0.7 logs after 30 min and 8 h treatment in ground chicken (Grant et al., 2017). The three serovars, *S.* Typhimurium, *S*. Newport, and *S*. Thompson, used in this study were isolated from retail ground chicken products and represented the real-life contamination of chicken products. Additionally, the same study also demonstrated that Salmonelex™ was effective against a combined challenge of *Salmonella* serovars Heidelberg, Enteritidis, and Typhimurium obtained from ATCC (Grant et al., 2017). A monophage preparation, consisting of SJ2, was able to significantly reduce *Salmonella* in both liquid egg and ground pork during storage at 4°C and 21°C (Hong et al., 2016).

Shiga toxin-producing Escherichia coli

Shiga toxin-producing *Escherichia coli* (STEC, also known as enterohemorrhagic *E. coli* or EHEC) are important foodborne pathogens that cause more than 2.5 million illnesses globally each year, resulting in 3,330 haemolytic uremic syndrome (HUS) cases and 269 deaths (Kirk et al., 2015). According to the CDC's National STEC Surveillance Program, an estimated 5,441 culture confirmed STEC infections were recorded in the US in 2016, which was a 26% increase over the previous year (Centers for Disease Control and Preventions, 2018). The clinical manifestations of STEC infections range from mild diarrhoea to hemorrhagic colitis and potentially fatal HUS (Besser et al., 1999). Beef and fresh produce are the two most common sources of STEC infections, implicated in about 75% of all STEC-related outbreaks in the US (Centers for Disease Control and Preventions, 2019; Interagency Food Safety Analytics Collaboration, 2018). Lately, poultry products have also been increasingly linked to STEC outbreaks (Chen et al., 2018; Mathusa et al., 2010). Additionally, STEC, in particular, *E. coli* O157:H7 has been identified in amphibian, fish, and invertebrate carriers (Ferens and Hovde, 2011; Sanath Kumar et al., 2001).

Phage biocontrol of STEC, particularly *E. coli* O157:H7, using commercial and noncommercial phage preparations has been reported by several investigators. As beef products are inherently at high risk for EHEC contamination, initial studies primarily focused on controlling EHEC contamination in beef products (Abuladze et al., 2008; Hudson et al., 2013a). For example, a single phage FAHEc1, isolated from raw sewage, was shown to reduce the STEC counts on beef slices by approximately 2 logs under conditions simulating hot boning and conventional carcass cooling (Hudson et al., 2013a). In another study, a commercial three phage preparation was shown to reduce *E. coli* O157:H7 *levels* by 1.2 logs in ground beef (Abuladze et al., 2008), and a different three phage preparation was reported to reduce the *occurrence* of *E. coli* O157:H7 on beef by approximately 77% (O'Flynn et al., 2004).

In addition to phage biocontrol of STEC on beef, several studies have also demonstrated the effectiveness of phage biocontrol in reducing *E. coli* O157:H7 contamination of other foods, such as fresh produce. A single bacteriophage, OSY-SP, that was isolated from sewage and livestock manure, was shown to reduce *E. coli* O157:H7 by 1-4 logs on cut green peppers and baby spinach (Snyder et al., 2016). Storage at 4°C for 72 h showed a sustained activity while at 25°C some bacterial regrowth was observed after 72 h (Snyder et al., 2016). Another study demonstrated the effectiveness of a phage cocktail on reducing *E. coli* O157:H7 on fresh-cut cantaloupes and lettuce (Snyder et al., 2016). The phage treatment resulted in ca. 2 logs reduction on lettuce and 2-3 log reduction in *E. coli* O157:H7 levels on cantaloupe, clearly demonstrating the effectiveness of phage biocontrol on fresh produce (Snyder et al., 2016).

Initially, most phage preparations, and therefore studies, were primarily focused on targeting *E. coli* O157:H7. However, with the increased frequency of non-O157 STEC associated disease, more recent research has focused on phage biocontrol that targets STEC in general. For example, the phage cocktail EcoShield PX™ was granted GRAS affirmation from the FDA (GRN 834) in the winter of 2019 and is effective against a broader range of STEC pathogens (Vikram et al., 2020). In a study using non-O157 strains, application of a three phage cocktail completely inhibited the *E. coli* strains ATCC 25922 and O127:H6

in ultra-high temperature treated (UHT) milk and raw milk at 4°C and 25°C (McLean et al., 2013). Furthermore, no regrowth of the two strains was observed following the phage treatment and storage at either 4°C or 25°C. In separate experiments, the authors demonstrated that, initially, a two-phage cocktail completely inhibited *E. coli* O5:H, an enterohemorrhagic strain, in UHT milk at 4°C and 25°C, but, in this case, regrowth of *E. coli* O5:H- was observed during storage at both temperatures. While multiple factors may contribute to the observed regrowth, two plausible causes could be strain-specific differences and use of three vs two phage cocktail. It is likely that a three phage cocktail may provide better activity and suppress the emergence of resistant mutants, as discussed later (Örmälä and Jalasvuori, 2013; Woolston et al., 2013; Yuan et al., 2019).

