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Overcoming barriers to phage application in food and feed

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Bacteriophages (phages) can play a useful role as narrow spectrum antimicrobials in food safety and in food production. Consumer attitudes towards traditional additives have led to a search for natural, potentially clean label, alternatives. At the same time, the rise in antimicrobial resistance has created a need for alternative antimicrobials for disease prevention and treatment in animal husbandry. Phages represent a viable option for both of these applications. We highlight important barriers which should be considered to improve the chance of a positive outcome when using phages in food and food production. These include the feasibility of adding high concentrations of phages, the physico-chemical properties of the food or target, how and when phages are applied, and which phages are chosen.

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Introduction

Bacteriophages (phages) are bacterial viruses and use bacteria to produce new phages. Phages can replicate using lytic or lysogenic lifecycles. Lytic phages replicate by attaching to a host cell, injecting phage nucleic acid, replicating the phage nucleic acid, assembling proteins, packaging nucleic acid with head and tail proteins, and lysing the host cell to release new phages [1]. Lysogenic phages integrate into the host genome and replicate along with the host. Interest is growing in the use of lytic phages as biocontrol agents of foodborne pathogens in food and food production [2]. Phages can be used in a number of ways in food and feed and can also be applied as preharvest or post-harvest interventions. Recently there has been a change in consumer attitudes towards how food should be processed and preserved with rising interest in the concept of 'natural foods' and 'naturalness' [3]. The importance of consumers' perception should not be overlooked as it has a significant effect on willingness to buy a product and can even affect how individuals enjoy and experience that product [4]. The importance of this changing consumer attitude has led to the introduction of the term 'clean label'; these products aim to have simple ingredient lists free from 'chemical-sounding' terms and negatively perceived ingredients [5•]. It has been found that consumers are more willing to accept additives from natural sources than the chemical additives that have been used for decades. This rise in clean label food has increased the demand for new methods to ensure food safety. Another reason for introducing phages is the problem of antimicrobial resistance. A WHO report on antimicrobial resistance drew attention to the contribution of antibiotic use in animals to antimicrobial resistance in general [6]. Antimicrobial resistance has been termed a One Health problem and requires an integrative approach targeting the environment and the health of humans and animals [7]. Historically the antibiotics used in treating livestock have been the same as, or closely related to, those used in human medicine and the use of long-term subtherapeutic doses of antibiotics, as they have been used in livestock as growth promoters, can increase the risk of emergence of antibiotic resistance.

Phage concentration

The success of phages for use in food and feed depends on the overcoming of a number of barriers. The number of phages used is of great importance; in general, the higher the concentration of phages the more significant the reductions in target bacteria. Bai *et al.* [8••] found that a phage cocktail at multiplicity of infections (MOIs) of 10^3 and 10^4 significantly reduced the *Salmonella* Typhimurium load of cucumbers and lettuce. The cocktail at an MOI of 10^4 led to a greater and more sustained reduction. Phage at MOIs of 1, 10 and 10^2 significantly reduced *S.* Typhimurium on lettuce with the reductions increasing as the phage concentration increased [9•]. This was also evident using SalmoFresh™, an FDA approved Salmonella lytic bacteriophage preparation, on chicken fillets [10]. SalmoFresh™ applied at MOIs of 10^2 and 10^3 both significantly reduced a combination of *S.* Typhimurium, *S.* Heidelberg and *S.* Enteritidis at an initial concentration of 3 log CFU/g on chicken fillets. SalmoFresh™ at an MOI of 10^3 was significantly better than phage at an MOI of 10^2. In other examples, a single phage was used to control *Escherichia coli* on raw and cooked beef [11], while ShigaShield™ reduced Shigella

