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A review of current methods using bacteriophages in live animals, food and animal products intended for human consumption

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Title: A review of current methods using bacteriophages in live animals, food and animal products intended for human consumption.

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Key words: Bacteriophage, food, animal, packaging, multiplicity of infection, storage.

Highlights

- A consideration of the use of bacteriophages in live animal research.
- A review of the uses of bacteriophages in raw and processed foods.
- Discussion of bacteriophage delivery routes in relation to type of food/ animal.
- Consideration of the ethical implications of bacteriophages in foods.
- Discussion of possible future bacteriophage-food research avenues.

Abstract

Bacteriophages are utilised in the food industry as biocontrol agents to reduce the load of bacteria, and thus reduce potential for human infection. This review focuses on current methods using bacteriophages within the food chain. Limitations of research will be discussed, and the potential for future food-based bacteriophage research.

1 Introduction

The use of bacteriophages to detect bacterial human pathogens has revolutionised modern microbiology. Their use represents a significant divergence from traditional cultivation, and could have the potential to rapidly provide information to diagnose and prescribe a treatment strategy for bacterial infections in humans and animals. However, their use has raised concerns from the public about the inclusion of live viruses in products destined for human consumption. It has also raised concerns about their propagation, safe transport and storage, as well as shelf life in foods that already have to conform to high safety standards.

Bacteriophages are obligate intracellular parasites of bacteria, and are usually specific to one species or even specific to just one strain of that species. Bacterial transmission through the food chain has been recognised as a significant threat to human health for many years. PCR-based technologies are commonly used for the detection of human pathogens in clinical samples (Speers, 2006), as well as for the detection of bacteriophages (del Rio *et al.*, 2008; Martin *et al.*, 2008). It might be possible for the existing technology platforms to be adapted to monitor shelf life, effective storage and thus to reassure the public over their inclusion in food products, in terms of biological safety.

This review will focus on current methods utilising bacteriophages as biocontrol agents for food products destined for human consumption. First, live animal research will be discussed. The methods include those where animals are directly inoculated with bacteriophage suspensions in their food and water, or indirectly in to their immediate environment where uptake is facilitated. Next, the review will discuss methods where bacteriophages are applied directly to foods or to their packaging. Finally, methods utilising bacteriophage enzyme preparations will be discussed.

2 Live Animal Research

The number of naturally occurring bacteria and bacteriophages are likely to fluctuate during passage through animal bodies, and be influenced by diet, contact with other animals, and incidents of disease. This might suggest that a dynamic predator-prey relationship exists within animal populations, and that animals could provide a valuable resource for the discovery of new bacteriophages against a range of pathogenic bacteria. Bacteriophages have been recovered as from live agricultural animals, particularly noted during incidents of disease, such as mastitis (Georgescu *et al.*, 2015). Han *et al.* (2013) isolated a bacteriophage from the *Myoviridae* family from a sewage outlet effective against *S. aureus* infections in agricultural cattle. The bacteriophage proved to have wide host range against strains isolated from incidents of clinical disease, including activity against

some methicillin-resistant strains, as determined by inoculating broth cultures with the bacteriophage at a range MOI of 0, 0.01, 1, 100, with host cell number determined by absorbance at 0 to 6 hours. The bacteriophage proved effective at reducing host cell numbers, and demonstrated good potential as a therapeutic agent against infectious disease in cattle. The optimum conditions were determined to be an MOI of 1 with 2 hours incubation at 38°C. This is an important piece of research, as it indicates that bacteriophages with broad host specificity are able to be isolated from the environment and be cultivated on a laboratory scale to demonstrable effect in agricultural animals. Application of this technology could mean a decreased disease load and animal death through infection might allowed for reduced disruption to milk or meat production, and thus to reduce economic burden on farmers through the loss that would be associated with bacterial disease. However, it must be noted that repeat application of bacteriophages could lead to a host immune response. It should also be noted that the co-evolution of bacteria and bacteriophages must be monitored if the effectiveness of the treatment is to be maintained.

The effective delivery of bacteriophages in to animal bodies is an important issue. The bacteriophage must be delivered to the site needed, remain viable during transport and delivery, and to exist in sufficient number to effect the host bacterial population. There are a number of methodological routes by which a bacteriophage culture can be delivered to a live animal (Figure 1). The most common route is oral delivery, where either the food or water is dosed with the viable bacteriophages, sometimes for individual animals, or sometimes by metaphylaxis.

Ma *et al*. (2012) reported a method to increase the survival of bacteriophage K through simulated gastric fluid, for use by oral delivery. Bacteriophage K is active against *Staphylococcus aureus*. The bacteriophage was encapsulated in to alginate microspheres with added calcium carbonate to counter acidic conditions, pH 2.5, and added in to the model gastric environment. Non-encapsulated bacteriophage was entirely deactivated, but the presence of the alginate-calcium carbonate resulted in just 0.17 $\log_{10}$ reduction after two hours. Increased viral survival could be the result of the associated pH increase due to the use of calcium carbonate, as well as the protection afforded by the alginate. This research indicates that the delivery of bacteriophages in to hostile body sites might be possible by adaptation of the delivery vehicle.
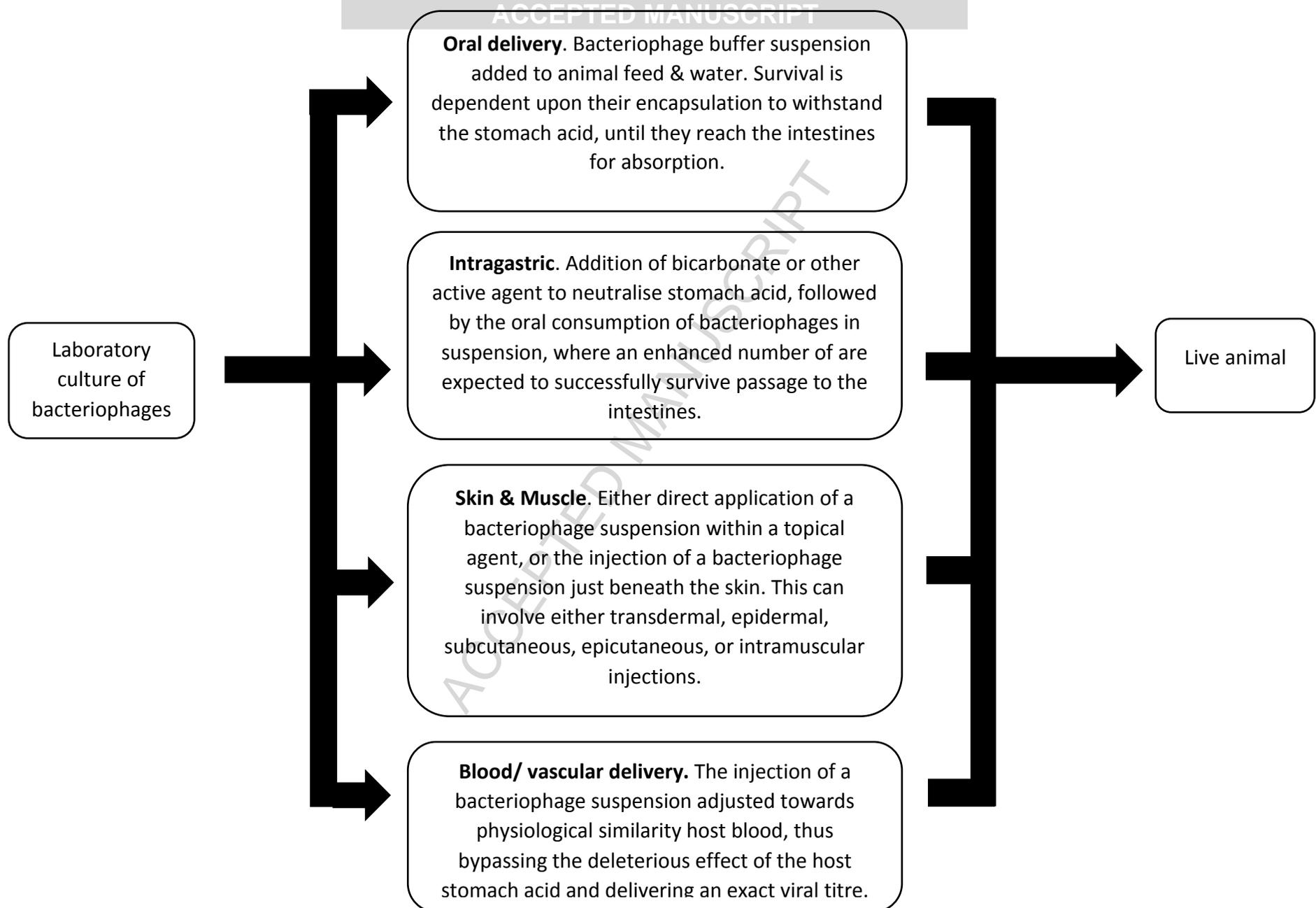
Figure 1. A brief summation of the methodologies for the delivery for bacteriophages to live animals.