Shigella spp.

Shigella species are major foodborne and waterborne pathogens (Tack et al., 2019). Shigellosis, the infection caused by the bacteria, usually results in diarrheal disease, with symptoms ranging from mild stomach cramps to vomiting and bloody diarrhoea. While the infection is self-limiting and generally clears within 5-7 days, people with compromised immune systems may suffer from more severe and debilitating illnesses. In addition, developing countries have a very high incidence of shigellosis and children under the age of 5 are disproportionately affected (Havelaar et al., 2015; Kotloff et al., 1999). In the US, approximately 13,000 culture confirmed cases of shigellosis were reported in 2016 (Enteric Disease Laboratory Branch, 2016), with one species *S. sonnei,* accounting for the largest (80.5%) percentage of infections (Enteric Disease Laboratory Branch, 2016). *Shigella* is not a frequent cause of foodborne outbreaks in the US, but two recent outbreaks have been linked to contaminated asparagus (2018) and raw oysters (2019) (Flynn, 2019; Food and Drug Administration, 2019). *Shigella* is also an issue for the US military, as travel to developing countries puts military members at an elevated risk for shigellosis (Magnone et al., 2013).

While several groups have evaluated phage biocontrol of *Shigella*, currently there is only one commercially available phage preparation, ShigaShield™. This

five-phage cocktail is GRAS-listed (GRN 672; Table 2) and has been shown to reduce the levels of *Shigella* by approximately 1 log in a variety of foods, including melons, lettuce, yoghurt, deli corned beef, smoked salmon, and chicken breast meat (Soffer et al., 2017). Notably, ShigaShield™ is also one of the few commercial phage biocontrol preparations that have been examined for its impact on the normal gut microflora in mice. In an article from the University of Florida, ShigaShield was reported to be well tolerated when administered to mice and, in contrast to an antibiotic (ampicillin), to have significantly less impact on the normal gut microflora (Mai et al., 2015). Other studies have also demonstrated the effectiveness of phage biocontrol in managing *Shigella* contamination of foods. For example, treatment with a phage, designated vB_SflS-ISF001, resulted in nearly a 2 log reduction of *S. flexneri* counts on raw and cooked chicken breast samples (Shahin and Bouzari, 2018). The same group examined vB_SflS-ISF001 and a second phage, vB_SsoSISF002, against a panel of *Shigella* that had been isolated directly from foods (Shahin et al., 2019). Combining the two phages showed increased efficacy in reducing the *Shigella* than when either phage was used alone. Phage biocontrol has also been shown to effectively control *Shigella* in contaminated water (Jun et al., 2016).

Campylobacter jejuni

Infections due to *Campylobacter* spp. are one of the most common causes of gastroenteritis worldwide, with an estimated 95 million foodborne illnesses yearly (Havelaar et al., 2015). In the US, *Campylobacter* has had the highest rate of yearly incidence since 2013 (Tack et al., 2019), with an estimated 1.5 million foodborne *Campylobacter* infections occurring every year (Centers for Disease Control and Prevention, 2019a) and an economic impact of about $1.9 billion (in 2014 dollars) (Economic Research Service, 2014; Hoffmann et al., 2012; Michael et al., 2014). *Campylobacter* is found in contaminated water and is frequently associated with animals, including farm animals such as poultry and cows.

