Chapter

Potential Roles for Bacteriophages in Reducing *Salmonella* from Poultry and Swine

Anisha M. Thanki, Steve Hooton, Adriano M. Gigante, Robert J. Atterbury and Martha R.J. Clokie

Abstract

This chapter discusses application of natural parasites of bacteria, bacteriophages (phages), as a promising biological control for *Salmonella* in poultry and swine. Many studies have shown phages can be applied at different points from farm-to-fork, from pre to post slaughter, to control the spread of *Salmonella* in the food chain. Pre-slaughter applications include administering phages via oral gavage, in drinking water and in feed. Post slaughter applications include adding phages to carcasses and during packaging of meat products. The research discussed in this chapter demonstrate a set of promising data that relate to the ability of phages to reduce *Salmonella* colonisation and abundance. Collectively the studies support the viability of phage as antimicrobial prophylactics and therapeutics to prevent and control *Salmonella* in the food chain.

Keywords: Bacteriophages, phages, swine, poultry, delivery

1. Introduction

The global problem of antimicrobial resistance (AMR) is driving the search for novel treatments to control multidrug-resistant (MDR) pathogenic bacteria. Infections caused by MDR pathogens impose a significant burden on healthcare systems and economic productivity and are a major cause of mortality. Globally, AMR is associated with 700,000 deaths annually, with the prospect of this reaching 10,000,000 by 2050 if no resolution is found [1].

A One Health approach, that considers the intrinsic associations between antibiotic use in livestock and agriculture, the emergence of MDR pathogens, and the societal impact of AMR in developed and developing nations is required [2, 3]. However, integrating these approaches is challenging as antibiotic use in agriculture is generally widespread [4, 5]. For example, prophylactic administration of antibiotics to pigs during the weaning process is a standard technique employed in many countries [6]. Over recent years, efforts to limit antibiotic use other than specifically to control active bacterial infections have been implemented. Consequently, the use of antibiotics as growth promoters in food

production animals was banned in the European Union (EU) in 2006 and in the United States of America (USA) in 2017 [7].

Gram negative *Enterobacteriaceae* are an important component of human, animal, and environmental microbiomes and can be associated with both health and disease. While the family contains several notorious pathogens (e.g. certain *E. coli*, *Klebsiella* spp., *Shigella* spp. *etc*.), the genus *Salmonella* presents a problem for AMR due to its ubiquitous distribution in food production environments and MDR phenotypes [8]. Worryingly, clinically important antibiotics are becoming ineffective, including colistin, which is a human critical antibiotic [9]. As such, alternative strategies to control/eliminate MDR *Salmonella* that may replace or complement antibiotics are needed.

Globally, dominant *Salmonella* serovars display a distribution pattern in pigs and poultry reflective of each industry. In pigs, *S*. Typhimurium (e.g. U288, U302, DT193, DT104), monophasic 4,[5],12:i:- and other variants such as 4,12:i:- are the dominant strains at both farm and slaughterhouse facilities in the UK and EU [10–13]. Other serovars such as *S*. Derby, *S*. Enteritidis, *S*. Bovismorbificans, *S*. Kedougou, *S*. Rissen, and *S*. Brandenburg are also reported [13, 14]. In the USA and China the dominant *Salmonella* serovars include *S*. Typhimurium, monophasic 4,[5],12:i:- *S*. Infantis, and *S*. Brandenburg [15].

For poultry, and in parallel with the global emergence of strains such as 4,[5],12:i:- the most prevalent serovar in UK production facilities is an *S*. Typhimurium derivative 13,23:i:- that accounted for almost a quarter of all isolations in 2019 [13]. Across the EU, the USA and China monophasic strains continue to expand throughout poultry production facilities. Other serovars such as *S*. Enteritidis, *S*. Berta, *S*. Typhimurium, *S*. Infantis, *S*. Hadar, *S*. Kentucky, and *S*. Heidelberg have all been isolated and/or linked to outbreaks [16–18]. The global diversity of *Salmonella* spp. within pig and poultry production constitutes a significant source of disease for humans and animals alike.

Controlling *Salmonella* requires intervention strategies capable of implementation at the national/international level. One such strategy is the targeted application of natural bacterial predators, bacteriophages (phages). Over the last decade, a robust body of evidence has demonstrated that phages can be applied at various points from farm-to-fork for pathogen control [19, 20]. Phage application could be implemented at the stage of rearing [21, 22], slaughter and processing [23], or at pre-retail/packaging [24, 25].

2. Phages

Phages are viruses that specifically infect and kill bacteria and with few reported side-effects in humans and animals. Phages are the most abundant biological entity on Earth, with estimated numbers ten times greater than bacterial cells [26]. Phages were independently discovered by Frederick Twort and Felix d'Herelle in 1915 and 1917 respectively. D'Herelle was the first to test phage efficacy in animals and showed phage treatment increased the survival of chickens suffering from fowl typhoid by 95–100% compared with 0–25% in untreated birds [27]. Despite this, phage therapy research slowed markedly following the discovery of antibiotics. However, research into phage therapy has been renewed since the emergence of AMR as it offers a promising alternative to antibiotics. Studies have shown phages are able to lyse MDR strains [28, 29] and there are multiple examples of successful phage therapy in humans [30] and animals [31]. Furthermore, phages can be applied to food to reduce bacterial loads and globally are being used commercially to improve food safety [32].