levels in smoked salmon and yogurt [12••]. In both cases the reduction was concentration dependent with the greatest reduction occurring at the highest phage titre tested. Using Listex™ P100, a commercially available phage against *Listeria monocytogenes*, a greater reduction was seen in tuna using an MOI of 10^2 than an MOI of 0.1 [13]. The greatest effect was seen with a lower starting concentration of *L. monocytogenes* combined with a higher concentration of Listex™ P100. However, phage concentration is not a definitive indicator of a positive outcome. A low titre phage cocktail decreased chickens' mortality and morbidity in a natural outbreak of Avian Pathogenic *E. coli* (APEC) but not in experimentally infected chickens [14]. This could have been due to the non-synchronous nature of the natural outbreak. In a natural outbreak all of the cases would be not at the same point in their infection cycle and the low titre phage cocktail may have controlled the infection at the early stages and interrupted the transmission. This would not have occurred in the experimental infection as all of the infections occurred at the same time.

Feasibility of adding high phage concentrations

The need for high numbers of phages for successful phage treatment brings up the question of the feasibility of generating phages at high enough titres to deploy in food or feed. *Salmonella* on post-chill chicken carcases has been suggested as a target as numbers do not usually exceed 3 log CFU/g [15]. The inoculation of 25 g samples of turkey fillet with 500 μl of a 10^9 PFU/ml bacteriophage preparation was sufficient to significantly reduce *S.* Heidelberg over a seven day period and represented a reasonable volume of phage preparation to be applied. In a study of the application of Finalyse™ hidewash, $\sim 3 \times 10^{10}$ phage/head of cattle in one gallon of water was used [16]. This was insufficient to reduce *E. coli* O157:H7 contamination of cattle hides and carcasses but given the one gallon quantity needed it may be difficult and cost prohibitive to increase the titre used. Recombinantly expressed endolysins isolated from phages could possibly be used in place of whole phages to avoid this issue [17], though once again the economic viability of this solution would have to be investigated. Issues with GMO legislative approval and consumer acceptance may limit the use of endolysins in food safety [18].

Properties of the target matrix

The properties of the targeted food or animal in question can be a barrier to phage success. The target matrix can affect the efficiency of phages. In a study of a single phage to reduce *S.* Typhimurium in whole milk, skimmed milk, energy drink, apple juice, and liquid egg the smallest effect of the phage was seen in liquid egg [19]. Phage titre increased in all foods except for liquid egg, in which a decrease occurred. The highly viscous matrix of egg limiting diffusion and homogeneous distribution of the phage particles was suggested as a reason for this reduced activity and the decrease in phage numbers. Similarly when looking at a phage cocktail to reduce *S.* Enteritidis in milk, cabbage, and chicken breast the greatest effect was seen in milk [20]. Again it was proposed that the liquid allowed greater diffusion of the phages. Using a single phage it was necessary to use an MOI of 10^5 to reduce *S.* Typhimurium load in liquid egg and fruit juice while an MOI of 10^7 was required to give a similar reduction in cooked beef and chicken [21]. Salmonelex™, a commercially available phage cocktail, was effective in reducing *Salmonella* on chicken [22]. Salmonelex™ diluted in tap water was more effective than in filtered tap water with reduced calcium, magnesium, and sodium. Temperature can also affect the activity of phages. The antibacterial activity of ListShield™, a commercially available phage against *L. monocytogenes*, was reduced at 12°C compared to 4°C [23]. When using phages to reduce *Staphylococcus aureus* during the manufacturing of cheese it was observed that phage titre decreased with decreasing pH [24]. Antacids can be given before or in combination with phages in animal application to improve the survival of phages in the gut by increasing pH [25,26].

