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Lytic Bacteriophages as Biocontrol Agents of Foodborne Pathogens

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ABSTRACT

The present review focuses on applications of bacteriophages (also called 'Phages') in biocontrol of foodborne pathogens. Food borne diseases caused by bacterial pathogens are a threat to the human health and national economy. Further, there is an increase in multidrug resistance among bacterial pathogens and the conventional methods of food safety, generally involve use of chemicals having certain toxicity issues. The bacteriophages in contrast are natural enemies of bacteria and have regained their popularity as a natural biocontrol agent of bacterial pathogens. The bacteriophages are viruses that kill bacteria and are the most ubiquitous (total number estimated to be 10^{30} - 10^{32}) known organisms on earth. These are part of the normal microflora of all fresh and unprocessed foods and play a key role in maintaining microbial balance in every ecosystem where bacteria exist. Recently, there is a gaining interest among researchers regarding practical applications of bacteriophages to improve food safety. Many bacteriophage based preparations have been approved by regulatory authorities for their usage in food safety as food additives and disinfectants. Mainly, the application of phages for biocontrol of food pathogens are classified into three categories: (1) Pre-harvest control of foodborne pathogens in food producing livestock and poultry, (2) Decontamination of inanimate surfaces in food-processing facilities and other food establishments and (3) Post-harvest control of foodborne pathogens by direct applications of phages onto the harvested/processed foods. Commercially available phage products being marketed by several companies for reducing the presence of foodborne pathogenic bacteria in food and food production environment have been described and reviewed here.

Key words: Bacteriophages, phages, food safety, foodborne pathogens

INTRODUCTION

Food borne diseases are of major concern worldwide. Two-third of the food borne disease outbreaks are caused by bacteria (CDC., 2005a, 2009). While the majority of bacterial strains are harmless or even beneficial to humans, several others, being pathogenic in nature can cause severe threats to health and safety and consequentially inflict tremendous burden on our socio-economic balance and health care systems. Bacteriophages (phages) can be used as one of the effective solutions for biocontrol of foodborne bacterial pathogens as these are natural enemies of bacteria commonly found in wastewater and soil (Hagens and Offerhaus, 2008; Tan *et al.*, 2014). The bacteriophages were discovered by Frederick Twort and Felixd' Herelle during the early 20th century (Duckworth, 1976). These are bacterial viruses that infect and multiply within their

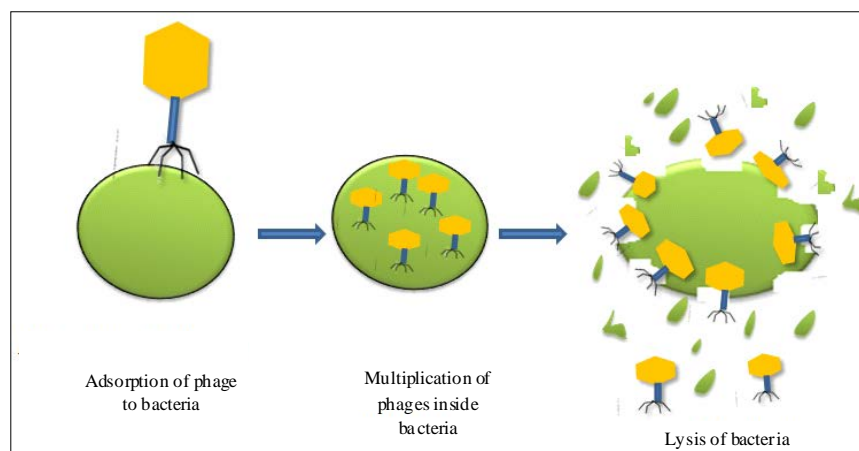


Fig. 1: Lifecycle of a lytic bacteriophage

specific hosts and are stable under varied pH and temperature conditions (Pearson *et al.*, 2013). These are the most ubiquitous (total number estimated to be 10^{30} - 10^{32}) known organisms on earth and play a key role in maintaining microbial balance in every ecosystem wherever bacteria exist. Also, they are highly specific for their host cell as their adsorption and entry are mediated by specific receptors (e.g., carbohydrates, proteins, lipo-polysaccharides etc.) present on host cells. Phages can exhibit one of the two types of life cycle: lytic or lysogenic (Lenski, 1988). The lytic phages cause rapid lysis and death of the host bacterial cell, whereas temperate or lysogenic phages spend part of their life cycle in a quiescent state called prophage.