2.1 Phage morphology and infection cycle

Phages are characterised based on their virion morphology, genome type and sequence, and the infection cycle they follow. Phages are approximately a hundred times smaller than bacterial cells by volume, and generally only infect a subset of strains within a host species. Over 5,000 phages have been viewed under the transmission electron microscope (TEM) [33] and over 96% of phages studied are tailed phages and belong to the order *Caudovirales*. Siphoviruses, myoviruses and podoviruses are the most common phage types and constitute 61, 25 and 14% of all isolated tailed phages respectively (**Figure 1**) [34].

Phages are obligate parasites of bacteria as they lack the capacity to replicate independently. Phage replication occurs through either a lytic or lysogenic cycle (**Figure 2**). Phages following the lytic cycle attach to receptor(s) on the host cell surface using tail fibres, after which they inject their DNA and sequester the host's metabolic processes to produce more phage, eventually leading to cell lysis and release of the virions for further cycles of infection [35]. In comparison, during the lysogenic cycle phage DNA is incorporated into the bacterial cell and is replicated along with the host. Under certain conditions, e.g. stress and DNA damage, the phage can enter a lytic cycle as above. The lifestyle of the phage is determined via sequencing where the absence of recognisable integrases and other genes involved in the process of integration can be taken as indicative of a strictly lytic life cycle [36]. As lytic phages kill their target cells directly, they are preferred for therapeutic applications.

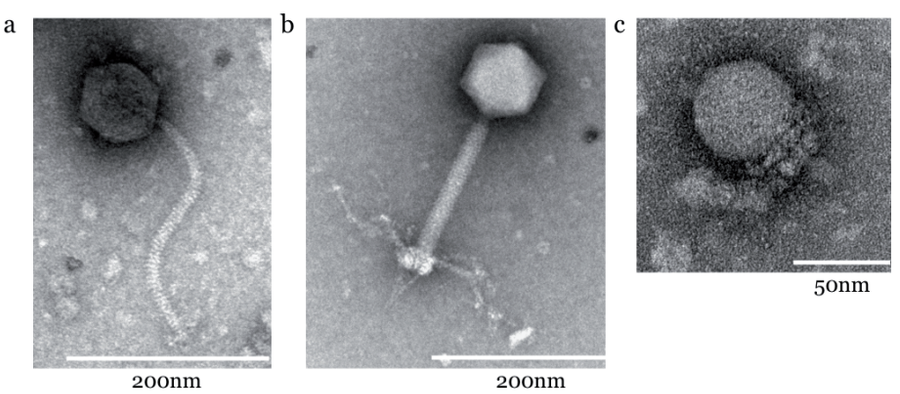

Figure 1.
Morphology of tailed phages viewed under TEM. The images show the typical structure of a (a) siphovirus, (b) myovirus and (c) podovirus. TEM images were taken by the Electron Microscopy Facility at the University of Leicester.

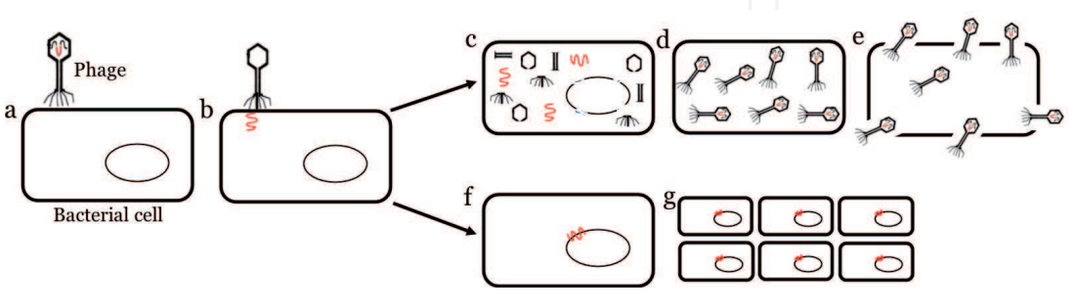

Figure 2.
Phage lytic and lysogenic infection cycle. (a) phages attach to a receptor on the bacterial cell, after which (b) they inject their DNA (red line) into the cytoplasm of the cell. Phages can then go on to follow the lytic cycle (c-d) or the lysogenic cycle (f-g). In the lytic cycle (c) phages take over the host cells machinery to replicate their nucleic acids and proteins (d) to form new phage progeny. This (e) leads to lysis of the bacterial cell to release the phage progeny and the phages go on to infect more target bacterial cells. In the lysogenic cycle (f) phage DNA is integrated into the bacterial genome and (e) as the bacterial cells are replicated the prophage is replicated simultaneously.