In 2014, Wong *et al*. isolated a *Salmonella*-specific bacteriophage, *st*1, from chicken faeces for potential use a biocontrol agent in live chickens. Analysis by Transmission Electron Microscopy (TEM) indicated that the bacteriophage appeared to resemble members of the *Siphoviridae* family, and demonstrated strong lytic activity against *S.* Typhimurium, and some lytic activity against *S.* Hadar. The live animal model used in this study presented the chickens with a challenge of 10^{10} CFU of *S.* Typhimurium, delivered by intracloacal inoculation. An effective 2.9 $\log_{10}$ reduction of bacterial cells was achieved within 6 hours, with no viable *S.* Typhimurium being detected 24 hours post-challenge, determined by post-necropsy swabbing and plating of visceral organs. Interestingly, the authors noted that bacteriophage *st*1 was shown to persist in the caecal wall up to 72 hours post-challenge at a level of 1.6 $\log_{10}$ PFU/ml. This could prove useful for the immediate prevention of bacterial re-infection or colonisation, and also suggests that the presence of the bacteriophage within the chickens is transient within several days, and that the effect would not be permanent. Further research is needed to demonstrate clearance from the animal body, but this could be useful to reassure concerned persons that inoculation with bacteriophages is time-limited, and that the animal will not be permanently host to the virus.

A study by Weiss *et al*. (2009) investigated the replication of T4 and T7 bacteriophages in germ-free mice that had been challenged with *E. coli*. Analysis indicated that the T4 bacteriophage titre increased 300-fold after oral inoculation with the bacteriophage within eight hours. However, the bacteriophage titre was transient, maximal at 8 hours post-inoculation, and decreasing rapidly over the next 48 hours. The T7 bacteriophage demonstrated a sustained, large-scale increase in titre. The authors noted that bacterial cells could potentially migrate within gastro-intestinal mucus layers to afford some degree of protection against the bacteriophage. It could be that the use of a bacteriophage active against a host bacterium is countered by the accessibility of the host bacteria within the animal body. However, of significance is that the T4 and T7 bacteriophages presented different growth profiles, *in vivo*. The US Food and Drug Administration (FDA) has already approved the inclusion of anti-*E. coli* and anti-*Salmonella* bacteriophages within animals prior to slaughter, meaning that bacteriophages could enter the human food chain, as well as disseminating in to the environment.

However, in order for the bacteriophages to do so, they must survive passage through the gastro-intestinal tract. The acid in the stomach acts as an effective defence to inactivate bacteria. Indeed, the use of antacids to neutralise the acid has been shown to effectively increase bacterial survival (Peterson *et al*., 1989). In 2001, Koo *et al*. published a useful study on the survival of *Vibrio vulnificus* and its bacteriophage within simulated gastric conditions. Pyrex beakers were used in combination with pH meters and fluid pumps to simulate gastric flow. Each beaker contained a magnetic stirrer

and were placed within a water bath to maintain temperature of 37°C. The researchers developed a simulated gastric environment using buffer solutions to mimic physiological conditions both chemically, and to physically manipulate the test medium using a pump. The pH was manually adjusted by the addition of hydrochloric acid and sodium hydrogen carbonate. Results indicate that counts of bacteriophage were reduced by only 1 log fold during the first thirty minutes of the experiment. However, none were recovered after forty-five minutes at a pH lower than 3.5. Further experimentation revealed that over nine hours of simulated intestinal conditions, a reduction of below 1.5 log of bacteriophage was observed. Interestingly, the authors note that the bacteriophage was more resistant to the acidic conditions than its host bacterium under the same conditions. These data indicate that bacteriophages can survive passage through the gastrointestinal tract. However, their effectiveness against bacterial species is likely to be dependent on the dose ingested.

3 Meat Products

Bacteriophages have been employed to effectively reduce the viable number of bacterial cells within meat products. Administration routes for raw meat samples and products such as milks are summarised in Figure 2. Key to the procedure is to ensure that the bacteriophages remain viable when applied post-slaughter, and perhaps post-processing. The bacteriophages must remain viable during storage conditions in order to reduce the bacterial load. However, once this has been achieved, the delivery of viable bacteriophages to the human consumer is not necessarily required. Lui *et al*. (2015) demonstrated that the application of a viable cocktail of T5-, T1-, T4-and O1-like bacteriophages to meat samples was effective at reducing the number of viable *E. coli* O157 cells after direct application on to air-dried beef samples. During storage at 4, 22 and 37 °C, the higher temperatures facilitated great lytic activity against the bacterium, presenting a 3.2 $\log_{10}$ CFU/cm^2 reduction in cell number after 144h at 4°C, the same reduction as presented after 6h at 22°C and 3h at 37°C, respectively. The research suggests that bacteriophages are best capable of affecting bacterial cell death at temperatures where bacterial cells will be more metabolically active. This is logical, as bacteriophage reproduction is dependent upon the biochemical processes of the host bacterial cell. It should be noted that at the lower temperature of 4°C, the bacteriophages were still able to induce bacterial cell lysis. However, an increased latency period was observed.

In 2013, Hudson *et al*. reported that the FAHEc1 bacteriophage was able to effectively reduce the number of viable *E. coli* O157 in 4cm^2 cuts of beef, *in vitro*. Briefly, 1.4 x 10^4 CFU of *E. coli* O157 and approximately 10^8 PFU bacteriophage were inoculated on to the meat, and incubated at 1 hour at 37°C. Next, the samples were homogenised in bacteriological broth, with bacterial and

bacteriophage numbers determined by spread plating and double overlay methods, respectively. Addition of the bacteriophages demonstrated almost complete removal of the 1.4 x 10^4 CFU of *E. coli* O157 on all but one test piece of meat, indicating a decrease of 2.5 log compared to the initial inoculum, and 3.1 log compared to control samples. Further, the authors reported that for meat samples prepared as above, but inoculated with 1.3 CFU *E.coli* O157, a significant reduction in host cell numbers was achieved by application of 3.2 x 10^7 PFU within samples chilled from 35 to 5°C over a 16-18 hour time period. This equates to a 2.3 log reduction in bacterial cell number. In support of this research, Moreira Hungaro *et al*. (2013) reported that bacteriophages characterised as belonging to the *Podoviridae* family isolated from chicken faeces that were able to elicit a significant reduction in the number of *Salmonella sp.* on chicken skin, *in vitro*. *Salmonella enteritidis* was inoculated on to sections of skin with sterile spreaders, and allowed them to dry for 25 minutes at 30°C. Next, the samples were dipped in to 100 mL of a five-bacteriophage cocktail for 30 minutes. The chicken skins were then placed in to buffer solution, and underwent stomaching prior to spread plating. The treatment proved effective, with a 1 log CFU/cm^2 reduction in the number of viable bacterial cells within the 30 minute test period. This is an important and easy to reproduce method. It is also a relatively low-cost means to investigate the use of bacteriophages on animal carcasses that could easily translate to further use in industry. The survival of bacteriophages on animal skin has implications for the rapid distributed between individual animals in given populations, especially given the preference for intensive farming practices leading to close confinement of animals, and the exchange of faecal and other biological materials between animals. It might also mean that humans might inadvertently act as vectors for their distribution between individual animals of the same species, for instance, chickens in the same coup, but also between animals of different species, where the farmer has direct contact with segregated animal populations.