The concept of fighting bacterial pathogens in foods using specific lytic phages has been around for many years (Wilkinson, 2001). The phages are also part of the normal microflora of all fresh and unprocessed foods. The principle behind the usage of lytic phages is that they specifically adsorb to their host bacterium, multiply and cause its lysis (Fig. 1), thus preventing spoilage of food by micro-organisms. Recently, the applications of phages in food safety are gaining momentum for several reasons, the most important of which is increased customer and regulatory pressures to ensure food safety while reducing the use of harsh chemical sanitizers and disinfectants. Indeed, the results of recent studies dealing with improving food safety and several recent regulatory approvals of various commercial phage preparations emphasize that lytic phages may provide a safe, environment friendly and effective approach for significantly reducing contamination of various foods with foodborne bacterial pathogens (Hagens and Offerhaus, 2008; Sulakvelidze, 2013; Singh *et al.*, 2013; Sharma, 2013; Tan *et al.*, 2014).

FOODBORNE ILLNESSES

Foodborne illnesses due to consumption of contaminated produce are an increasing problem worldwide. The Centers for Disease Control and Prevention (CDC) estimated that 76 million foodborne illnesses, including 325,000 hospitalizations and 5,000 deaths occur in the United States each year (Nyachuba, 2010). According to the Centers for Disease Control and Prevention (CDC., 2011), 48 million cases of food poisoning occur each year in the United States alone, of which 128,000 result in hospitalization and 3,000 in deaths. Recently, in 2013, the Foodborne Disease

Active Surveillance Network (FoodNet) reported around 19,056 cases of foodborne infections causing 4,200 hospitalizations and 80 deaths in United States (Crim *et al.*, 2014). Regarding financial burden posed by foodborne pathogens, it has been estimated that the cost of illnesses caused by 14 major foodborne pathogens is \$14 billion per year in USA alone, 90% of which is caused by the five most common causes of foodborne diseases (Hoffmann *et al.*, 2012). Out of these, *Salmonella enterica* is the most common (\$3.3 billion), followed by *Campylobacter* spp. (\$1.7 billion), *Listeria monocytogenes* (\$2.6 billion), *Toxoplasma gondii* (\$3 billion) and norovirus (\$2 billion). As per CDC reports, the leading cause of deaths among humans is caused due to foodborne bacterial diseases caused by *Listeria* sp., *Salmonella* sp., *Escherichia coli* O157:H7 and *Campylobacter jejuni* (CDC., 2005b, 2007a, b, 2008a, b, 2010).

Keeping this in view, food processors worldwide implement various approaches to ensure the safety of foods they produce. Currently, the conventional pathogen decontamination protocols in food-processing facilities focus primarily on using chemicals, physical disruption techniques and irradiation to remove a broad spectrum of foodborne bacterial pathogens from those facilities and from the foods produced in them (Gomez-Lopez, 2012). However, no single approach is 100% effective and the above-mentioned approaches possess some significant drawbacks, including their ability to corrode food-processing equipment, the toxic effects of chemical residues and their ability to damage the organoleptic properties of some foods. These broad-spectrum approaches also kill potentially beneficial bacteria that are important components of foods. In contrast, the bacteriophages offer an environmentally safe, non-corrosive and effective modality for eliminating or significantly reducing the levels of their specifically targeted bacterial pathogens in various foods, without a deleterious impact on the organoleptic properties and without disrupting the normal and often beneficial microflora of foods (Endersen *et al.*, 2014; Hagens and Offerhaus, 2008; Tan *et al.*, 2014).

METHODS TO CONTROL FOODBORNE PATHOGENS

Contamination of foods with foodborne bacterial pathogens may be reduced by three main types of phage treatments (Fig. 2):

- Pre-harvest control of foodborne pathogens in food producing livestock and poultry
- Decontamination of inanimate surfaces in food-processing facilities and other food establishments
- Post-harvest control of foodborne pathogens by direct applications of phages onto the harvested/processed foods

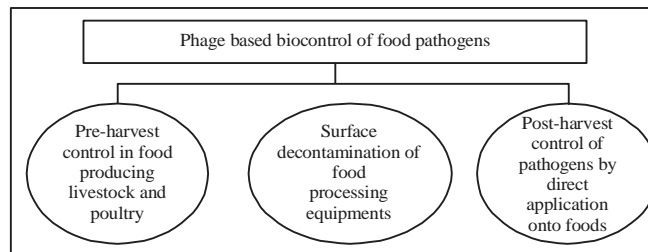


Fig. 2: Phage based approaches for biocontrol of foodborne pathogens

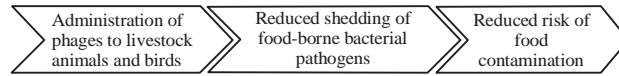


Fig. 3: Pre-harvest control of foodborne pathogens

PRE-HARVEST CONTROL OF FOODBORNE PATHOGENS IN FOOD PRODUCING LIVESTOCK AND POULTRY

Phage therapy has shown promise as an effective pre-harvest intervention by controlling foodborne pathogens in animals and birds (Raya *et al.*, 2006, 2011; Lim *et al.*, 2012; Carvalho *et al.*, 2010). The proposed rationale for this approach is that phages may be used to prevent and/or significantly reduce colonization of pathogenic bacteria in livestock animals and birds. In other words, the phages are administered in order to reduce shedding of specific foodborne bacterial pathogens and thus reduce the risk of subsequent contamination of food products containing the animals' or birds' products (Fig. 3).