2.2 Phage isolation, host range and resistance

Phages can be isolated from any environment their hosts inhabit. *Salmonella*-specific phages have been isolated from faecal material obtained from pig and chicken farms, food processing plants, wild boar reserve [29], slurry lagoons [37], and sewage [22, 23]. Consequently, as phages are found in nature, humans and animals are continuously exposed to them, which is a major advantage in using them, as new entities would not be introduced into biological systems when phages are applied therapeutically [38].

The lytic spectrum (host range) of a phage is determined by screening against multiple strains of the target pathogen. Both narrow and broad host range phages have potential uses as therapeutics [39], for example a highly-specific, narrow host range phage can be applied with minimal perturbation to other residual microbial populations. Broad host-range phages provide a better scope of lysis and are therefore the desired components of most phage therapeutic applications. Multiple phages can be combined as a cocktail to improve phage coverage of the target species [40].

Emergence of resistance against therapeutic phages is a possibility as both phages and bacteria are in a continuous arms race. The mechanisms of phage resistance include altering the phage receptor, blocking phage DNA injection or inhibiting phage replication. This resistance can be countered by using cocktails of phage which bind to different receptors, as its unlikely resistance to all phage in the cocktail will emerge concurrently. Moreover, phage resistance can lead to a fitness cost for the bacterial cells [41]. Different multiplicities of infections (MOI's), which is ratio of phages to bacterial cells can also be trialed to limit resistance [42].

3. Experimental phage studies in chickens and pigs

In this section, the application of phages pre- and post-slaughter to reduce *Salmonella* numbers in chickens and pigs is discussed. Studies have varying levels of success in reducing *Salmonella* in challenge models, but with each study, valuable information is gained on phage dose, route of administration and resistance.

3.1 Experimental phage studies in chickens

3.1.1 In vivo *phage studies in chickens at farm level*

One of the first studies that investigated phage therapy against *Salmonella* challenged chickens dates back to 1991 [43]. The authors orally challenged one day old Rhode Island Red chickens with *S*. Typhimurium (10^8 Colony Forming Units (CFU)) and 10 minutes later administered a single phage orally at dose 10^{12} Plaque Forming Units (PFU)/mL. The mortality of untreated chickens was 56% 21 days post-challenge but in chickens treated with phage mortality was reduced to 20%. The authors demonstrated phage transition and replication in the gut at sites of *Salmonella* colonization such as the crop, intestine and caecum. Similarly, Atterbury et al. [21] showed in two different broiler chicken studies phage treatment (10^{11} PFU/mL) administered two days after challenge (10^8 CFU/mL), reduced ceacal colonisation by 4.2 and 2.2 $\log_{10}$ CFU/mL in birds challenged with Enteritidis P125109 or Typhimurium 4/74 respectively after 48 hours.

Goncalves and colleagues [44] compared the efficacy of three different phage cocktails in 45-day-old broiler chickens. The phages were administered at a dose of 10^9 PFU/mL via oral gavage, 1 hour post challenge with *S.* Enteritidis at

10^7 CFU/mL. Two of the three phage cocktails reduced caecal *Salmonella* counts by ~2 $\log_{10}$ CFU/mL in 12 hours, and *Salmonella* counts were below the detectable limit in the crop.

Toro et al. [45] designed a cocktail of three phages which could infect the top seven serotypes commonly associated with chickens. This cocktail was administered orally on days 4, 5, 6, 18, 19 and 20 at a dose of 5.4×10^6 PFU/bird and birds were challenged on day 7 with *S*. Typhimurium (10^5 CFU/mL). The phage treatment reduced *Salmonella* colonisation in the caeca by ten-fold, 4 days post-challenge, and 48 hours after treatment phages were isolated in the birds' faeces. Interestingly, the authors found phage treatment had a beneficial effect and chickens given the treatment gained more weight in comparison to challenged birds.

Delivering phages to chickens individually, via oral gavage, would be impractical commercially, however they could be administered easily through drinking water. Clavijo and colleagues [46] added a six-phage cocktail (named SalmoFREE®) at dose of 10^8 PFU/mL to drinking water on days 18, 26 and 34 (chickens were slaughtered on day 35), which was sufficient to reduce ceacal *Salmonella* counts to below the detectable limit (below 100 CFU/mL). The trial was conducted at a commercial farm where there was a record of *Salmonella* outbreaks and included 34,680 broiler chickens. This is the biggest and the only trial to date evaluating phage efficacy against *Salmonella* in a commercial setting. There was no difference in mortality or productivity measurements between untreated control birds and those treated with phage only, suggesting the cocktail was safe. Furthermore, the authors conducted a microbiome study and showed phage treatment had no detrimental effect on the chicken's microbiota [47]. Their studies provide further valuable evidence into the effectiveness and safety of phage treatment.