Application method of phages

The method of phage application can also be a barrier to a positive outcome. Although dipping and spraying are common methods for phage application, they can have negative effects [27]. Dipping and spraying can release phage particles into the environment, dipping liquid can be a source of cross contamination and spraying equipment may not be available in the processing environment. Different methods can be used to suit the situation or to expand the conditions of activity of the phages. Microencapsulated phages significantly reduced *E. coli* on tomatoes and maintained the reduction for five days [28]. Microencapsulation reduced the UV sensitivity of the phages and increased the survival of phages at pH 3–7 and extreme temperatures. Free phages, paper impregnated with phages, and encapsulated phages all immediately reduced *E. coli* on alfalfa seeds and sprouts [27]. After five days free phages and encapsulated phages had a significant effect compared to untreated controls. This can be case dependent as in the same study free phages reduced *L. monocytogenes* by 3 log CFU/g in cantaloupes while encapsulated phages reduced numbers by only 1 log CFU/g over the same period. This was suggested to be due to how the bacteria attached to different food matrices represented by cantaloupe and alfalfa sprouts. A phage cocktail applied to chicken feed was as effective as phage introduced by crop gavage and represents a much easier mode of inoculation [25]. Phages can also be applied in more unexpected ways such as in the depuration of bivalves where phages were added to the depuration tank water [29]. Longer treatment time was required in control tanks to obtain comparable reductions to those achieved when using phage tanks (Table 1).

Table 1

The application of phages to eradicate target microbes. In the case of ListShield™ against *L. monocytogenes* in Spanish dry cured ham bacterial numbers increased after treatment at 12°C but decreased after treatment at 4°C, this increase was represented by a (+) [23]

Phage cocktail	Target organism	Food matrix	MOI	Application method	Bacterial reduction	Reference
BSPM4, BSP101, BSP22A	*S.* Typhimurium	Iceberg lettuce	10^3 10^4	Applied to surface	1.1–1.9 log CFU/cm^2 2.8–3.9 log CFU/cm^2	[8••]
		Cucumber	10^3 10^4		0.7–1.2 log CFU/cm^2 2.5–2.8 log CFU/cm^2	
LPST10	*S.* Typhimurium	Lettuce	1 10 10^2	Applied to surface	0.7–1.7 $\log_{10}$ CFU/cm^2 1.1–1.7 $\log_{10}$ CFU/cm^2 1.9–2.7 $\log_{10}$ CFU/cm^2	[9•]
SalmoFresh™	*S.* Typhimurium, *S.* Heidelberg, *S.* Enteritidis	Chicken breast fillets	10^2 10^3	Applied to surface	0.6 log CFU/g 1.1 log CFU/g	[10]
FAHEc1	*E. coli*	Raw beef	1 5×10^2	Applied to surface	1 log CFU/cm^2 2.7 log CFU/cm^2	[11]
		Cooked beef	10^2 10^4		1 log CFU/cm^2 5 log CFU/cm^2	
ShigaShield™	*Shigella sonnei*	Smoked salmon	2.25×10^2 2.25×10^3 2.25×10^4	Spraying	0.16 log CFU/g 0.50 log CFU/g 1.098 log CFU/g	[12••]
		Yogurt	4.5×10^2 4.5×10^3 4.5×10^4	Mixed in to food	0.07 log CFU/g 0.26 log CFU/g 1.01 log CFU/g	
Listex™ P100	*L. monocytogenes*	Tuna sashimi	0.1 10^2 10^3 10^6	Applied to surface	0.62 log CFU/g 1.11 log CFU/g 1.08 log CFU/g 2.35 log CFU/g	[13]
phi F78E	Avian pathogenic *E. coli*	Experimentally infected chickens Naturally infected chickens	30	Oral gavage and spraying Oral gavage and spraying	No reduction in morbidity or mortality Decreased mortality to below 0.5% in no more than three weeks	[14]
SalmoFresh™	*S.* Heidelberg	Turkey breast	10^4	Applied to surface	1.3 log CFU/g	[15]
Finalyse™	*E. coli*	Live cattle	$\sim 3 \times 10^{10}$ phage/head of cattle in one gallon of water. Looked at prevalence in cattle not bacterial numbers.	Applied to hide surface	6.6 % reduction in prevalence	[16]
P22	*S.* Typhimurium	Whole milk Skimmed milk Energy drink Apple juice Liquid egg	10^8	Mixed in to liquid	4.45 log CFU/ml 4.32 log CFU/ml 2.09 log CFU/ml 2.06 log CFU/ml 0.96 log CFU/ml	[19]
PA13076, PC2184	*S.* Enteritidis	Milk Cabbage Chicken breast	10^4	Applied to surface Applied to surface Mixed in to liquid	4 log CFU/sample 3.86 log CFU/sample 2.5 log CFU/sample	[20]