Control of food-borne pathogens in animals: Phage based interventions have been aimed at controlling *E. coli* serotype O157:H7 in cattle and other ruminants as these are considered to be the principal reservoirs of *E. coli* O157:H7 and the contents of intestines and fecal material on their hide may contaminate meat during slaughter (Lim *et al.*, 2010). This bacterial pathogen causes a myriad of foodborne disease manifestations, including diarrhea, hemorrhagic colitis, hemolytic uremic syndrome and thrombotic thrombocytopenic purpura (Banatvala *et al.*, 2001). Animals that shed high levels of this pathogen may pose an elevated risk of contaminating the food chain if presented to slaughter. Thus, 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. Raya *et al.* (2006) 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 days after challenge with *E. coli* O157. Sheep receiving a single oral dose of CEV1 showed a 2-3 log-unit reduction in caecal and rectal *E. coli* O157:H7 levels within 2 days compared to the controls. Again, to evaluate the antibacterial effect of a single phage over a mixture of phages, Raya *et al.* (2011) 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 group (Raya *et al.*, 2011). The authors concluded the phage cocktails are more effective than individual phages at reducing *E. coli* O157:H7 populations in the ruminant gastrointestinal tract. 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 reduce *E. coli* O157:H7 in live animals immediately before slaughter.

The *Salmonella* sp. is also one of the principal causes of food-borne diseases causing diarrhea worldwide and is commonly found in chickens, turkey, pigs etc. Wall *et al.* (2010) investigated the efficacy of phage mixture in reducing *S. typhimurium* colonization of pigs during their transportation and prior to being slaughtered. The researchers administered phages orally and in microencapsulated form that led to efficient reduction in *Salmonella typhimurium* contamination of ileum, cecum and tonsils of pigs. Also, the researchers reported that the microencapsulated

phages were more efficient than the orally administered phage mixture. Similarly, Callaway *et al.* (2011) evaluated the ability of a phage mixture to reduce and control *Salmonella* contamination in swine. The phage cocktail (10^9 PFU mL⁻¹) was administered after 24 and 48 h of *S. typhimurium* infection in pigs and fecal samples were collected every 24 h upto 4 days. A significant reduction was observed in the *S. typhimurium* numbers in both cecal and rectal contents. Albino *et al.* (2014) indicated the use of phages in reducing the colonization of *Salmonella* sp., in pigs as foodborne illnesses due to consumption of *Salmonella* contaminated pork products are an important public health problem. The orally administered bacteriophages (10^7 and 10^9 PFU mL⁻¹) significantly reduced ($p < 0.05$) *S. typhimurium* counts under *in vitro* as well as *in vivo* conditions. However, the prevalence of *Salmonella* sp. and *Campylobacter* sp. has been found to be more associated with poultry products such as chicken skin, eggs etc (Lurette *et al.*, 2008; Hagens and Loessner, 2010). *Listeria monocytogenes* is also one of the important foodborne pathogens responsible for causing the deadly disease listeriosis. It is an opportunistic pathogen and is commonly associated with fresh and ready to eat products contaminated via infected humans, equipment or factory environment. Thus, as *Campylobacter* sp., *Salmonella* sp. and *Listeria monocytogenes* are not commonly associated with livestock contamination, researchers have focused largely on reduction of these bacteria in poultry, fresh or processed foods by direct application of phages onto contaminated foods (Endersen *et al.*, 2014).