These findings are supported by research by Hooton *et al*. (2011), where *Salmonella*-specific bacteriophages isolated from sewage were used to effectively reduce the carriage of *Salmonella* Typhimurium U288 on porcine skin, *in vitro*. After artificial inoculation with host bacteria onto aseptically retrieved sections of porcine skin, application of bacteriophage cocktail was able to reduce carriage by between 4.1 to 4.3 log_{10}. This method applied a three-bacteriophage cocktail at a concentration of 10^8 PFU/mL to section of post-slaughter porcine skin at a concentration of log_{10} 5.4 CFU/cm^2 *S.* Typhimurium U288, distributed using sterile disposable spreaders. The samples were subsequently dried, and then incubated at either 4°C or -20°C, and then subjected to stomaching at 300 rpm for 3 minutes, followed by serial dilution and spread plating on to XLD medium. Bacteriophage suspensions were applied at an MOI range of 0.01 to 10,000, and allowed to dry for 30 minutes. Subsequently, samples were tested for viable bacterial growth compared to

uninoculated controls by serial dilution and spread plating on to XLD medium. Results indicated that when applied at an MOI of ten or above, the addition of bacteriophage cocktail resulted in a 2 $\log_{10}$ reduction over 96 hours in the number of viable *Salmonella* Typhimurium U288 recovered via spread plating. The authors noted that whilst storage of meats at 4°C limits the metabolic activity of the host bacterium and thus bacteriophage replication, the utilisation of a single exposure to the bacteriophage cocktail might limit the development of host resistance. The topics of resistance and coevolution of bacteria and bacteriophages represents a knowledge gap investigating this phenomenon *in vivo.* This could be another important research avenue for zoonotic and bacterial disease encompassing a larger sphere of food technology and healthcare.

Research indicates that bacteriophage infection and reproduction requires the presence of metabolically active host cells. To this end, the refrigeration of foods would appear to allow for bacteriophage infection of host cells, but at sub-optimal rates. Also of critical importance is the ratio of bacteriophage to host cell needed for the reduction of bacterial cell numbers. Studies agree that the use of a higher concentration of bacteriophages is optimal, but it must be noted that the studies published only usually refer to one to two species of bacteria. If such techniques are to be effective, then the use of bacteriophage cocktails is perhaps an avenue for further research, given the potential for foods to contain a range of bacterial pathogens from multiple sources. However, the selection of appropriate bacteriophages would be critical to the success of such an endeavour, requiring a full investigation of their host range. Finally, it is, important to remember that the use of bacteriophages as biocontrol agents might mean that viable bacteriophage enter the human food chain post-cooking/ processing, which presents several consumer health related issues (Abedon *et al*., 2011). This will require an effort to allay fears in the lay community about the consumption of virus-containing products. Table 1 provides examples of commercially available bacteriophage products approved for use within humans and animals.

Research has long established that environmental factors such as temperature are crucial to the survival of bacteriophages in food, and therefore the effect of cooking on bacteriophage survival must be evaluated for each food item. DiGirolamo *et al*. (1972) demonstrated that the temperatures used for cooking experimentally contaminated crabs was insufficient to inactivate the bacteriophages therein. Briefly, the crabs were exposed to the T4 bacteriophage for 48 hours, in seawater at a concentration of 4 x 10^4 PFU/ml of seawater. Subsequently, the crabs were frozen at -20°C until needed. Next, the crabs were allowed to thaw to room temperature, and cooked in five litres of boiling water, with 0.5g sodium chloride added per litre. Samples of crab meat were taken at 0, 5, 10, 15, and 20 minutes. Boiling was relatively effective at inactivating the bacteriophage within the crabs, with only 10% of active bacteriophages recovered after ten minutes of boiling, and 2.5%

after twenty minutes of boiling. These data suggest that whilst cooking might eliminate bacteriophages from meat samples, the duration of the cooking is key to reducing viral load. Processing of foods and the duration and temperature needed for cooking would have to be effectively communicated to the consumer.

Animal or food type	Product (manufacturer/ supplier)	Target bacterial species	Mechanism of delivery	Reference
Live crops	Agriphage (Omnilytics Ltd)	*Xanthomonas campestris* pv. vesicatoria, or *Pseudomonas syringae* pv. tomato	Suspension of species-specific bacteriophages sprayed on to infected crops, either by hand or mechanical device.	www.omnilytics.com/
Meat	ListShield® (Intralytix Ltd)	*Listeria monocytogenes*	Suspension of species-specific bacteriophages added to meat samples, post-slaughter. Incubated at temperatures used for refrigeration of food to reduce bacterial cell numbers.	www.intralytix.com
	EcoShield® (Intralytix Ltd)	*E. coli* O157		
	SalmFresh® (Intralytix Ltd)	*Salmonella* enterica		
	Listex (Micreos, Ltd)	*Listeria monocytogenes*	Suspension of *Listeria-specific* bacteriophages. The manufacturer states that the product can be applied by any of the following methods: spray onto product prior to packaging; spray in to package; spray on to slicer blade/dicer blades; or by dipping/immersion within Listex suspension.	www.listex.eu
Other bacteriophage-containing	New Horizons Diagnostics (US patent	*Streptococcus sp.*	Bacteriophage preparation for incorporation in to chewing gum or toothpaste for the treatment of dental caries.	US patent No. US006399098B1

products licensed for human use	6,399,098 B1)			
Other bacteriophage-containing products licensed for animal use	Delmont Laboratories, USA	*Staphylococcus pseudintermedius*	Contains the products from a bacteriophage-treated bacterial suspension, including: peptidoglycan, bacterial DNA, lioptechoic acid. Product used to treat staphylococcal hypersensitivity, or polymicrobial skin infections of dogs.	www.delmonstlabs.com