Delivering phages as feed additives has been investigated. Sklar and Joerger. [48] added a single phage dose (A) and a three-phage cocktail (B) to starter broiler feed at a dose of 10^7 PFU/g. The treated feed was available throughout the trial and chickens were challenged with *S.* Enteritidis at 10^4 CFU on day 1. After 14 days phage A reduced caecal colonisation by 1.9 $\log_{10}$ CFU/g and cocktail B by 0.6 $\log_{10}$ CFU/g. The authors found that the process of mixing phage with feed and storing feed in bird rearing conditions over 14 days caused a 2 $\log_{10}$ PFU/g reduction in phage numbers. Phage stability in feed could be a limitation and further research is needed to determine the impact storage conditions have on phage stability, such as factors as humidity and temperature.

3.1.2 Experimental post-slaughter phage studies in chickens

Following processing and packaging, meat is refrigerated to avoid bacterial growth, but *Salmonella* can survive under these conditions and phages could be used to reduce surface contamination of *Salmonella*. Goode et al. [38] applied a single phage to chicken skin artificially contaminated with *S*. Enteritidis at 10^3 CFU/cm^3. Phage applied at doses above 10^5 PFU/mL reduced bacterial numbers by over 98% and phages amplified on the surface of the infected skin by three-fold over 48 hours. In comparison, in the uninfected samples the phage titre reduced by 1 $\log_{10}$ PFU/cm^3, which suggests phages don't linger in absence of their target pathogen.

Atterbury et al. [49] showed phage treatment at dose 10^9 PFU/mL reduced levels of *S*. Enteritidis and *S*. Typhimurium by 72.2% and 38.9% respectively on spiked chicken skin samples (10^6 CFU/ml). The authors confirmed phage infection was occurring on the surface of the chicken skin by spreading a bioluminescent *S*. Typhimurium strain on the surface of chicken skin and then monitored its growth using photon counting. Further studies have shown the efficacy of phage treatment to reduce *Salmonella*

numbers on chicken skins are comparable to the typical chemical agents used by the food industry [50]. In addition, combining phage and chemical treatment was able to further decrease *Salmonella* counts to below detection levels [51].

To date only one study has investigated phage application on whole carcasses. Higgins et al. [52] spiked chicken carcasses with *S*. Enteritidis at 20 CFU, after which carcasses were sprayed with phage at different doses. The authors found only high phage doses of 10^8 and 10^{10} PFU/ml were effective and after 24 hours, *Salmonella* was only isolated from one out of fifteen carcasses. The phage counts were not monitored in the study, therefore it's unclear if there was phage amplification.

Phage treatment of raw meat samples has been shown to be effective at reducing bacterial load and consequently reducing its presence in the final consumer product. Duc et al. [53] tested the lytic activity of a five-phage cocktail at dose 10^9 PFU on chicken breasts inoculated with either *S*. Enteritidis or *S*. Typhimurium at 10^5 CFU. The phage cocktail reduced counts of both strains by ~1.6 $\log_{10}$ CFU/piece of chicken breast, when stored at 8°C, over 24 hours. However, when the meat was stored at 25°C phage treatment was more effective and reduced *S*. Enteritidis or *S*. Typhimurium by 3.1 and 2.2 $\log_{10}$ CFU/piece respectively over 24 hours. This could suggest phage activity is temperature dependent. However, another study showed phage activity was unaltered when spiked chicken breasts (10^5 CFU/ml) were treated with phage at doses 10^6 and 10^7 PFU/mL and stored at 4°C and 25°C. Under both conditions, phage treatment reduced bacterial counts to undetectable levels after just 12 hours [54]. The studies suggest phage temperature stability can vary between phages and its stability needs to be tested to determine which are more effective at food storage temperatures.

3.2 Experimental phage studies in pigs

3.2.1 Phage therapy in pre-market and market-weight pigs

Very few studies have examined the efficacy of phage treatments to control *Salmonella* in live pigs and this is largely due to the inherent difficulties of performing longitudinal studies from piglets to finished pigs. One pioneering study did exactly that and the efficacy of a fifteen-phage cocktail were tested in challenged piglets and market-weight pigs [22]. In the first study, the phage cocktail (10^9 PFU/mL) and challenge strain *S*. Typhimurium γ4232 (5×10^8 CFU/pig) were co-administered via oral gavage to piglets. Piglets were euthanised 6 hours post-inoculation in order to mimic the amount of time spent in a holding pen. Overall, the activity of the phage cocktail was sufficient to achieve 2–3 $\log_{10}$ CFU (~99%) reductions in the ileum, tonsils and caecum. In collected ileum and caecal samples, in five out of six phage-treated pigs *S*. Typhimurium counts were reduced to below the limits of detection (~100 CFU/mL).

The authors next assessed the efficacy of the phage cocktail in market-weight pigs. Four pigs (in three replicates) were inoculated via oral gavage with 5×10^9 CFU *S*. Typhimurium and allowed to contaminate a holding pen for a period of 48 hours. Following this, sixteen naïve pigs (non-*Salmonella* infected – eight phage-treated/eight mock treatments controls) were introduced to the holding pens and allowed to co-mingle with the seeder pigs for 6 hours. Phage cocktail administration involved an initial oral gavage of 10^9 PFU/mL followed by further identical doses every 2 hours for a total of 6 hours. After 6 hours of co-mingling between *S*. Typhimurium γ4232-infected, phage cocktail-treated, and mock control-treated pigs, each cohort was euthanised. In phage treated pigs there was 1 to 1.5 $\log_{10}$ CFU/mL reductions in *Salmonella* colonisation in ceacal and ileal samples. The role phages can play in controlling *Salmonella*

infection in pigs at a critical stage of the production process is evident from the work performed by Wall et al. [22].