Control of food-borne pathogens in poultry: The phage therapy applications to control foodborne pathogens in live animals have also been conducted in poultry, since poultry and egg products are important sources of the human pathogens. Poultry has been recognized as a major reservoir of two most prominent food pathogens, *Salmonella* sp. and *Campylobacter* spp. (Endersen *et al.*, 2014). With respect to *Salmonella* sp., Fiorentin *et al.* (2005) isolated a cocktail of phages from free range chickens and used them to reduce the concentration of *Salmonella enteric* serovar Enteritidis Phage Type 4 (PT4) in the ceca of broilers. Five days post phage treatment, the concentration of *S. Enteritidis* PT4 per gram of cecal content in the phage-treated group was reduced by 3.5 log units and samples collected up to 25 days after treatment revealed that the treated birds still had lower Colony Forming Units (CFU) of *S. Enteritidis* PT4 per gram of cecal content compared with untreated broilers. Borie *et al.* (2008) administered a mixture of three phages to chickens 24 h post-infection with *Salmonella enteritidis* by aerosol spray or oral administration (in drinking water). The results indicated that the phage therapy was effective in reducing *S. enteritidis* in chickens over a period of 20 days. Also, Bardina *et al.* (2012) evaluated the therapeutic potential of phages in reducing *S. typhimurium* concentrations in intestines of mice and chickens both pre- and post-infection. The *Salmonella* concentrations were significantly reduced in both mice and chickens highlighting the potential of phages in prevention and treatment of bacterial infections. Lim *et al.* (2012) measured the effectiveness of phages to control *S. enteritidis* infection in 1 day old chicks. The study reported that phage (10^9 CFU mL⁻¹) dosage as a feed additive significantly lowers (70%) bacterial counts in cecal tissue and proposed their usage in control of *Salmonella* infections in poultry and the incidence of food poisoning cases caused by this bacteria. Hungaro *et al.* (2013) compared the effectiveness of bacteriophages with the traditionally used chemical agents in reducing the number of *S. enteritidis* counts in chicken skin. Similar reductions of about 1 CFU cm⁻² were obtained with both phage cocktail as well as the chemical reagent suggesting that phage cocktails can be employed as an alternative agent in reduction of poultry contamination caused by *S. enteritidis* under industrial conditions.

Henriques *et al.* (2013) suggested that, bacteriophages can be applied as an aerosol spray for reducing the horizontal transfer of *Salmonella* sp. in poultry during transportation of eggs from incubators to hatchers.

For biocontrol of *Campylobacter* sp. contamination in poultry, Loc Carrillo *et al.* (2005) investigated the effect of phage administration in controlling *Campylobacter* sp. infection of broiler chickens and observed an efficient reduction in bacteria in cecal contents of phage treated birds. A similar study was conducted by Wagenaar *et al.* (2005) for controlling *C. jejuni* colonization in broiler chickens using single phage and phage mixtures. The experiments using phage mixture provided a notable decrease of *Campylobacter* levels in the caeca of the treated chickens compared to the single phage treated chickens. El-Shibiny *et al.* (2009) demonstrated the phage efficacy in reducing *C. jejuni* and *C. coli* numbers in caeca of *Campylobacter*-colonized chickens. Carvalho *et al.* (2010) tested a phage cocktail consisting of three phages for the control of *C. coli* and *C. jejuni* through two routes of phage administration (i.e., by oral gavage and in feed). The phage cocktail was able to reduce the titre of both *C. coli* and *C. jejuni* in faeces by approximately $2 \log_{10}$ CFU g^{-1} when administered by oral gavage and in feed. This reduction persisted throughout the experimental period and neither pathogen regained their former numbers. Further, it was observed that administration of phage in feed to *Campylobacter* infected chicks leads to an earlier and more sustainable reduction of *Campylobacter* compared to the oral gavage.

While these studies indicate the possibility of using phages to control bacterial colonization in cattle and poultry, 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 and birds.

DECONTAMINATION OF INANIMATE SURFACES IN FOOD-PROCESSING FACILITIES AND OTHER FOOD ESTABLISHMENTS

The second approach (also called 'Phage bio-sanitation') involves using phages to improve food safety by decontaminating various inanimate surfaces in household kitchens, food processing facilities and other food establishments, so that the foods contacting those surfaces are less likely to become contaminated with foodborne bacterial pathogens (Gibson *et al.*, 1999). Although most of the foodborne bacterial pathogens are inactivated during cooking, some of them may survive on the surfaces on which the foods were processed before cooking and other foods which are ready-to-eat may come in contact with those surfaces, get contaminated and lead to foodborne diseases (Bloomfield and Scott, 1997; Kusumaningrum *et al.*, 2003). Similarly, foodborne bacteria may persist on various surfaces in food-processing facilities and contaminate foods that are being processed or packaged in those facilities. To address this problem, food processors commonly use various chemicals to remove a broad spectrum of bacteria from various surfaces in their facilities with significant drawbacks associated with them (Maukonen *et al.*, 2003). Bacteriophages can be a natural and non-toxic alternative to eliminate or reduce levels of foodborne bacterial pathogens on various hard surfaces commonly used in food-processing facilities and in home kitchens (Sulakvelidze and Pasternack, 2010). The contamination of hard surfaces with pathogenic bacteria such as plague causing *Yersinia pestis* is particularly significant as it is highly contagious in nature and can spread rapidly via aerosol route (Perry and Fetherston, 1997). Recently the potential of phage cocktail having lytic activity against *Y. pestis* in decontaminating various hard surfaces experimentally contaminated with *Y. pestis* has been evaluated (Rashid *et al.*, 2012). The

cocktail consisted of five phages (designated YPP-100) capable of lysing 59 *Y. pestis* strains. YPP-100 was examined for its ability to decontaminate three different hard surfaces (glass, gypsum board and stainless steel) experimentally contaminated with a mixture of three genetically diverse *Y. pestis* strains CO92, KIM and 1670G. Phage concentrations of 10^9 , 10^8 and 10^7 PFU mL⁻¹ completely eliminated all viable *Y. pestis* cells from all three surfaces with only 5 min of exposure to YPP-100. Further, even at diluted phage concentrations (10^6 PFU mL⁻¹), significant reduction in *Y. pestis* levels was observed.