Table 1. Examples of commercially available bacteriophage-containing or associated products.
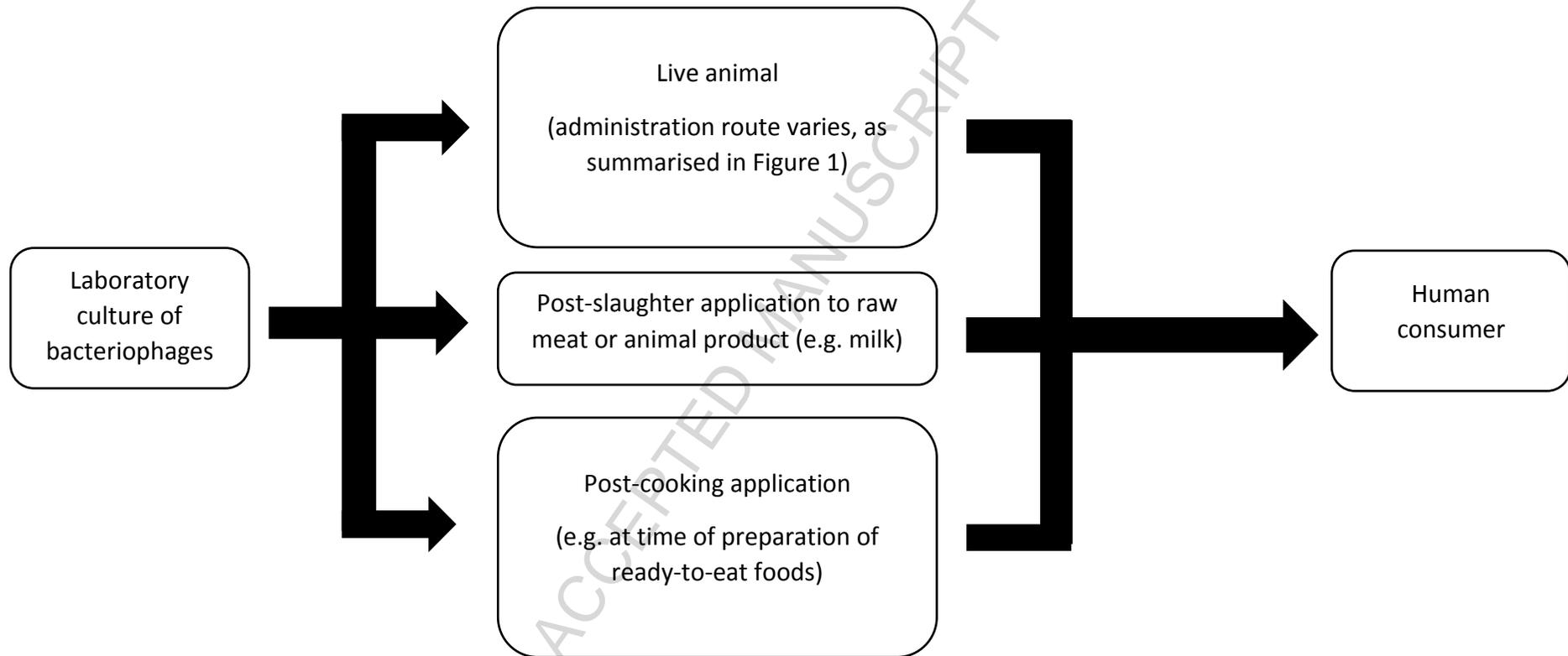
Figure 2. Summary of methods for the inclusion of bacteriophages in to food samples.

4 Milk and Dairy Products

Milk and related products, such as cheeses, might suffer contamination from bacteria associated with the normal flora of the animal, or if the milking equipment is contaminated. Hence, the milk must be processed to reduce the microbial load prior to the point of consumption. Throughout the chain from collection to consumption, there are several key stages at which bacteriophages might be added to the product, as summarised in Figure 3. It must be noted that alongside any added bacteriophages, the presence of viable bacteria suggests that there might be other, naturally occurring bacteriophages in the milk. The effect of any process, such as Pasteurisation, on the presence of a range of bacteria and bacteriophages should be evaluated at each stage of handling and storage prior to consumption.

In the dairy industry, the use of bacteriophages has proved effective at reducing the number of viable bacterial human pathogens in samples destined for human consumption, where the process of Pasteurisation has been shown to directly impact the efficacy of the bacteriophages within the milk. García *et al*. (2009) demonstrated that the a cocktail of two *Staphylococcus aureus* bacteriophages inhibited the bacteria in UHT and pasteurised whole-fat milk, but notes a reduced activity when the bacteriophages are applied in raw or semi-skimmed milk. This indicates that the treatment the milk undergoes must be assessed in order to maintain efficacy of the bacteriophages. Such treatments are highly controlled so as to provide biological safety for consumers, but also to retain nutritional quality and taste. Any proposed adaptations to increase biological safety would have to demonstrate negligible impact on the nutritional status of the product, as well as not detracting from consumer attractiveness at the point of retail.

Thermal inactivation is often used to reduce the number of naturally occurring *Lactococcus* bacteriophages within dairy products. Common thermal treatments include 72°C for 15 seconds and 63°C for 30 minutes for raw milk; whilst in yoghurt production, the parameters range from 80°C for 30 minutes to 95 for 10 minutes (Soukoulis *et al*., 2007). The reason these treatments are applied is to prevent the bacteriophages interfering with the fermentation process of the milk. However, this represents an obvious barrier to the use of bacteriophages as a biocontrol agent within milk or related products. Whilst it has been established that bacteriophages can withstand the Pasteurisation process, despite a significant reduction in viable count, this might suggest that their inclusion represents a viable alternative allowing bacteriophages to enter the food and remain viable post-processing. Madera *et al*. (2004) reported that *Lactococcus lactis* bacteriophage c2 presented no loss of viability when treated for 30 minutes at 60°C in raw milk enhanced cultures, but that after 10 minutes at 70°C no viable bacteriophages were recovered. However, the researchers also

reported that media containing another *L. lactis* bacteriophage 936 still contained viable particles after 30 minute treatments at 75°C. Therefore, the effectiveness of bacteriophages in milk and related products might be dependent upon the post-processing number of viable bacteriophages being able to deliver an effective reduction in bacterial load, and thus affect an increased product shelf life, and thus increasing product safety.
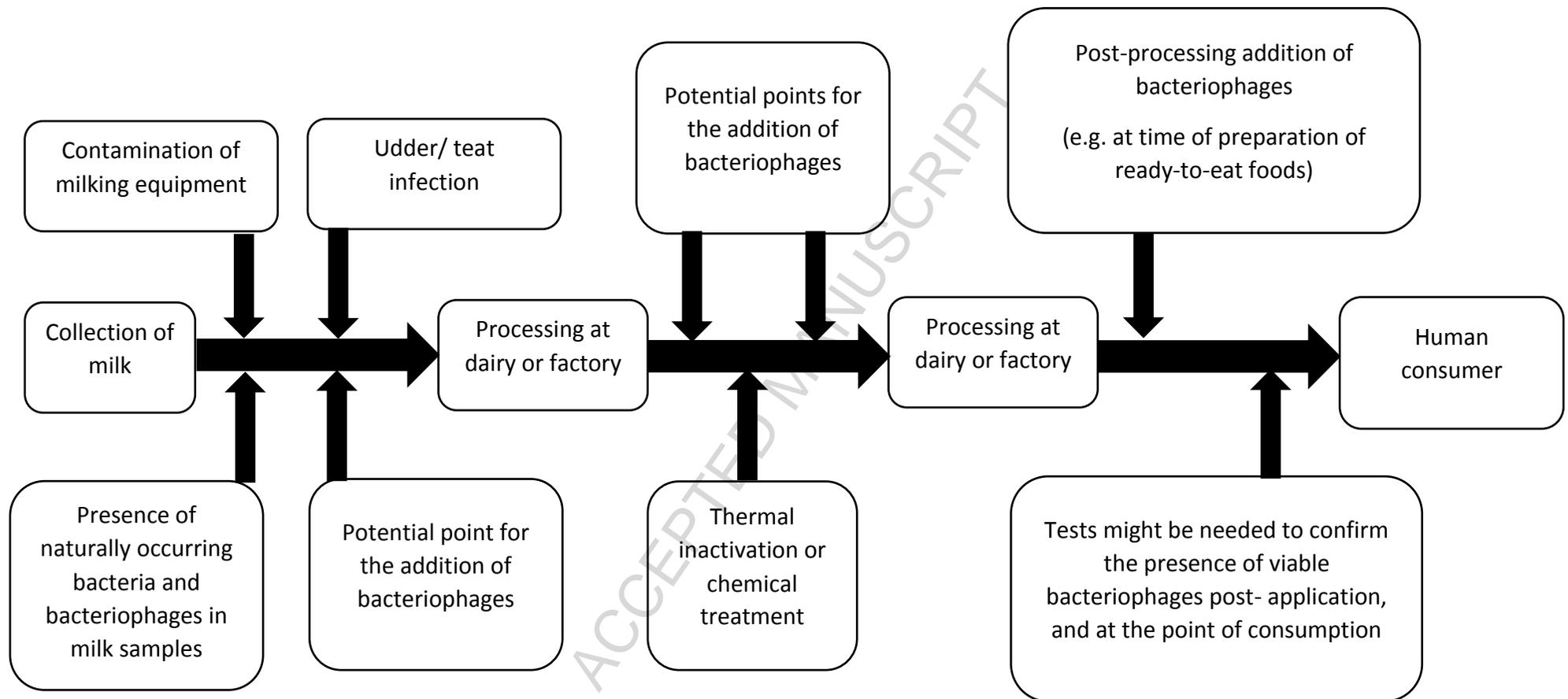
Figure 3. Summary of the critical points associated with the inclusion of bacteriophages within milk and cheese products prior to consumption.