Table 1 (*Continued*)

Phage cocktail	Target organism	Food matrix	MOI	Application method	Bacterial reduction	Reference
SE07	*S.* Enteritidis	Liquid egg	10^5	Mixed in to liquid	1.96 log CFU/ml	[21]
		Fruit juice	10^5	Mixed in to liquid	2.06 log CFU/ml	
		Beef	10^7	Spraying	2.03 log CFU/ml	
		Chicken	10^7	Spraying	2.18 log CFU/ml	
Salmonelex™	*S.* Newport, *S.* Typhimurium, *S.* Heidelberg, *S.* Enteritidis	Skinless chicken legs and thighs	10^3	Applied to surface in tap water Applied to surface in filtered water	0.39 log CFU/cm^2 0.23 log CFU/cm^2	[22]
ListShield™	*L. monocytogenes*	Spanish dry cured ham	10^2	Applied to surface 4 °C	1.5 log CFU/cm^2	[23]
			10^2	Applied to surface 12 °C	+1.5 log CFU/cm^2	
			10^3	Applied to surface 4 °C	2 log CFU/cm^2	
			10^3	Applied to surface 12 °C	+3 log CFU/cm^2	
			10^4	Applied to surface 4 °C	3.5 log CFU/cm^2	
vB_SauS-phi-IPLA35, vB_SauS-phi-SauS-IPLA88)	*S. aureus*	Fresh cheese	6	Added during cheese manufacture	3.83 log CFU/g	[24]
phiCcoIBB35, phiCcoIBB37, phiCcoIBB12	*C. jejuni*	Live chickens	15	In feed Oral gavage	1.96 log CFU/g 1.69 log CFU/g	[25]
CP68, CP14	*C. jejuni*	Live chickens	10	Oral gavage	3 log CFU/g	[26]
LinM-AG8, LmoM-AG13, LmoM-AG20	*L. monocytogenes*	Cantaloupe	10^5 10-10^2	Applied to surface Encapsulated phage	3 log CFU/g 1 log CFU/g	[27]
EcoM-HG2, EcoM-HG7, EcoM-HG8	*E. coli*	Alfalfa seeds and sprouts	10^4 10-10^2 N/A	Applied to surface Encapsulated phage Impregnated paper	1.5 log CFU/g 1.3 log CFU/g 0.6 log CFU/g	
FJLA23, FKP26, FC119, FE142	*E. coli*	Tomatoes	10^4	Spraying microencapsulated phage	2.5 log CFU/tomato	[28]
phT4A, ECA2	*E. coli*	Cockles	1	Added to depuration tank water	0.6 log CFU/g	[29]
SalmoFresh™	*S.* Newport	Cucumbers	7.9×10^4	Sprayed before slicing Sprayed unsliced	1 log CFU/sample 1.83 log CFU/sample	[30]
Nepra, Lelidair, Nobby, Slant, Gaspode, Momine	*Pectobacterium atrosepticum*	Potatoes	10^2	Phage wash	Reduction in disease incidence 61.3%	[31•]
DT6	*E. coli*	Milk	2.4×10^4	Added during milk fermentation	1.1 log CFU/ml	[36]
wksl3	*S.* Enteritidis	Chicken skin	5×10^3	Spraying	2.43 log CFU/cm^2	[39]
Team1, P68, LH1-MUT	*S. aureus*	Cheddar cheese	150	Added during cheese manufacture	2 log CFU/ml	[41]