Escherichia coli O157:H7 is a highly prevalent pathogen with low infectious dose commonly found on surfaces. The potential of a cocktail of *E. coli* O157:H7-specific phages (BEC8) against *Escherichia coli* (EHEC) O157:H7 strains has been investigated on various food processing surface materials i.e., Stainless Steel Chip (SSC), Ceramic Tile Chip (CTC) and High-Density Polyethylene Chip (HDPEC) (Viazis *et al.*, 2011, 2015). The results demonstrated that the phage cocktail effectively killed EHEC mixture on all 3 hard surfaces in 1 h above room temperature conditions. Further, the phage cocktail (BEC8) reduced EHEC number on hard surfaces following first-order kinetics. However, it was found that longer times (around 2-4 h) may be required to achieve a 5-log reduction of bacterial cells in liquid culture at 23°C.

Foodborne *Salmonellosis* caused by *Salmonella* is also an international health risk, causing an estimated 93.8 million illnesses globally and approximately 155,000 deaths, each year. Although, it is inactivated during cooking on food surfaces but may survive on surfaces of equipment used for food processing and contaminate ready to eat foods (Majowicz *et al.*, 2010). The potential of phages has been demonstrated in reducing the number of *Salmonella* strains from stainless steel and glass surfaces using two phage cocktails SalmoFresh™ and SalmoLyse™, possessing lytic activity against a broad range of *Salmonella* strains (Woolston *et al.*, 2013). During the surface decontamination studies, the *Salmonella* strains were exposed to the phage preparations on the hard surfaces for 5 min, after which the phages were washed away and the levels of residual *Salmonella* on the surfaces were enumerated. Significant ($p < 0.05$) reductions in *Salmonella* counts were observed from stainless steel and glass surfaces (2.1-4.3 log units). The authors concluded that the routine use of SalmoFresh™ or SalmoLyse™ (or any other technically equivalent phage preparation) may provide an effective and sustainable solution for development of a bacteria-free environment in food processing facilities. However, additional long-term studies are needed to investigate the validity of such phage-mediated “Eco-management” approach.

Phage immobilization over packaging material is another way to prevent food spoilage in ready to eat foods (Anany *et al.*, 2011). The researchers used modified positively charged cellulose membranes as the support for phage immobilization in their studies. Cocktails of phages active against *Listeria* or *E. coli* immobilized on these membranes were shown to effectively control the growth of *L. monocytogenes* and *E. coli* O157:H7 in ready-to-eat and raw meat, respectively, under different storage temperatures and packaging conditions. The phage storage stability was also investigated to further extend their industrial applications. It was shown that lyophilization can be used as a phage-drying method to maintain their infectivity on the newly developed bioactive materials. Such bioactive materials can be used further for surface decontamination of foods during their storage and transportation.

POST-HARVEST CONTROL OF FOODBORNE PATHOGENS BY DIRECT APPLICATION OF PHAGES ONTO THE HARVESTED/PROCESSED FOODS

The outbreaks of foodborne illness associated with the consumption of raw and processed foods, including both vegetarian and non-vegetarian products emphasize the need for an effective natural

antimicrobial agent which can be directly applied to the food to kill pathogenic micro-organisms (CDC., 2010, 2011). US Food and Drug Administration (FDA) has mentioned a few bacterial pathogens viz., *Staphylococcus aureus*, *Shigella* sp., *E. coli*, *Salmonella* sp. and *Listeria monocytogenes* that can be found in fruits, vegetables, fresh cut products that make salads, dairy and meat products. Chemical disinfectants used during the processing of fresh cut leafy green commodities are not sufficient to kill foodborne pathogens attached to intact or cut surfaces of leafy greens and are primarily used to prevent cross contamination of leafy greens through water (Behrsing *et al.*, 2003). The bacteriophages are naturally present in all fresh and non-processed foods, including fresh ground beef, fresh fruits and vegetables, raw skim milk, cheese and frozen mixed vegetables and thus, is a natural way of preventing bacterial contamination in foods (Viazis *et al.*, 2011; Luo *et al.*, 2012). The approach of using phages involves directly applying them to the food surfaces to eradicate or significantly reduce the number of specifically targeted foodborne bacterial pathogens contaminating the foods. Also, the concept of using phage biocontrol protocols involving the direct application of phages to various foods is essentially based on using a microorganism that may already be present in those foods and simply applying the appropriate number of appropriate phages at appropriate location. Thus, if a food is contaminated with a bacterial pathogen that is the host for the lytic phages applied to the food's surface, the phages should eliminate or significantly reduce the contamination, thereby making the food safe for consumption without deleterious effects on its normal, beneficial microflora and organoleptic qualities.