Listeria monocytogenes is another significant bacterial human pathogen associated with cheese and cheese-containing products. *L. monocytogenes* is responsible for 130 average annual infections in the UK (Anon., 2015), and 1600 in the USA, per annum (Scallan *et al.*, 2015). In 2011, Guenther & Loessner reported a novel method for the addition of bacteriophage effective against *L. monocytogenes* to cheeses. Cheeses were inoculated with the host bacterium at a concentration of 1×10^1, 1×10^2 or 1×10^3 CFU/cm^2, and then purified bacteriophages were inoculated in either a single dose of 3×10^8 PFU/cm^2 one hour after the bacterial inoculation; or 1 h and 20 h at two single doses of 3×10^8 PFU/cm^2 each; or after 1 h as a single dose of 3×10^9 PFU/cm^2. The addition of 3×10^8 PFU/cm^2 bacteriophage produced a two-log decrease of *L. monocytogenes* within 6–8 days. The researchers noted that bacterial re-growth was evident when the surface pH of the cheese rose above 6.5, again suggesting that post-application storage is essential for the correct use of bacteriophages in foods. Silva *et al*. (2014) reported a method for the experimental inoculation of commercially available cheeses with *L. monocytogenes*-specific bacteriophages. This method involved the manual homogenisation of cheeses in lambda buffer, then subsequent inoculation of bacteriophage P100 and the host bacterium, followed by air drying in a microbiology air hood. The number of viable host bacteria was determined by spread plating and incubation for 48 hours at 35°C. Results indicated a significant decrease in bacterial cell number during incubation at 10°C for seven days, by a factor of 1 log in Minas Frescal and 0.8 log in Coalho cheeses, respectively. However, the authors noted that the number of *L. monocytogenes* cells is an important factor in subsequent assays, and that a high initial concentration of bacteriophages per host cell is required in order to maintain a sustained effect. This point underlines the importance of delivering a high enough initial inoculum of viable bacteriophages to deliver an initial effect, as well as the potential to maintain bacterial numbers below the threshold to cause infection or depreciate food qualities, such as taste and consumer appeal. This method, whilst perhaps not appropriate for large scale industry, does indicate that bacteriophages can indeed survive and reduce host bacterial cell numbers in commercially available cheeses.

The effective application of viable bacteriophage to post-processed (i.e. ready to eat, RTE) foods is supported by the research of Guenther *et al*. (2009). Cultures of *L. monocytogenes* and bacteriophages P100 and A511 were applied to chocolate milk drink and cheese brine, and facilitated a decrease in host bacterial number to below the 1 CFU/ mL limit of detection. Similarly, application of *L. monocytogenes* and bacteriophages P100 & A511 to solid foods such as hot dogs, turkey and salmon resulted in a reduction in bacterial count up to 5-log within 24h at 30°C. Interestingly, the authors noted that storage of bacteriophages in samples of animal origin at 6°C for 6 days resulted in the maintenance of infective bacteriophage particles, whereas the presence of

plant materials reduced bacteriophage numbers by greater than 1-log. Again, the authors also noted that the application of a higher initial titre of bacteriophages led to a greater effect than the use of lower doses. This reinforces the need for an effective evaluation of the needed MOI for a specific bacterial pathogen, in relation to what is achievable in the food product, as well as the potential inclusion of other bacteriophages active against other pathogens, and the associated interactions that might occur within.

Alongside the presence of nutrients and bacterial cells, the potential for additives to interfere with bacteriophage activity is worth researching. Tomat *et al*. (2016) published a method for investigating the impact of acetate and lactate on the activity of *E. coli*-specific coliphages in milk. The bacteriophages were entirely deactivated by 4% acetate and lactate sodium salts in pH-unadjusted milk at 25°C after just one minute of incubation. However, when the pH was adjusted to 4.5 for lactate and 5.0 for acetic acid, respectively, the bacteriophage activity remained unchanged. Further research indicated that exposure to 0.25 mg/ml nisin after 24h affected no change in the activity of the bacteriophages. This suggests that modification of the chemistry of the food product might affect the sustained efficacy of any bacteriophages present. This is one potential option to optimise bacteriophage delivery, but might affect taste and nutrients within the sample. Data will be needed in order to fully evaluate how this might work. But, the resistance of coliphages to nisin, lactate and acetate is suggestive of resilience to chemicals currently used in food production, and could indicate that the composition of food preparations in order to provide effective delivery of viable bacteriophages.

In summary, these collective works demonstrate that the critical factors for optimising bacteriophage efficacy, and thus for reducing the risk of bacterial infection, include a high enough bacteriophage titre post-processing, and a temperature facilitating effective bacterial metabolism and thus viral replication. These factors must be thoroughly investigated for the development of bacteriophages in RTE foods.

5 Food Packaging

A new development in the use of bacteriophages as biocontrol agents in foods is the incorporation of viable bacteriophages in to absorbent pads which can be placed directly on to foods. These pads allow for the diffusion of bacteriophages in to the substrate, and could offer a novel means of keeping foods fresh for longer, with obvious advantages to both retailers and consumers. Meireles Gouvêa *et al*. (2015) successfully isolated bacteriophages active against *Salmonella typhimurium*

from chicken and pig excrement and exudates, and inoculated the bacteriophages in to an absorbent pad, which was placed on to the top of agar plates inoculated with a susceptible host bacterium. Concentrations of 10^6, 10^8 and 10^9 bacteriophages were used, and plates were investigated for the presence or absence of bacterial growth at 6, 12, 24, 36 and 48 hours. Analysis revealed that at 10°C, bacteriophage activity was less efficient than at 37°C. This again supports the point that the metabolic activity of the host is crucial in order for bacteriophage adsorption on to the host cell surface. Refrigeration of foods might be effective at controlling bacterial growth, but prove to be a limiting factor for bacteriophage activity. Lone *et al*. (2016) successfully developed a protocol for the biocontrol of *L. monocytogenes* and *E. coli* in ready to eat foods. Phage cocktails were applied by either the use of either immobilised phages on to positively charged cellulose membranes, encapsulating phages within alginate beads, or direct application on to paper. When directly applied to cantaloupe, after 24 hours at 4°C and 12°C, a 2-log reduction in *L. monocytogenes* counts was observed after 5 days of storage. Treatment with immobilised bacteriophage on cellulose membranes led to a 1-log reduction in bacterial cell numbers. Further, for *E. coli* cultured on alfalfa seeds, a 1-log reduction in bacterial numbers was observed within 1 hour after application, and a 1-log reduction was also observed in germinated sprouts after 5 days.

In a new direction, in 2014, Chai *et al*. (2014) published data from a technique where glass coupons were coated with bacteriophage exopolymerase enzyme as a means of reducing the health risk from the build-up of bacterial biofilms. The authors reported that thermal stability of the bacteriophage enzyme preparation decreased to minimal amounts when treated at 75°C for 10 minutes. After 4 hours treatment with the enzyme preparation, 80% of the biofilm bacteria were reported to be eliminated, which increased to 92% following an additional 30 min treatment with chlorine dioxide. The authors noted that the use of the depolymerase enzyme could reduce the number of planktonic bacteria in liquid media, as well as in the biofilm mode of growth. This might thus perhaps increase dissemination throughout the enzyme inside the glass vessel. The enzyme was determined to cleave sugar residues from the exopolymeric matrix of *Klebsiella aerogenes*, as confirmed by HPLC analysis. This might suggest that enzyme and bacteriophage activity could be measured by detecting increasing sugar residue in solution. Further, the activity of the enzyme against sugar residues might suggest that the spectrum of cleavage activity might be extended to the capsule layer surrounding these Gram negative cells.