When phages should be applied

Phage interventions must be carried out at the correct stage of processing for a positive outcome and to avoid reintroduction of bacteria. For example, cucumbers that were sprayed with SalmoFresh™ and then sliced did not show a significant reduction in *S.* Newport while unsliced cucumbers did show a significant reduction [30]. It is believed that insufficient numbers of phages were transferred by cutting to achieve a significant reduction. A cocktail of six phages reduced the symptoms of *Pectobacterium atrosepticum* soft rot in potato tubers significantly [31•]. However, phage alone increased the disease severity in uninfected control tubers. This was suggested to be caused by the presence of enzymes or metabolites from phage production. This could limit the use of phages as a prophylactic treatment but not as an intervention as the cost to uninfected tubers could outweigh the benefit of reduction of symptoms in uninfected tubers. Regarding the issue of reinfection pigs can become infected with *Salmonella* after just two hours in a contaminated abattoir environment so would need to be treated at the correct time to avoid this [32]. To reduce *Campylobacter* in chicken, interventions should be performed at multiple stages as *Campylobacter* can re-enter the food due to its ubiquitous nature on farms [33].

Phage selection

There are a number of disadvantages associated with the use of phages but this barrier can be overcome by careful selection of phages. The emergence of phage resistant bacteria is a risk [34]. To decrease the issue of resistance treatments can be rotated or cocktails of phages can be used instead of single phages. Phages should only be applied when there is no risk of treated bacteria being reintroduced into the processing environment and causing issues with resistance. The highly specific nature of phages to their host has historically been viewed as a disadvantage in that they may only infect a limited number of strains [35]. This view is changing as it is recognised that their specificity limits negative effects to the surrounding bacterial community. For example, it would be possible to use phages to reduce enteropathogenic or Shiga toxigenic *E. coli* during milk fermentation without compromising the performance of starter cultures [36]. Cocktails of multiple phages can also be used to increase the number of strains targeted [37•]. Phages can carry virulence genes or antibiotic resistance genes but this can be largely avoided by using lytic rather than lysogenic phages [38]. There are also concerns about the immunogenicity of phages and the cytotoxicity which could result by lysis of target bacteria. Phages have been found to cause no adverse reactions in rats and mice suggesting they are safe for human use [39,40]. Phages against *S. aureus* in cheddar manufacture did not increase enterotoxin production [41]. Phages are constantly encountered by humans since they are found naturally in the gut of humans [42], on their skin [43], in animals [44], in sewage treatment systems [45], and during the breakdown of food fermentations [46] among others.

Conclusion

Phages show promise for use in controlling bacterial pathogens as additives from natural sources, which may be more readily accepted by consumers than traditional additives, and also in the growing fight against antibiotic resistance. Phages may only reduce bacterial populations in food and not eliminate them completely, but this is not a serious issue. Criteria are put in place by groups, such the European Union, for acceptable levels of pathogens in food depending on the pathogen, food, and intended consumer with some required to be absent and some acceptable at low levels [47]. There is zero tolerance for *Salmonella* in foods such as precut vegetables, *L. monocytogenes* in food for infants and for medical purposes, and *E. coli* O157:H7 on sprouts. Phages reduced *S.* Typhimurium in whole milk and skimmed milk to undetectable, and therefore authorised, levels [19]. Phages reduced *L. monocytogenes* to undetectable levels on tuna and Spanish dry cured ham [13,23]. Coagulase-positive staphylococci are permitted in cheeses during manufacture up to 10^4 CFU/g. Phages have been successful in reducing *S. aureus* in cheddar cheese to safe levels [41].

Bacteria use a number of methods to defend against phage infection at various points in the infection process [48]. Bacteria can inhibit phage adsorption by altering or blocking receptors. Superinfection exclusion occurs when a prophage causes the expression of proteins which stops phage injection. Restriction modification methylates host DNA and cleaves invading unmethylated DNA. Bacteria using the CRISPR/Cas system (clustered, regularly interspaced, short palindromic repeat) integrate small fragments of invading DNA and cleave DNA with this sequence. Abortive infection limits the spread of the phage by the death of infected host cells. Care must be taken in the application of phages and their use to ensure they achieve a favourable outcome before they become a widely used and accepted aid in food processing. Phage concentration, the feasibility of adding a high concentration of phages, the properties of the food or animal to be treated, how phages are applied, when they are applied, and what phages are used are all barriers which must be overcome when designing a process using phages for decontamination or bacterial control.

Conflict of interest statement

Nothing declared.

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