Listeria monocytogenes has been reported as one of the serious foodborne pathogens as after entering the host body, it is recognized as an intracellular pathogen and therefore cannot be reached by the host's immune system of a person suffering from *listeriosis*. Due to its high pathogenicity, zero tolerance policy (no detectable level permitted) has been declared by FDA for *L. monocytogenes* in food products (Zaczek *et al.*, 2015). Bacteriophages have emerged as a natural alternative to conventional products in reducing food contamination with *L. monocytogenes*. With respect to the regulatory issues associated with the use of phages for treatment of bacteria in foods, a mixed *Listeria* phage preparation has received approval to be used as a food additive in the production of ready to eat meat and poultry products and another phage preparation comprising a virulent single *Listeria* phage Listex P100 has received the highly desirable GRAS (generally recognized as safe) status for its usage in all food products. The efficacy of bacteriophage Listex P100 has been investigated to control *L. monocytogenes* growth on melon, pear and apple products (juices and slices) stored at 10°C to evaluate the effect of pH and physical form of food matrix on phage efficacy (Oliveira *et al.*, 2014). Phage treatment was found to be more effective on melon followed by pear, but no effect on apple products was observed. Reductions of about 1.50 and 1.00 log CFU per plug for melon and pear slices were found, respectively. In juices, higher reductions were obtained in melon (8.00 log CFU mL⁻¹) followed by pear (2.10 log CFU mL⁻¹) after 8 days of storage. However, *L. monocytogenes* in apple juice was unaffected by phage treatment in which the phage decreased to almost undetectable numbers. The results highlighted that Listex P100 could avoid pathogen growth on fresh-cut and in fruit juices with high pH during storage at 10°C. Further, to improve the phage application on high acidity fruits, a combination with other technologies may be required.

Salmonellosis caused by *Salmonella* sp. through consumption of contaminated food also constitutes a significant risk worldwide with typical symptoms ranging from diarrhea to systemic typhoid fever (McGhie *et al.*, 2009). The disease outbreaks caused by *Salmonella* sp. have been found to be commonly associated with seed sprouts, cantaloupes, unpasteurized fruit juices,

tomatoes and fruits such as watermelon, mango etc (Ye *et al.*, 2010). In 2009, another group of researchers evaluated the potential of phages in preventing *Salmonella* contamination of sprouted alfalfa seeds (Kocharunchitt *et al.*, 2009). For this, alfalfa seeds were first immersed in 10^7 CFU mL⁻¹ of bacterial suspension and then dried. The dried seeds were then soaked in *Salmonella* sp. specific phage suspension (SSP5 and SSP6) for 12 h at 25°C daily. The seeds sprouted within 5 days and it was found that there was no reduction in *Salmonella* number in the phage-treated seeds under *in vivo* conditions. However, the *Salmonella* growth on sprouting seeds was two orders of magnitude lower under *in vitro* conditions. The authors concluded that environmental changes and background microbiota may have altered the phage attachment sites. In 2010, similar studies were conducted for biocontrol of *Salmonella* on sprouting mung bean and alfalfa seeds (Ye *et al.*, 2010). The researchers used lytic phages isolated from pig or cattle manure effluent together with the *Enterobacter asburiae* (JX1) strain which exhibits an inherent antagonistic activity against *Salmonella* sp. The combined treatment significantly reduced the growth of bacteria on mung bean sprouts and alfalfa sprouting seeds. However, individual treatments with phage or *E. asburiae* (JX1) did not cause any reduction in *Salmonella* counts corresponding to the previously obtained data of Kocharunchitt *et al.* (2009). In 2013, it was reported that combined treatment of fresh vegetables with bacteriophages (before storage of vegetables at 10°C) and levulinic acid (after vegetable storage at 10°C) is more successful in reducing *E. coli* O157:H7, *Shigella* sp. and *Salmonella* as compared to the single step treatments (Magnone *et al.*, 2013). Researchers have also evaluated the effectiveness of phage cocktail mixture (containing three phages of concentration 10^9 PFU mL⁻¹) in reducing contamination of fresh cut lettuce of *Salmonella enterica* Enteritidis and *Salmonella typhimurium* at room temperature (Spricigo *et al.*, 2013). The phage cocktail significantly ($p < 0.05$) reduced the number of *Salmonella* cells on lettuce after 30 and 60 min of phage treatment. Another group of researchers investigated the ability of specific bacteriophages FSP-1 and FSP-3 to lyse *Salmonella* sp. in spiked samples of cooked chicken meat at three different temperatures i.e., 4, 28 and 37°C (Augustine and Bhat, 2015). Significant reductions in bacterial counts were observed at all the studied temperatures after 3 days of treatment. The efficiency of bacterial reduction increased when bacteriophages were applied as a cocktail mixture with high Multiplicity of Infection (MOI) with an average reduction of 79, 92 and 78% at 4, 28±0.5 and 37°C, respectively.