Several *Campylobacter* bacteriophages have been isolated, generally from poultry sources (e.g., faecal matter, surfaces, and internal tissues such as livers

and ceca), and some of them have been examined for their ability to reduce contamination of various foods by *Campylobacter* (Firlieyanti et al., 2016; Hammerl et al., 2014; Kittler et al., 2013; Zampara et al., 2017). Interestingly, isolation of *Campylobacter*-specific phages has almost exclusively been performed utilizing just two *C. jejuni* isolates as a host strains for phage isolation. Most of those were with *C. jejuni* NCTC 12662 and the isolated phages generally specifically targeted the capsular polysaccharide, termed Group III phages (Sorensen et al., 2015). In contrast, a few studies used *C. jejuni* RM1221 as the target strain and these phages, termed Group II phages, typically utilize the flagella as a route of entry (Sorensen et al., 2015). Most reports of using phages for biocontrol are for pre-harvest interventions and studies examining the ability to reduce post-harvest contamination of various foods by *Campylobacter* are limited. In a study on chicken neck skin (Zampara et al., 2017), two Group III phages were able to reduce contamination by ca. 0.4 logs each when applied as single phages, but their efficacy increased to 0.7 log reduction of *Campylobacter* when used in combination with one another. The application of a Group II phage showed no effect on the *Campylobacter* levels. Another group of investigators reported 1-3 logs reduction in *Campylobacter* levels on artificially contaminated cooked and raw beef slices (Bigwood et al., 2008). The effects were more pronounced when higher levels of bacteria were present and when the phage was applied at a higher MOI. Reductions were maintained for up to 8 days at 5°C storage. Currently, there are no commercial phage biocontrol products available in the US but, given the importance of *Campylobacter* from the standpoint of food safety, it seems likely that such products would be made commercially available in the not too distant future.

Considerations for Phage Biocontrol

As interest in bacteriophage biocontrol for food safety purposes increases, several considerations need to be taken into account for the optimal implementation of this novel approach. Some of these aspects are briefly discussed below.

Regulation of Bacteriophage Preparations

Despite the more than 100-year history of using phages therapeutically in humans, their use for food safety applications is a relatively novel concept pioneered by a US-based company Intralytix, Inc., which was the first company in the world to obtain FDA-approval for bacteriophage product for the phage biocontrol applications (note: the FDA does not endorse or approve any interventions; however, the term “approval” is commonly used – including throughout this review - to indicate that the agency allows the product to be used commercially). That first approval was for the *L. monocytogenes* specific phage cocktail called ListShield™ (formerly LMP-102; Figure 1) and it came in the form of an amendment of the FDA’s “food additive regulations” in 2006 (Food and Drug Administration, 2006). The same product was also listed by FSIS as a suitable antimicrobial intervention for ready-to-eat meat and poultry products (FSIS Directive 7120.1). The success of this original petition paved the way for other companies in the US and abroad to invent and develop new phage products for food safety applications – and other approvals indeed soon followed. For example, later that same year, the FDA issued a no objection letter for the *Listeria*-specific preparation Listex™ (currently PhageGuard Listex™) as a Generally Recognized as Safe (GRAS) substance. As of the day of this writing (December 31, 2019), fourteen phage biocontrol products have been approved for food safety applications under various regulatory frameworks in the United States (Table 2). Of these fourteen products, eleven products were approved as GRAS by the Center for Food Safety and Applied Nutrition (CFSAN) of the FDA, and application for GRAS designation now is the most commonly used route for regulating phage biocontrol products for post-harvest food applications. The approval process includes engaging the USDA, if necessary, to determine if the phage preparation should be included in their guidelines for safe and suitable ingredients used in the production of meat, poultry, and egg products. The FSIS Directive 7120.1 includes both pre- and post-harvest applications, such as the application of phage to the hides of cattle and targeted phage application onto poultry or meat. While the FSIS guidelines are developed based upon specific phage preparation efficacy data, in general, any phage product that meets the description in the directive may be considered to be compliant. The use of phage preparations for food safety purposes has also received approval in

several other countries, such as Israel, Canada, Switzerland, Australia, and New Zealand (Table 2); these approvals are frequently issued based upon US regulatory approvals. One notable exception is the European Food Safety Authority (EFSA) which has been slow in adopting this new intervention and has been delaying the introduction of phage biocontrol in the European Union.