Korehei & Kadla (2014) reported a method for the entrapment of T4 bacteriophages on electrospun poly(ethylene oxide) (PEO) fibres for use in food packaging. The T4 bacteriophages, active against *E. coli*, were able to effectively deliver a sustained release of bacteriophage in to buffer medium, as well as delivering a substantial initial release of bacteriophage particles. Transmission Electron

Microscopy (TEM) data reveal that bacteriophages become distributed randomly on the fibres, as well as deeper within the core of the fibres. TEM of fibres from different stages of the dissolution process reveal that the randomly distributed bacteriophages become detached rapidly, whilst bacteriophages associated deeper within the fibres become dissociated in to buffer more slowly. Analysis revealed that for the T4 bacteriophage, during 30 minutes of immersion of the PEO fibres in to aqueous buffer, 100% of particles were released. The authors speculate that this is likely to be due to the hydrophobic nature of PEO fibres dissolution in aqueous medium. Further experimentation indicated that bacteriophage can be modulated by altering the thickness of the fibres, where a change of 100k to 600k molecular weight. This resulted in a 1 log reduction of bacteriophage release during a test period of 10 minutes. These data support the observations of high initial followed by slow sustained bacteriophage release. The authors note that the molecular weight and composition of fibres is crucial to the release of bacteriophage, where increased fibre diameter and weight results in decreased bacteriophage release. Further, the authors report that the combination of PEO fibres with cellulose diacetate fibres also decreased release rate. These findings indicate that the incorporation of bacteriophages within materials used for food packaging is possible, but that the precise nature of the materials used will greatly affect bacteriophage release. This must be balanced with the effect of different packaging on food quality and storage, where no depreciation in either quality is observed.

Combining bacteriophage activity with luminescence has also drawn attention in terms of its rapid detection for biological agents. Schofield *et al*. (2009) developed a reporter assay, where the presence of *Yersinia pestis* was determined through the evolution of a bioluminescent signal. Maximised detection of *Y. pestis* was achieved through a positive bioluminescent signal within sixty minutes for 1×10^2 cells after incubation at 28°C in the presence of serum. This is an important piece of research, as it indicates that, firstly, the presence of complex biomolecules in serum does not interfere with the infection cycle of the bacteriophages; and, secondly, that a bioluminescent signal can be observed rapidly. This is crucial for the safe consumption of food, and could become important for retailers and consumers alike, if it can be developed in to an easy to read technology able to be used at multiple points in the food chain, and to prevent retail of unsafe products. The authors also note how crucial temperature is in this type of bioassay. From the perspective of the bacteriophage, there must be metabolically active cells present. Hence, the sensitivity of such as assay must be determined in relation to an achievable signal within a given timeframe, accounting for host cell number and activity.

These data suggest that bacteriophages retain their viability when incorporated in to packaging materials to provide a significant antibacterial effect against important bacterial human pathogens.

The use of bacteriophages within packaging materials could well develop further, in a similar way to which the incorporation of antibacterial materials has developed in to packaging in recent times. Their potential to indicate food quality and thus consumer safety by sight could be a significant step towards reassuring people about the safety of these products. A summary of the research presented on food packaging in this review, along with potential advantages and disadvantages is presented in Figure 4.
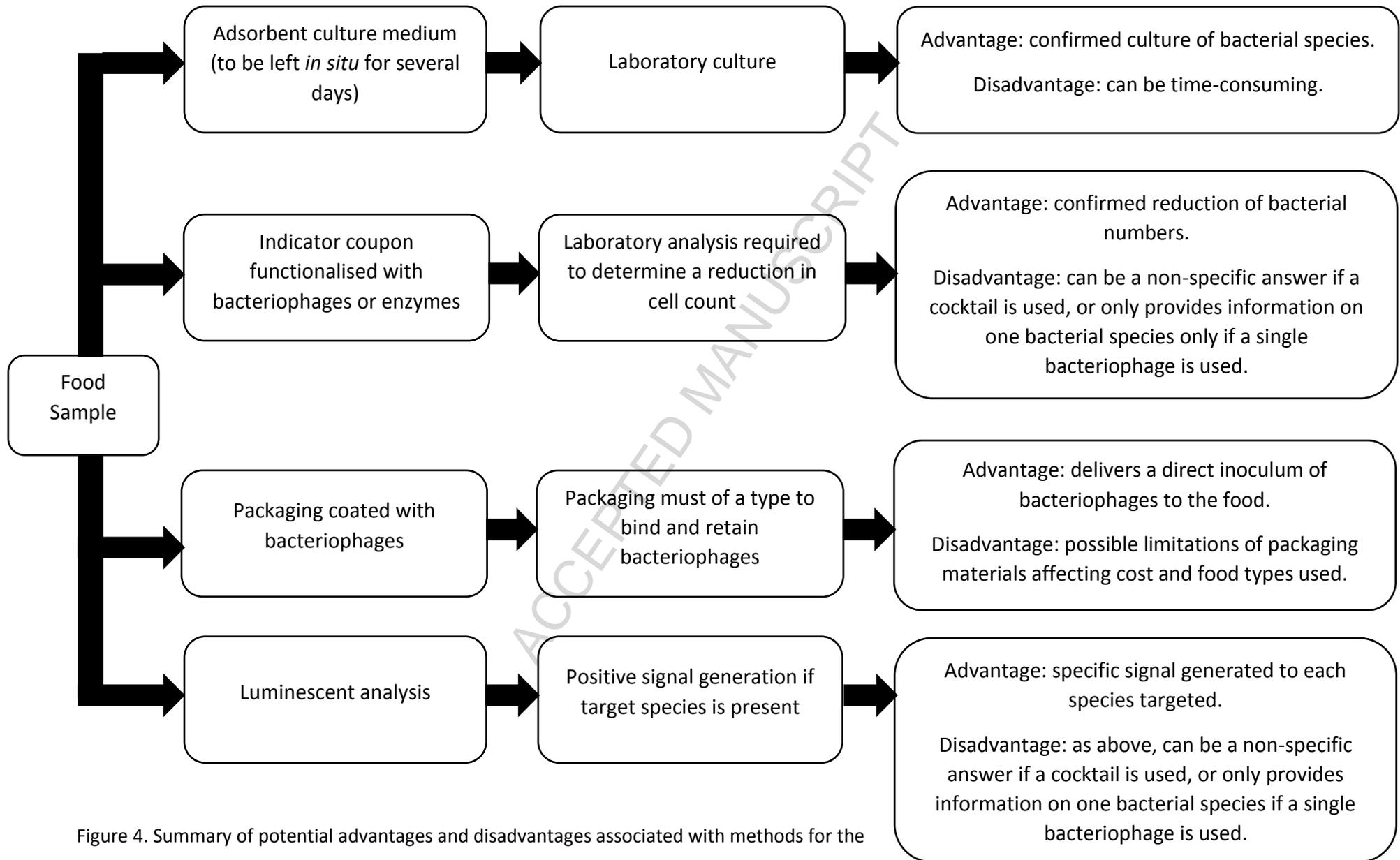
Figure 4. Summary of potential advantages and disadvantages associated with methods for the reduction of food-borne bacterial pathogens using packaging modified with bacteriophages.

6 Aquaculture

In aquaculture, the risk of infection spreading throughout a stock animal population is high due to the use of communal water tanks, for instance. Typical infectious agents of fish species include *Aeromonas hydrophila* (Austin & Austin, 1993; Cason *et al*., 2000) and *Vibrio cyclitrophicus* (Deng *et al*., 2008), both noted to cause significant infection and associated economic loss to farmers. *Vibrio cyclitrophicus* is a significant pathogen of *Apostichopus japonicas*, the sea cucumber, causing skin ulceration in the animal (Deng *et al*., 2008). In 2016, Li *et al*. published a method for the biocontrol of *V. cyclitrophicus* using a bacteriophage designated as phage vB_VcyS_Vc1 to effectively reduce bacterial cell numbers. The bacteriophage was identified as a member of the *Siphoviridae* family by morphological analysis, and the method involved introducing the bacteriophage in to the test system as a freeze-dried skimmed milk powder within the feedstuff. Research revealed that supplying viable bacteriophage particles in this manner effectively increased the number of viable sea cucumbers to 81% (from 18%), or to 63% when the animals were suspended in a phage-containing suspension, and 58% when the animals were physically injected with purified bacteriophages at an MOI of 10. During the freeze-drying, the bacteriophage titre decreased by approximately 2 log.