A similar degree of efficacy was observed when applying a microencapsulated phage cocktail treatment to control shedding of *S*. Typhimurium during a holding period of 6 hours [55]. Saez et al. found that shedding of *Salmonella* from pigs in the phage-treated group (PT) was less common than non-phage treated pigs (nPT) at 2 hours (% pigs shedding PT-38.1%, nPT-71.4%) and 4 hours (PT-42,9% - nPT-81.1%). Sampling of caecal and ileal contents 6 hours post-infection showed that phage-treated pigs had significantly less *S*. Typhimurium levels at both anatomical sites by 1 $\log_{10}$ CFU/mL. Another study produced some promising results by showing how dietary supplementation with probiotics (*Saccharomyces cerevisiae*, *Lactobacillus acidophilus*, and *Bacillus subtilis*) and phages can positively influence growth performance of pigs. A phage cocktail (~10^9 PFU/g) designed to target a diverse selection of bacteria (*S*. Typhimurium, *S*. Enteritidis, *S*. Choleraesuis, *S*. Derby, *Staphylococcus aureus*, *Escherichia coli*, and *Clostridium perfringens* types A and C) was administered as part of a feed supplement. Interestingly, the addition of phage was found to be more effective than probiotics. Phages may therefore offer an attractive alternative to replace the use of antibiotics as growth promoters in pigs [56].

3.2.2 Phage decontamination of pigskin

Post-slaughter application of phages has the potential to reduce risks associated with pork contaminated with *Salmonella* prior to general retail. An investigation into the stability of phages at retail temperatures (fresh 4°C and frozen −20°C) and also their ability to control the endemic UK pig pathogen *S*. Typhimurium U288 was examined [23]. Hooton et al. tested killing activity of *Salmonella*-specific phages against a diverse panel of *Salmonella* serovars prior to formulation as a four phage cocktail (PC1). PC1 consisted of three novel *Salmonella* phage isolates (ΦSH17, ΦSH18, and ΦSH19) combined with the broad-host range *Salmonella* phage Felix 01 in equal volumes/titres for a final concentration of 10^8 PFU/mL. Initially it was shown that both *S*. Typhimurium U288 and the phage components of PC1 are both stable on experimentally-contaminated pigskin pieces stored at temperatures reflective of those at retail. The efficacy of PC1 was subsequently tested on spiked pigskin over a five-day trial under fresh conditions (4°C). A 3 × 3 matrix of CFU (10^6, 10^4, and 10^3) versus PC1 PFU (10^7, 10^5, and 10^4) was used to examine a range of MOIs (0.01–10,000) to determine the most effective combination.

The phage cocktail applied at MOI's of 1000 (10^7 PC1 V 10^4 U288) and 10 (10^5 PC1 v 10^4 U288) reduced *S*. Typhimurium U288 levels by ~92% after 1 hour post challenge. After 48 hours *Salmonella* counts were significantly reduced by ~1.4 $\log_{10}$ CFU/4 cm^2. The first reductions of *S*. Typhimurium U288 below the limits of detection were also reported at the 48 hour timepoint, specifically when an MOI of 10 was employed against low level contamination. At 96 hours post-inoculation it was evident that MOIs in excess of the target bacterium could reduce low-level bacterial contamination to below the limits of detection. The results reported here indicate that phages may provide useful tools for the post-harvest reduction of *S*. Typhimurium U288 on pork products [23].

4. Commercial phage products

A handful of phage products that target *Salmonella* in pre- and post- slaughter stages of the food chain are commercially available and summarized in **Table 1**.