Efficacy

Phage biocontrol typically reduces the levels of targeted bacteria by 1-3 logs, which is lower than the up to 5 logs reduction claimed for some other, harsher interventions, e.g., irradiation. However, several of the studies reporting such a high log reduction have also used a very high inoculum dose for the challenge. For example, Nagel et al. (Nagel et al., 2013) used an 8 log inoculum of *Salmonella* and *Campylobacter* to achieve a 5.5 log reduction in poultry carcasses treated with various antimicrobials (including chlorine and peracetic acid) in a post-chill immersion tank; whereas the majority of phage biocontrol studies have used much lower challenge inoculum (e.g. Soffer et al. (2017) used a 3 log inoculum). Thus, higher reductions may be possible if a very high dose of bacterial inoculum is used to experimentally contaminate foods during phage efficacy studies; e.g., a reduction of up to 5 logs was reported as a result of phage treatment by several authors (Guenther et al., 2009; Leverentz et al., 2004)). Notably, when a lower inoculum dose is used in challenge studies, many chemical antimicrobials (e.g. PAA) also demonstrate lower reduction (Moore et al., 2017) – similar to reductions achieved by phage biocontrol. These potentially lower log reductions by phage biocontrol may also be more of a perception problem than a real technical issue. For instance, ListShield™ reduced the levels of *Listeria monocytogenes* by approximately 1 log in artificially contaminated smoked salmon fillets, but when the same cocktail was applied to naturally contaminated salmon fillets in a commercial processing facility, the *L. monocytogenes* was completely eliminated (no detectable *L. monocytogenes*) (Perera et al., 2015). Few, if any, foods are contaminated with 5 logs of foodborne bacteria; nevertheless, a 5-log reduction sounds much more effective than a 1-3 log reduction and the companies marketing phage preparations for the food industry may have to overcome this perception challenge. Thus, future phage biocontrol studies may need to (i) utilize comparably high initial bacterial

challenge levels to demonstrate that phage biocontrol can (or cannot) also result in 4-5 log reduction similar to chemical sanitizers, and/or (ii) provide compelling evidence that lower reductions (1-3 log) still provide a strong, real life-pertinent protection of foods (e.g., result in a significant reduction in the incidence/ occurrence of foodborne bacteria in foods contaminated with the levels of foodborne bacteria commonly found in real-life settings). In support of the second point, a 2003 risk-assessment study included a model predicting how reductions in *L. monocytogenes* contamination of pre-retail deli meat would affect the mortality rate associated with that pathogen. The predictions indicated that even when only reduced by 1 or 2 logs, the mortality rate of elderly people due to *L. monocytogenes* would decrease by ca. 50% - 74% (Food and Drug Administration, 2003), suggesting even small reductions in contamination may yield significant improvements in food safety and public health.

Additional Technical Challenges

Phage biocontrol often significantly reduces the levels of the targeted pathogen but does not always eliminate it completely from foods. This issue is not phage biocontrol-specific and many other antimicrobial treatments of food products exhibit similar shortcomings (Kalchayanand et al., 2016; Penney et al., 2007), but the possible explanations for the incomplete inactivation by phages differ from standard interventions. Three major causes are discussed below.

Physical Impedance

A number of factors related to application methods on food and certain inherent properties of bacteriophages may hinder the efficacy of phage biocontrol. One of the key factors restricting phage efficacy is the food matrix. Phages are required to physically adsorb onto the bacteria to exert their lytic activity. This interaction can be impeded by the food matrix. Unlike some bacteria, phages are immobile and depend on Brownian motion to reach their target (Kasman and Porter, 2019). In an event where phages are not evenly sprayed or mixed with the food to ensure direct contact of phages with their targeted bacterial cells, the efficacy of phage treatment may be significantly reduced. Efficacy may also be lower in drier foods where not enough liquid is available to enable Brownian motion. Even in the case of foods with adequate moisture, such as meats and cut fruits

and vegetables, the phages may get trapped in microscopic surface structures and not reach the target bacteria, rendering them ineffective. Thus, ensuring thorough coverage of the food surface area with phages is perhaps the most critical prerequisite for phage biocontrol efficacy. Therefore, carefully designed application of the phages for the particular food processing facility is necessary. Various approaches, such as adjusting the phage concentration and/or the spray volumes, using fine (mist-like) sprays, thoroughly mixing foods during phage application, and otherwise ensuring thorough phage application should enhance the effectiveness of phage biocontrol.

Resistance

Another important challenge is the potential emergence of phage-resistant bacterial isolates. The emergence of phage-resistant isolates has not yet been reported to be an efficacy-hindering problem during industrial phage biocontrol applications, but it remains a valid, and perhaps guaranteed, an eventuality which has been documented under laboratory conditions (Hong et al., 2016; Sillankorva et al., 2012). For example, in a study of *Salmonella* in raw eggs and ground pork, the frequency of phage resistant isolates was higher in the raw eggs treated with phage than in the untreated eggs; however, the ground pork did not show a similar trend (Hong et al., 2016). The food matrices may have played a role, as the liquid nature of the raw egg could have allowed more opportunities for the phage and *Salmonella* to interact, which was likely limited on the more solid ground pork. Additionally, this study used a single bacteriophage preparation, as opposed to a phage cocktail, which might also have contributed to the higher phage resistance rates observed. The use of phage cocktail is reported to reduce the frequency of emergence of phage resistant mutants (Örmälä and Jalasvuori, 2013; Woolston et al., 2013; Yuan et al., 2019), as phages can use a variety of bacterial surface structures to adsorb (Lindberg and Holme, 1969; Marti et al., 2013; Zhang et al., 2016). Thus, the smart design of phage preparations, ones that contain lytic bacteriophages capable of targeting multiple strains of the same species and/or distinct bacterial receptors, can strengthen the potency and effectiveness of phage biocontrol. To this end, a PhageSelector™ program has been recently reported to help design an optimally effective phage preparation with a broad target range against *E.*