Staphylococcus aureus is also a relevant pathogen to the food processing industry since it causes food poisoning because of certain heat-stable enterotoxins (Anany *et al.*, 2011). Bovine mastitis caused by *S. aureus* is also a major concern because of its resistance to antibiotic treatment and its propensity to recur. Bacteriophages were investigated as antibacterial agents as far back as the 1920s as a means of eliminating bacteria, including Staphylococci in human infections. Keeping this in view, a collection of bacteriophages of dairy origin infecting *S. aureus* as a preliminary approach has been made to develop phage-based antimicrobial strategies having applications in food biopreservation (Garcia *et al.*, 2009). Bovine *S. aureus* strains isolated from mastitic milk samples were used as hosts to assess the prevalence of *S. aureus* phages in the dairy environment. Phages ΦH5 and ΦA72 were used in bacterial challenge tests against *S. aureus* in milk. Preliminary challenge trials were performed in Ultra High Temperature (UHT) whole-fat milk to assess the ability of the phages to lyse *S. aureus* Sa9 individually or in combination. Further, *Staphylococcus aureus* Sa9 was also challenged with the phage mixture in pasteurized, semi-skimmed and whole fat raw milks to determine to what extent commonly used milk treatment procedures (i.e., heating and skimming) affected the ability of the phages to inhibit *S. aureus*.

Phage mixture (1:1) was found to be significantly more effective ($p < 0.05$) than each single phage. The effect of phage inhibition on growth of *S. aureus* in UHT and pasteurized whole fat milk was comparatively higher than semi-skimmed and whole fat raw milk. However, it is worth noting that in all cases, the phage mixture kept *S. aureus* counts low enough to avoid toxin accumulation over concentrations that cause food intoxication, even in raw milk (Waldvogel, 2000). Although the work has a great relevance to the food industry to prevent accumulation of toxins that are not easily inactivated by heat treatments, yet, a more detailed analysis is required to determine the effect of milk components on phages themselves.

Another pathogen identified in 1983, *E. coli* O157:H7 is one of the most important foodborne pathogen. The bacterium causes a variety of human diseases such as diarrhea, hemorrhagic colitis, hemolytic uremic syndrome, gastroenteritis and thrombotic thrombocytopenic purpura (O'Flynn *et al.*, 2004). Abuladze *et al.* (2008) reported for the first time the effectiveness of a phage cocktail, ECP-100 (ECML-4, ECML-117 and ECML-134) in reducing contamination of vegetables with *E. coli* O157:H7. The phage cocktail lysed 90% of the bacterial strains on broccoli samples during storage at 10°C for 24, 120 and 168 h. Similar results were found with ECP-100 in reducing *E. coli* O157:H7 number on tomato slices and spinach samples. The potential of ECP-100 in reducing *E. coli* contamination of cantaloupe slices and fresh-cut lettuce stored at refrigeration temperature 4°C was also evaluated (Sharma, 2013). Viazis *et al.* (2011) investigated the efficacy of a mixture of eight lytic *E. coli* O157:H7 specific phages (10^7 PFU mL⁻¹) with different MOI levels (1, 10 and 100) over *E. coli* O157:H7 contaminated organic baby spinach and baby romaine lettuce leaves under various conditions (8, 23 and 37°C for 10 min, 1 and 24 h). The authors concluded that bacterial inactivation efficiency of phages increase with increasing temperature, MOI of bacteriophage and incubation period. Also, bacteriophages in combination with naturally occurring antimicrobial compound trans-cinnamaldehyde can be an environmentally friendly and effective way of reducing bacterial contamination in food compared to bacteriophage alone.

Bacteriophages have been largely evaluated for their effectiveness in killing the surface borne bacteria on leafy greens and other produce commodities, while avoiding damage to leafy or edible portions of produce desired for their unblemished appearance and fresh consumption (Viazis *et al.*, 2011; Luo *et al.*, 2012). However, the role of lytic bacteriophages in preventing cross-contamination i.e., applying phages before the introduction of the pathogen to the produce surface has not been evaluated. Thus, the effectiveness of using bacteriophages (EcoShield™) to prevent contamination of lettuce with *E. coli* O157:H7 introduced through cross-contamination was investigated (Ferguson *et al.*, 2013). EcoShield™ is a commercial phage preparation containing a mixture of *E. coli* O157:H7-specific bacteriophages which has been approved through Food Contact notices by FDA and has been granted a temporary exemption by the Food Safety and Inspection Service (FSIS) for use on meats and on food contact surfaces. Ferguson *et al.* (2013) introduced bacteriophages to fresh cut lettuce before the introduction of *E. coli* O157:H7 onto the surface of lettuce by different modes of application (immersion and spraying) to determine if a pre-treatment of lytic bacteriophages can protect against cross-contamination. Their findings suggested that pretreatment of phages does not cause an immediate reduction of *E. coli* O157:H7 on the surface of fresh cut lettuce, but reductions do occur during several days of storage at 4°C. Also, the mode of application (immersion and spraying) affected the efficacy of lytic bacteriophage treatments to reduce *E. coli* O157:H7 on fresh cut lettuce. Although both methods provided a degree of protection