Product name and developers	Phages	Approval	Notes	Reference(s)
GPI Biotech VAM-S Gum Products International, Inc. (Newmarket, Canada)	Three lytic phages: Phi_16, Phi_78, Phi_87	FDA approved, GRAS (GRN000917 Sep 2010)	• For use on poultry, red meat, eggs, fruits, vegetables, fish and shellfish	https://www.fda.gov/grasnoticeinventory
Bafasal Proteon Pharmaceuticals (Poland)	Four lytic phages: 3sent1, 8sent65, 8sent1748 and 5sent1	EURL approved (FAD-2017-0039 - CRL/170007 Oct 2018)	• Approved as feed additive • Awaiting approval in the EU	[57] https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2017-0039-bafasal.pdf
Biotector® SCJ CheilJedang Research Institute of Biotechnology (South Korea)	Non-disclosed	Patented	• On feed to control *Salmonella* in poultry	[20]
SalmoFresh™ Intralytix Inc. (USA)	Six lytic phages	FDA-approved, granted GRAS status (GRN000435) Feb, 2013 Approved by Israel Ministry of Health; Health Canada	• Food treatment • Effective against over 900 *Salmonella* strains representing more than 50 serotypes.	https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices&id=435&sort=GRN_No&order=DESC&startrow=1&type=basic&search=INTRALYTIX [58]
SalmoPro® Phagelux (Canada)	Two lytic phages: BP-63, BP-12	FDA-approved, granted GRAS status (752) Jul 2018	• For use as an antimicrobial processing aid to control *Salmonella* on food, when applied onto food surfaces	https://www.fda.gov/grasnoticeinventory [58]
Salmonelex™ (Former PhageGuard) Micreos Food Safety BV (The Netherland)	Two lytic phages: Fo1a and S16	FDA-approved, granted GRAS status (GRN000630)	• For use as an antimicrobial on foodstuffs to control *Salmonella* • Can be sprayed topically or added to chill tank water	Micreos Food Safety BV. Salmonelex™. Available online: https://www.fda.gov/media/98485/download [58]
SalmoFREE® Sciphage (Colombia)	Six lytic phages	Patented only (patent number WO2017089947A2)	• For therapy and control *Salmonella* in poultry frm • Phages are added to water	[46, 47]

Table 1.
Patented and approved Salmonella *phage products.*

Some products (SalmoFresh® and SalmoPro®) have already obtained clearance from specific regulatory agencies, such as FDA, and are available to purchase, while others are patented but not approved by any regulatory authority, at the time of writing. However relevant scientific data about the product has been published, such as for SalmoFree® [20].

5. Phage/antibiotic synergy (PAS)

The efficacy of combinatorial medicinal treatments is well-documented and have proven successful in treating a range of human diseases such as cancer, HIV, and malaria [59]. Similarly, the use of phages and antibiotics synergistically (PAS) has been explored and experimental studies have shown using phages and antibiotics in combination could enhance bacterial suppression and lower emergence of bacterial resistance. Furthermore, a combined approach can lead to re-establishment of antibiotic sensitivity, for example in cases where phages bind to bacterial drug efflux pumps [60]. *In vitro* studies have investigated PAS activity for the control of *S*. Typhimurium with the well-studied phage P22 and antibiotics ceftriaxone and ciprofloxacin. The study found pre-treatment of *S*. Typhimurium with phage P22 prior to antibiotic addition was the most effective approach in comparison to treating with phages 6 hours after antibiotic treatment [61]. The timing and order of phage and antibiotics needs to be considered as it can influence PAS activity. It was also reported that the presence of antibiotics did not negatively influence phage binding to *Salmonella* cells, and a significant increase in phage lytic activity was observed [62].

To date, no *in vivo* PAS studies have been conducted in *Salmonella* challenged chickens and pigs. Therefore, further *in vivo* work is required for the underlying dynamics of PAS to be understood and developed into useful combinatorial therapies. Within the context of phage therapeutics in agricultural settings (and potentially in the clinic) PAS may well provide an exciting route of research for development into a parallel treatment with antibiotics. The emergence of resistance from the target bacterium to both antibiotics and phage treatment, choice of antibiotics and phage combinations, and potential efficacy-improving interactions with immunological responses will be important factors for consideration [59].

6. Potential challenges of using phages in poultry and pigs

The use of phages against *Salmonella* in farming, either pre or post-slaughter, have some challenges. Some of those difficulties are common to phage therapy in general and fall in to four categories initial phage selection; phage delivery; resistance development; and regulatory approval.

6.1 Phage selection

On the initial phage selection, potential phage candidates need to be virulent and propagate via the lytic cycle as opposed to temperate (can propagate via lysogenic or lytic cycle), which need to be confirmed by sequencing. This is to ensure the phage will not integrate on host genome avoiding transduction and horizontal gene transfer [21, 63, 64].

6.2 Phage delivery

The topic of phage therapy pharmacodynamics and pharmacokinetics is complex and more specific reviews have recently been published on this topic [65, 66]. In brief, phages need to reach the site of bacterial colonisation, and in poultry and pigs, *Salmonella* initially colonises the gut. Many studies have been designed to establish if phages can be delivered to the gastrointestinal tract and beyond via oral administration, either in feed or drinking water. For post-slaughter application, phage preparations can be applied by directly applying to carcasses, meat, skin,

packaging materials as well as surfaces in the abattoir or meat processing facilities. Both pre- and post-slaughter applications present challenges to phage delivery [36].

A particular challenge to phages delivered orally to control *Salmonella* is to ensure they will be active in the gut pH, despite the fact that they are sensitive biological entities and will encounter changes in pH (**Figure 3**) and temperature. Phages are typically stable between pH 4 and pH 10 [67]. However, the studies discussed in Section 3 highlight natural phages retain lytic activity through the passage of the gut and do reach the focal point of infection.