coli (Cieplak et al., 2018). Additionally, applying phages as close to the end of food processing as possible could help reduce the emergence of phage-resistant mutants by limiting the exposure of the bacteria to the phages.

Even when efficacy-hindering resistance evolves, it should be possible to quickly update phage preparations by replacing old phages that are no longer effective against their targeted strains with new phages that have lytic potency against those strains. The technical feasibility of such a “product update” has been demonstrated for a *Salmonella* phage preparation (Woolston et al., 2013). The study examined the ability of a six-phage *Salmonella* phage cocktail to reduce the levels of *Salmonella* on glass and stainless steel surfaces which are commonly used as food contact surfaces in the food processing industry (Woolston et al., 2013). Initial studies demonstrated that the bacteriophage cocktail significantly reduced the population of *S.* Kentucky and *S.* Brandenburg by ca. 2-4 logs, on all surfaces examined. But the phage preparation was ineffective in reducing *S.* Paratyphi B S661 (which was resistant to this phage preparation) levels on the hard surfaces contaminated with that strain. Updating the phage preparation by replacing two of its original component phages by two new phages with lytic potency against the *S.* Paratyphi B S661 strain, instantly restored the efficacy of the preparation. This new phage preparation reduced the *S.* Paratyphi levels by ca. 2 logs on glass and stainless steel surfaces, while also maintaining efficacy against the Kentucky and Brandenburg serotypes (Woolston et al., 2013). The study provided compelling evidence that phage cocktails could be easily modified when needed, e.g., if and when efficacy-hindering phage-resistant mutants emerge against a given commercial phage preparation. Implementation of this approach in a real-life setting, however, is still not fully delineated, including regulatory approvals. Encouragingly, the FDA and USDA have started to allow such updates of some commercial phage preparations without the need to go through the entire regulatory approval process with every update; e.g., GRN 834 for an *E. coli*-targeted phage cocktail EcoShield PX™ (Table 2) allows the product manufacturer to modify the phage preparation to include three to eight lytic phages in order to achieve optimal efficacy, including in the event of bacterial resistance emerging against the original preparation.

Temporal resistance to phages on the food matrix may also contribute to incomplete elimination of the target bacteria (Hoskisson and Smith, 2007; Tokman et al., 2016). Several studies tested this conjecture by evaluating randomly selected isolates recovered following phage treatment, however, a clear answer is still elusive. The observance of transient phage resistance in studies using pure bacterial culture led to the speculation that temporal resistance may contribute to the survival of bacteria on the food matrix (Orquera et al., 2015), but the evidence from food application of commercial phages is lacking. For example, two studies found that the recovered *L. monocytogenes* isolates following phage treatment were susceptible to the phage preparations suggesting that temporal resistance was either a very short-termed and an unstable phenomenon or not a major contributor to incomplete eradication of the target bacteria on food (Carlton et al., 2005; Chibeu et al., 2013).

Implementation

Phage biocontrol is envisioned to be a part of a multi-hurdle approach for improving food safety. Therefore, it requires planning to achieve optimal efficacy when combining bacteriophages with other food safety interventions. As almost all commonly used chemical sanitizers are capable of inactivating phages, therefore phages must be applied separately to ensure that they remain effective (Sukumaran et al., 2015). This was illustrated by the decreased efficacy of a simultaneous application of bacteriophages and chemical preservatives when compared to either treatment individually (Rodríguez et al., 2004). But, if phage preparations are carefully incorporated with other approaches, the efficacy of each could be – and often is – improved. For instance, in the presence of high organic loads, pretreatment of fruits and vegetables with a bacteriophage preparation boosted the efficacy of a produce wash by up to 2 logs (Magnone et al., 2013). Similarly, studies suggest an additive effect also occurs when phages are applied after chemical treatments, as higher reductions were observed in the combined treatments than when the interventions were used alone on apples, cantaloupes, lettuce, and chicken breast (Moye et al., 2020; Sukumaran et al., 2015). Phage biocontrol has also been shown to be effective when combined with modified atmospheric

conditions, having better reductions in bacterial counts on chicken breast compared to their storage under aerobic conditions (Sukumaran et al., 2016). As modified atmosphere packaging is widely used by the various food industries, this observation has direct implication to improve product safety. In summary, proper integration of phage biocontrol in the existing Hazard Analysis and Critical Control Points (HACCP) protocols is key to it becoming an integral part of an effective multi-hurdle approach for improving food safety.