from introduction of *E. coli* O157:H7 to fresh cut lettuce, spray application of lytic bacteriophages was found to be more effective in reducing *E. coli* O157:H7 populations on lettuce surfaces compared with immersion of lettuce in EcoShield™ solutions. Further, a synergistic effect between lytic bacteriophage treatment and sodium hypochlorite wash was also observed, indicating the suitability of using lytic bacteriophages in the presence of such sanitizers. Their findings suggest that use of lytic bacteriophages can reduce cross-contamination of *E. coli* O157:H7 on fresh cut lettuce, however, more research is needed to optimize the application of *E. coli* O157:H7-specific bacteriophages against *E. coli* O157:H7 on leafy greens under field and facility-processing conditions.

COMMERCIALY AVAILABLE PHAGE PRODUCTS

Several companies have developed phage based products for their applications in food safety. Intralytix Inc., a USA based company has developed three phage based products viz., ListShield™, EcoShield™ and SalmoFresh™ that has been approved by FDA for their usage in food additives (Intralytix, 2014). Besides this, they have two phage preparations for veterinary applications: PLSV-1™ and INT-401™. The company has developed and licensed bacteriophage-based animal health care products effective against *Salmonella* (PLSV-1™) and *Clostridium perfringens* (INT-401™) in poultry (Miller *et al.*, 2010). Further, ListShield™ has been approved by United States Environmental Protection Agency (EPA) for its usage in reducing *L. monocytogenes* contamination of inanimate surfaces in food-processing facilities. Also, SalmoFresh™ which is specific against *Salmonella enterica* has been given a GRAS (Generally Regarded as Safe) designation in 2013. On August 2014, the National Food Service, Ministry of Health in Tel Aviv, Israel, issued 'Guidelines: Use of Bacteriophages (bacteria killing viruses) in Food' which approve the use of all FDA-approved phage preparations for their applications in food in Israel (Intralytix, 2014). Listex™ P100 developed by Microcos Food Safety, Wageningen, Netherlands has also received a GRAS designation for its applications in food safety (Sulakvelidze and Pasternack, 2010). OmniLytics, Inc. is a bacteriophage company that is focused on developing safe, natural solutions for infectious disease control. With emphasis on bacteriophage technology, OmniLytics is pioneering research and development of bacteriophage solutions for pathogen control in the agricultural, food and water, industrial, pharmaceutical and defense markets. OmniLytics, Sandy, UT, USA has developed an antibacterial composition, AgriPhage™ that consists of phages against *Xanthomonas campestris* pv. vesicatoria and *Pseudomonas syringae* pv. tomato for the treatment of bacterial disease in agricultural crops (especially tomato and pepper plants) in a safe and eco-friendly manner (Swensen, 2006). Further, the company is working in collaboration with Elanco (a division of Eli Lilly and Company) for development of commercial phage based products against various foodborne pathogens, such as *E. coli* O157:H7 and *Salmonella*. Also, a phage-based product to prevent *E. coli* O157:H7 infections on hides i.e., Finalyse™ is being distributed by Elanco Company.

The regulatory approvals given by federal agencies such as FDA, EPA and GRAS for application of bacteriophages in foods indicate that bacteriophages are a natural, safe and effective modality to prevent food contamination caused by bacterial pathogens at all stages of food production (Sulakvelidze and Barrow, 2005; Sulakvelidze and Pasternack, 2010; Hagens and Loessner, 2010; Endersen *et al.*, 2014). However, further studies may be required which evaluates the mode of application, concentration and compositional aspects of phage based products.

CONCLUSION

The possibility of using phage therapy in biocontrol of foodborne pathogens which is highly beneficial for food safety and public health. However, additional long-term studies are required concerning the emergence of phage resistant mutants, concentration and mode of phage application depending on the types of foods used so that phage biocontrol protocols can become an integral part of routine food safety intervention strategies implemented by food industries. Also, the pharmacokinetics of phages must be analyzed as they may vary for each phage family. The concerted efforts may help in progress of phage applications in food safety at large scale and provide a natural alternative to conventional artificial preservatives used by food industries.

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