Alternative solutions have been developed to protect phages from the acidic conditions by using dry or liquid formulation solutions. For example, it was shown that Felix O1 microencapsulation in chitosan-alginate microspheres could fully preserve phage viability upon 1 hour exposure to simulated gastric fluid (pH = 2.4 with 3.2 mg ml^{-1} pepsin) and 3 hour exposure to 2% (wt/vol) porcine bile extract [68]. Other studies have shown that liposome-encapsulated phages (UAB_Phi20, UAB_Phi78, and UAB_Phi87) were significantly more stable in simulated gastric fluid (pH = 2.8 with 3.0 mg ml – 1 pepsin) when compared to free phages *in vitro* while the preparation was stable at 4°C for at least 3 months [69]. These data show that the challenge of gut pH range that the phage has to endure, when administered orally, can be overcome by selecting phages that remain viable and withstand wide pH variations or, in addition or as an alternative, shield the phages by means of pH resistant pharmaceutical formulation development.

6.3 Overcoming phage resistance

Phages are no different from other antimicrobials that are used to kill bacteria, and can become resistant to them following exposure. Often, the use of phage cocktails and rotation schedules is used to limit or avoid the development of resistant mutants. When phages are used post-slaughter as disinfectants, they can be deployed at a high titer, to reduce the build up of phage-resistant bacteria [70]. In order to reduce the accumulation of phages on surfaces after their intended use in

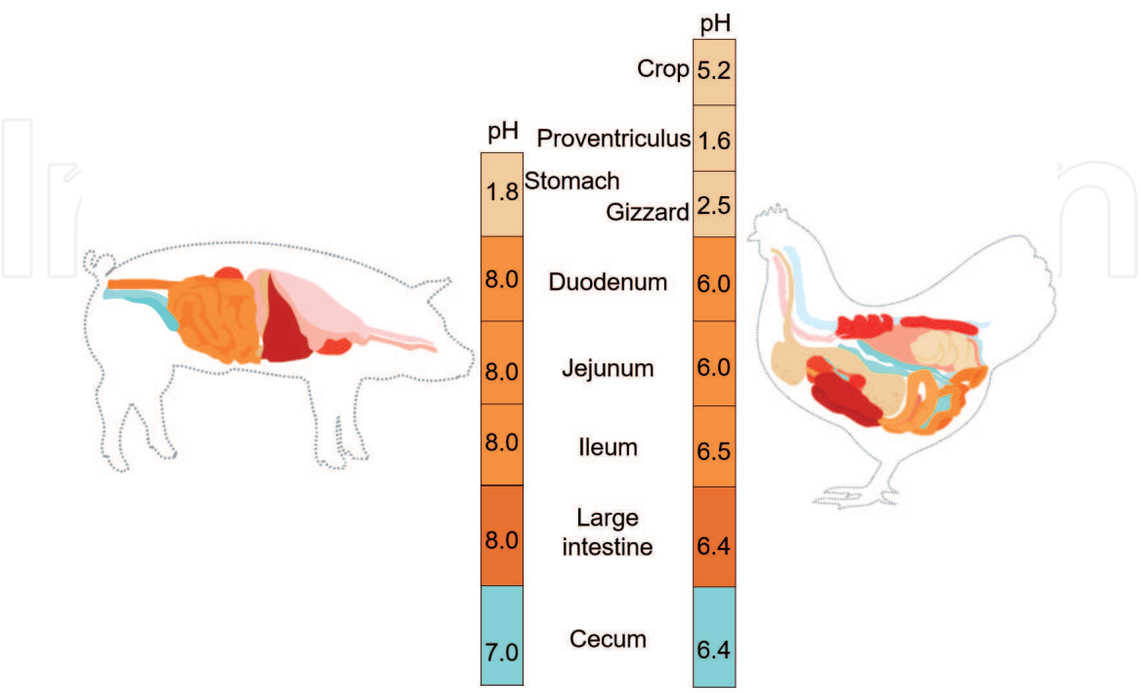

Figure 3.
The gastrointestinal pH changes in the gut of pigs and chickens.

the food industry, some disinfectants were tested and proved to be successful at neutralizing phages, such as peracetic acid [71].

When using phages within farm settings the challenge of phage persistence, spread and resistance development is more significant because successive animals will be housed in the same facilities and disinfection must be thorough. A recent study using a patented six phage cocktail against *Salmonella* (SalmoFREE) showed that after the first trial, SalmoFREE phages were detected from the beginning of the second trial in treated and control groups houses, showing that even after the cleaning and disinfection process, phages persisted in the environment and survived between trials. As a consequence, birds in the second trial (control and treatment group) showed unexpected reduction of *Salmonella* counts even before treatment/placebo administration at day 17 [46].

Effective disinfection practices and phage cocktail rotation may be the solution to the phage persistence challenge, however the effect of disinfectants should be tested on a case-by-case basis to determine their efficacy at neutralizing the respective phage(s) cocktail [72].

6.4 Regulating phage products

As discussed above, phages may be used as a feed additive to prevent or treat infection, as a medicine to treat infection or as a post product treatment for carcasses or meat. Phages could also be used to decontaminate either the environment that the animals are living in, or facilities regarding production of the final product. Phages go through specific regulatory pathways depending on which of these intervention points that they are used in, and on the level of claims associated with their use. To take a product to market requires the developer to know which regulatory route they will take, in order to gather appropriate data on safety and efficacy [73].