Vibrio parahaemolyticus is another important human pathogen that is associated with raw seafood. Jun *et al*. (2014) evaluated the effectiveness of bacteriophage pVp-1, a member of the *Siphoviridae* family, against the multi-drug resistant *V. parahaemolyticus* strain CRS 09-17. Tanks holding 35L water were challenged with *V. parahaemolyticus* for 24 hours, and then drained, with new water being added. Subsequently, free-living oysters were added and challenged with either sterile saline water or with pVp-1 suspension at a concentration of 1.6×10^7 PFU/ml. Randomly selected oysters were collected at 0, 0.5, 1, 2, 3, 6, 12, 24, 36, 48, and 72 h, whilst leaving all other oysters *in situ*. The animals were homogenised in alkaline peptone water with added sodium chloride, with subsequent plating on to CHROMagar™ to determine bacterial counts, and use of the double-layer agar method (Adams, 1959) to determine the titre of bacteriophage present. This approach proved effective in monitoring a post-treatment decrease in recoverable bacterial cells by plating. Bacterial counts decreased from 6.6×10^3 to 1.9 CFU/g in the presence of bacteriophage. At the same time, an increase of bacteriophage titre of 3.3×10^5 to 1.2×10^7 PFU/g was observed at 6 hours, decreasing to 1.7×10^6 PFU/g after 12 hours. These data suggest that the bacteriophage are able to persist within the tanks for up 12 hours, and to elicit an effective antibacterial response within this time.

This research is important because it suggests that the bacteriophage can persist within the water tanks post drainage to affect the bacteria present in association with oysters in subsequent waters. The potential carry-over for bacteriophages demonstrated in these selected studies has implications for the environmental spread of the virus through drainage of aquaculture tanks, as well as suggesting that a thorough cleaning regime would be required to demonstrate their removal from any such system prior to the introduction of other animals.

7 Bacteriophage enzymes

A promising alternative approach to the use of viable bacteriophages in foods is the addition of preparations containing highly purified suspensions of bacteriophage enzymes, known as endolases. These enzymes are peptidoglycan hydrolases, where their function is to degrade the peptidoglycan layer of bacterial cell membranes leading to rupture of the bacterial cell, and the subsequent release of viral progeny in to the surrounding environment.

The distinction must here be made between the mode of action of bacteriophages active against Gram positive and Gram negative cells. Gram positive cells present an externally-facing layer of peptidoglycan, which is easily accessible to bacteriophages expressing endolysins on the capsid. However, Gram negative cells present an outer cell membrane external to the peptidoglycan. This means that whilst Gram negative endolases must access the peptidoglycan from within the cell cytoplasm, the Gram positive enzymes might present potential for activity against cells from external sources, facilitating lysis from without, and perhaps able to be delivered in a similar manner to antibiotics. It should be noted that whilst the outer membrane of Gram negative cells is an effective barrier to externally delivered bacteriophage enzymes, research has indicated that Gram negative membranes damaged by detergent or other action, allows for entry of the enzymes to facilitate peptidoglycan degradation (Loessner, 2005).

The SPN1S bacteriophage, which infects *Salmonella sp.*, produces endolysins possessing a superfamily domain similar to that of lysozyme, but presenting a much stronger activity than that eukaryotic enzyme (Lim *et al*., 2012). *In vitro* research has demonstrated that the inclusion of EDTA in preparations with the endolase leads to increased permeability of the outer membrane, and increased enzyme activity and *E. coli* cell lysis. Lim *et al*. (2012) demonstrated that the enzyme preparation was effective against Gram negative, but not Gram positive species, perhaps suggesting a limited spectrum of activity to a particular type of host cell wall. This might indicate the need for preparations containing endolysins active against more than one species are required for effective

food safety. Optimum enzyme activity was reported at pH 9.5 and 37°C. This is important, as it must be remembered that bacteriophages are intracellular parasites of bacteria, and hence an optimum temperature of 37°C could be an advantage. Also, during the assembly of bacteriophage particles within a host cell will significantly alter its internal pH and other associated conditions. Therefore, enzymes with a substantial activity range would present an advantage for their inclusion as biocontrol agents.

Research by Schmelcher *et al*. (2012) revealed that bacteriophage endolysin activity can be modulated by the concentration of divalent cations within the test medium. It was reported that optimum endolysin activity was achieved at a concentration of between 3 to 10 mM Ca^{2+}. However, it must also be noted that they reported that the concentration of Mg^{2+} had a negligible effect on enzyme activity, whilst the presence of Mn^{2+} ions demonstrated an inhibitory effect, *in vitro* (Schmelcher *et al*., 2012). These data suggest that the chemical composition of food must be evaluated, as the activity of the endolysins, and hence the efficacy of the bacteriophages themselves could be adversely affected if not taken in to consideration. This is also a consideration for the use of additives in foods, where the presence of additional compounds intended to improve shelf life or palatability of the foods might in fact deliver a detrimental effect on the antibacterial nature of the bacteriophages.

The prospect of including bacteriophage enzymes rather than the entire viral particle might be a viable alternative acting to reassure the public that about safety concerns associated with the consumption of bacteriophage-containing material. Research by Park *et al*. (2011) indicates that the enzymes are effective against stationary phase bacterial cells, whilst entire bacteriophage particles only demonstrate high efficacy against metabolically active, replicating cells. This could represent a viable strategy for the maintenance of biological safety of foods during conditions that restrict the activity of the bacteriophages themselves. Specific examples would be storage temperature, moisture content, and pH of the food.

8 Examples of commercially available products containing bacteriophages

Listex® is a commercially available preparation containing bacteriophages active against *L. monocytogenes*, manufactured by Micreos, based in the Netherlands. Chibeu *et al*. (2013) evaluated the potential for Listex® to reduce the number of viable *L. monocytogenes* associate with samples of cooked turkey and beef. Overall, the research indicated that Listex® was capable of reducing the microbial load when used in combination with antimicrobials, which the authors suggest might be a

potentially useful adjunct for the increased safety of RTE foods. When applied singly, Listex®
provided a 2-log reduction in the number of viable *L. monocytogenes* cells at 4°C over a 28 day
period. However, when used in combination with antimicrobials, the reduction increased to 4.5 log
at 4°C, and up to 7.5 log at 10°C. Interestingly, the number of viable bacteriophage particles were
noted to remain approximately constant at $1x10^7$ to $1x10^8$ PFU/ cm^2, which is comparable to the
$1x10^7$ to $1x10^8$ PFU/ cm^2 initially applied to the meat samples. These findings suggest that active
bacteriophage replication within the host cells can maintain the overall PFU load during cold
temperature storage. Research by Oliveira *et al*. (2014) also supports the potential for *L.
monocytogenes* bacteriophages to be included within food samples. The researchers tested the
ability of bacteriophages to reduce the bacterial load on pear and melon slices during storage at
10°C. They determined that the application of Listex® resulted in a reduction of 1.5 and 1.0 log
CFU/mL of bacterial cells on melon and pear slices, respectively. Further, their research
demonstrated that a reduction of 8 and 2.1 log cell reductions were obtained from the respective
fruit juices, after storage at 10°C over eight days.