Acceptance of phage biocontrol

Today's consumer is increasingly looking for "green appeal" in their food products (Atchley, 2019). There is a growing trend towards the purchase of food products that are not treated with chemical sanitizers or antibiotics and that are sustainably and naturally grown and processed, including not "genetically modified" (Lewis and Hill, 2020). Demand for health and wellness foods, including organic and locally produced food products, such as available at local farmer's markets and community-supported agriculture, has been on the rise (Reganold and Wachter, 2016; Woods et al.). While this is a welcome trend from the perspective of improving consumer health, one of the major challenges for the food industry is to keep this minimally treated food safe from microbial contamination. Phages have multiple properties that make them the perfect microbial control tool to improve the safety of minimally treated food including (i) organic nature of wild-type lytic phages, (ii) specific target pathogen control as opposed to generalized killing by antibiotics and chemical sanitizers, (iii) minimal or no residual effect on the natural microflora of food products, and (iv) no effect on food sensory or health-promoting qualities. However, the use of phages is not without challenges. Among these hindrances, besides the technical challenges described above, are (1) consumer perception and (2) willingness by food producers to adopt this new green technology. Consumer perception is perhaps the biggest challenge, as there is a potential risk that the phage technology can be misconstrued as "viruses on my food," where viruses are being inherently identified as "bad" by the general populace. Food producers take their cue from consumer trends and may be reluctant to use phages for microbial control if phage technology is miscomprehended. On the other hand, a recognition of the true benefits of phage mediated biocontrol of foodborne

pathogens, including reduced chemical sanitizer use while controlling the pathogens, should encourage consumers and food producers to adopt the phage technology.

Although the science behind the phage technology is unambiguous, overcoming the problem of consumer perception and adoption by industry will require a fair amount of education of consumers and food processors on basic facts about phages and their use. While a limited amount of research has been conducted regarding the perception of phages, current evidence suggests that the phage biocontrol technology is viewed favourably (Naanwaab et al., 2014). Generally, consumers were supportive and even willing to pay a higher price for phage treated fresh produce when they were properly educated on the nature of lytic bacteriophages and the phage biocontrol approach (Naanwaab et al., 2014). Thus, for the phage technology to be embraced by consumers and food producers, the basic essential facts, such as the ubiquitous nature of phages, their natural presence in all the foods ingested, and their contribution to human health (Dalmasso et al., 2014; Guglielmi, 2017; Hatfull, 2008; Sulakvelidze and Barrow, 2005), need to be conveyed clearly. Ideally, phages will gain the recognition that the now well-received bacterial probiotics have earned, that phages are already a part of our ecosystem and can be beneficial to our health, leading to wider acceptance of phage biocontrol and improving the safety of our foods.

Concluding Remarks

Despite some lingering challenges, phage biocontrol is increasingly being recognized for its effectiveness in controlling foodborne pathogens in the food processing industry. Food producers are adopting phage biocontrol as part of a multiple hurdle approach to achieve a greater reduction in target pathogens and improve food safety. Moreover, a supportive consumer perception will likely further propel the adoption of phage biocontrol. A number of commercial phage preparations have been approved or are under consideration by the regulatory bodies in the US and other countries. The flexibility of commercial phage preparations from an application standpoint is helpful for food producers, as phages can be employed at either pre-harvest or post-harvest stages, at a

variety of processing steps, and through various mechanisms such as spraying, dipping, etc. The biggest advantage of using phage biocontrol is that wild type lytic phages are natural antimicrobials that allow targeted elimination of problem foodborne pathogens in foods without deleteriously impacting the natural microflora of foods and other nutritional or organoleptic qualities of foods.

Acknowledgements / Conflict of Interest Statement

All the authors are employees of Intralytix, Inc., a Maryland-based corporation engaged in the development and commercialization of bacteriophage preparations for various applications, including phage biocontrol. AS and JW also hold an equity stake in Intralytix, Inc.

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