Baños *et al*. (2016) investigated the potential for bacteriophage P100 (Listex®) to reduce *L.
monocytogenes* numbers in fish samples. Overall, results indicate a decrease of bacterial load during
1 to 15 days post-application at 4°C. But, after this time period, the number of bacterial cells
increased. This might suggest that the complex bacteria-bacteriophage dynamics are dependent
upon correct storage and handling protocols being adhered to, outside of which bacterial regrowth
is a distinct possibility. Regrowth would have obvious adverse implications for retailers and
consumers alike. Baños *et al*. compared the effectiveness of P100 to another laboratory
bacteriophage strain, AS-48, applied both singly and in a dual cocktail by direct application on to the
meat. On raw hake, single application of AS-48 decreased *L. monocytogenes* by 1.68 - 3.13 log
CFU/cm^2, and on salmon between 1.9 - 2.8 log, as determined at 1 to 7 days incubation. In contrast,
application of P100 production reductions of *L. monocytogenes,* but in all cases, lower reductions
than AS-48 under the same time and incubation conditions. The dual-cocktail approach entirely
removed viable host cells from all hake samples within two days and within one day incubation,
within the threshold of the tests.

EcoShield® is manufactured by Intralytix Ltd, in the United States of America, and it is composed of
bacteriophages active against *E. coli* O157:H7. Research by Boyacioglu *et al*. (2013) demonstrated
that when applied to spinach leaves contaminated with the target bacterium over a thirty minute
period, the product was able to effect a reduction of 2.38 and 2.49 log CFU during storage at 4 and
10°C, respectively. Further research indicated that over seven days storage at 4 and 10 °C, this lower
level of viable bacterial cell number was maintained. The authors observed that differences

observed in cell reductions reported by different research groups could be due to the use of different bacterial strains during laboratory testing. This indicates that the inclusion of a bacteriophage or a multiple bacteriophage cocktail might only be effective if the target bacterium is present, leading to the potential for culture or other technique to be employed in order to determine if the bacteriophages have sufficiently broad host range to counter the pathogens likely to be present in the food samples. Carter *et al*. (2012) investigated the potential for EcoShield® to reduce the number of *E. coli* O157:H7 on lettuce and beef samples. Application of the bacteriophage preparation resulted in no significant increase in bacterial load during up to seven days storage at 4°C. The researchers also investigated the potential for recontamination of the samples to lead to an increased bacterial load, despite the presence of the previously applied bacteriophages. The authors reported that no statistically significant increase in *E. coli* O157:H7 number occurred during storage at 10°C for 24h, suggesting that a previously applied load of bacteriophages might be effective to reduce future contamination events leading to an increased health risk to consumers.

9 Limitations of assays

The inclusion of bacteriophages within food or related products requires further research to overcome problems associated with their use. Primarily, their use as an antibacterial agent must be demonstrated to be effective in the final product, without post-treatment impairment. Research shows that after the application of the bacteriophages, significant decreases in host cell and bacteriophage titre can be evidenced within food samples. Analysis has revealed that crucial factors for the maintenance of viable bacteriophages include the correct storage temperature, pH, and duration of storage as example conditions, as well as any subsequent treatments to the food. Research must also be undertaken to ensure that any additives or even antibiotics added to the products do not inhibit the efficacy of the bacteriophages against their target species. It has been shown that treatments currently applied to foods, such as Pasteurisation, can reduce the titre of viable bacteriophages within the sample, thus raising doubts as to their ability to deliver an antibacterial effect. Research has clearly demonstrated that bacteriophages require both metabolically active host cells, and correct ambient pH and temperature in order to maintain an effective bacteriophage titre. The titre has been shown an important factor for effective bacteriophage delivery, where a high initial concentration is needed in order to account for degradation during processing, storage, and delivery to the consumer. This requires research in to bacteriophage survival from food chain, as well as within the cold chain used by the food industry, post collection of milk or post-slaughter.

It must also be noted that the majority of research to date has been conducted in the laboratory on already processed meats or food products. At present, information on the dissemination of bacteriophages within a human or animal body represents a barrier to their use in foods. More information on the interactions occurring within the body, e.g. with intestinal mucus layers, is a clear limitation for their use. Such work would also act to determine the environmental fate of bacteriophages introduced in to animal populations on farms. Subsequently, if the effective absorption rate from the gastro-intestinal tract and dissemination within the body can be realised, interactions within the body tissues and the environment can be contextualised within the larger sphere of interactions. Finally, there is relatively little known about the coevolution of bacteria and bacteriophages in live tissues. This is an important limitation for the use of bacteriophages in humans and animals. While studies have looked at this phenomenon in the laboratory, as it is poorly understood how the relationship evolves inside animal bodies, and needs to be investigated further. Finally, the effect of endolysin preparations on animal and human cells must be evaluated in terms of cell viability, and on their ability to effect the growth of these cells. Ideally, the enzymatic preparations should be constituted so as not to adversely affect cell viability, tissue development, or alter substances such as mucus layers within the animal body.

10 Conclusions & Future Directions

In order for bacteriophages to become as commonplace within food as lactobacilli are in health drinks, the public perception must be challenged by more in-depth *in vivo* studies. At present, there are relatively few studies on the consequences of the long-term consumption of products containing bacteriophages by humans and the food animals themselves. This research will be necessary in order to promote any potentially beneficial results from their consumption. Further, detail on their passage through the human and animal gastro-intestinal tract will be needed to ascertain their longevity within the host, as well as their rate of absorption in to the blood. In conjunction with these points, the fate of bacteriophages excreted by humans in to the sewage and by animals directly in to environmental matrices such as soil and water, will be of pivotal importance in the safe introduction of bacteriophages in to human food or medicine. The environmental fate of bacteriophages must be fully determined, as the introduction of viable viruses in to the natural ecosystems is a potential cause for concern. Bacteriophages must be proved to be safe to the environment, the animal, and the human at both the point of consumption, and post-consumption.

The use of purified enzymatic preparations could represent a viable alternative to the inclusion of intact bacteriophage particles, as they might be more acceptable to members of the public. The term "good bacteria" has been effectively utilised to great success in the promotion of yoghurt-based health drinks. A significant amount of work remains in order for bacteriophages or their products to become as acceptable on a large-enough scale to both members of the medical community and the public.

Artliysins are bacteriophage lysin enzymes that have been structurally modified in the laboratory. To date, research suggests that they might represent a novel means to control bacterial cell numbers, by adapting existing bacteriophage-bacteria interactions. Yang *et al*. (2015) report the effectiveness of a modified lysin, PlyA, against *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. Stationary phase cells suspended in 20 mM Tris-HCl (pH 7.4), and added to enzyme preparations, with or without EDTA and citric acid, which would act as membrane permeabilisers, and incubated for 15 – 60 min at 37°C. This artilysin proved effective at reducing the number of bacterial cells in buffer solution when target cells were in the logarithmic phase of growth, suggesting that the biochemical status of the cell is crucial to delivering the antibacterial effect. An enhanced bactericidal effect was observed when *A. baumannii* was cultured with EDTA, producing a decrease of between 2.3 - 3.2 logs. For *P. aeruginosa*, culture in the presence of EDTA increased enzyme activity against stationary phase cells, producing a reduction of 4.4 log. Culture with citric acid indicated that a dose-dependent reaction occurred. The authors suggest that factors other than the pH change afforded by the citric acid must relate to enzyme activity. Supporting this work, Briers *et al*. (2014) report that artlilysins are capable of delivering a rapid antibacterial effect against *P. aeruginosa*, *in vitro*. The authors report that these compounds can affect the viability of cells by between 4 -5 log units in the presence of EDTA, *in vitro*. The authors also report that through the use of protein engineering techniques, it is possible to engineer artilysins which are capable of passing through the membranes of *A. baumannii* and *P. aeruginosa*, to deliver a toxic effect from within the cell.

This could represent a new avenue of antimicrobial research based on bacteriophages. If artilysins can be determined to be effective against bacterial human pathogens, as well as presenting minimal complications in the human and animal bodies, then these novel compounds might offer a means to enhance food safety without the inclusion of entire bacteriophage particles. This in itself might affect consumer perception, as the presence of intact virus particles is a notable barrier to the inclusion of bacteriophages in foods at present.

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