In recent years there has been a significant amount of engagement from regulating bodies, who are also acutely aware of the need to find novel antimicrobials. They are also aware that this is often seen as a major hurdle to developing the technology and are keen to help. It is important to state that by working with regulators there is an opportunity to impose a regulatory system that will allow the exploration of this technology whilst hopefully mitigating against many of the mistakes that we have previously made in terms of overusing antibiotics from the outset. Antibiotic stewardship was largely implemented after extensive bacterial resistance to antibiotics had already been achieved however sensible regulation could work hand in hand with a stewardship program to maintain effective phage use for future generations [74].

Establishing how phages fit into traditional drug/veterinary medical product regulatory systems is not always trivial. In the USA, phages are regulated by the Food and Drug Administration (FDA), regardless as to whether they are to be used in humans or animals, although they go through different parts of this depending on exactly how they are being used. Interestingly the FDA regulates phages in the same way regardless of whether they are 'natural' or engineered [73].

In Europe phages are currently regulated by the European Commission through the European Medicines Agency. Unlike the system in the USA, if phages are genetically altered, they are regulated differently. In the UK if phages are to be used within animals, they are regulated by the Veterinary Medical Directorate but if their end use is in humans they are regulated by the Medicines and Healthcare products Regulatory Agency and in food they are regulated by the Food Standards Agency. The different regulatory authorities do communicate with each other to identify

commonalities and routes forward. Clearly there are parallels with other biologicals such as monoclonal antibodies, which will inform how phages are effectively regulated [73].

7. Future work: machine learning tools

Phage characterisation based on host range analysis, studying phage host interactions, phage infection kinetics and designing phage cocktails is resource-intensive. Machine learning (ML) based tools can be developed to predict these interactions, and the application of computational biology, artificial intelligence (AI) and modelling in phage research is rapidly developing [75]. The combination of these techniques with high-throughput Next Generation Sequencing promises greater insights into phage biology alongside the development of new tools to address previously intractable problems in phage therapy [76]. Computational tools applied to phage research are based on:

1. Homology-based methods: comparing the features (e.g. DNA/RNA/protein sequences) of an unknown phage with comparable information from databases of known phage. Examples include HostPhinder [77], VirHostMatcher [78] and ILMF-VH [79].

2. Machine Learning (ML) methods: these use combinations of algorithms and statistical techniques such as logistic regression and support vectors to find patterns in large datasets which are then used to make predictions [80].

3. Deep Learning (DL) methods: a subset of machine learning in which the key features used for pattern recognition and classification are identified by the computer algorithm directly and do not require human input [81].

A key aim of these approaches, as applied to phage therapy, is to facilitate or automate the matching of phages to target bacterial pathogens. This would revolutionize the field as it would reduce or eliminate the need for extensive host range profiling in the laboratory and would allow the rapid countering of resistance.

Homology-based methods have been used more extensively than ML so far, but more for the identification and annotation of phage DNA from metagenomic data than for phage host matching. Homology-based approaches have used genomic similarity (e.g. HostPhinder [77]), oligonucleotide frequency (e.g. VirHostMatcher [77]), and phage abundance profiling [82]. However, the success of these methods varies widely, with correct identification of the host to genus or species level only occurring between 9.5% and 75% of the time.

Phage host matching using ML has also met with varied success. Approaches include using chemical parameters of all phage and host proteins [80], or focusing on a subset of these, such as receptor binding proteins [75], which have accurately predicted phage hosts 30 to 90% of the time. Relatively few studies have used DL methods. As with the homology-based methods above, some studies have focused on the use of DL to identify and separate phage sequences from metagenomic data. DL was used by Li et al. [79] to accurately to match phage and host species 81% of the time using 27 features of phage and host proteins.

A disadvantage of ML and DL is that large datasets are required, and these are often skewed heavily towards phage which infect a small number of well-studied bacteria. For example, in one study approximately 86% of phage used in the ML model infected a single species (*M. smegmatis*). Moreover, DL methods are not

readily interpretable and regarded as 'black boxes' due to the lack of human involvement in feature selection and application. Additionally, even the best performing ML and DL models are currently unable to predict phage hosts at the strain level, which will a necessary step in real-world therapeutic applications.

Phage host matching is likely to be more useful when using phage therapy for highly diverse pathogens, such as *Salmonella* and *E. coli*, than for more homogenous bacteria such as *Staphylococcus aureus*. ML and DL have the potential to automate the process of phage selection of their predictions are shown to be reliable, and potentially in the future could help design personalised phage therapeutics for human and agricultural use.

8. Conclusions

Phages could provide a natural alternative to traditional antimicrobial therapies in pig and poultry production. Multiple intervention points exist from farm-to-fork allowing for the development of targeted phage therapeutic strategies. The promising results obtained from diverse experimental approaches demonstrate the potential of phages to reduce *Salmonella* in live animals, as well as in finished retail products. With correct stewardship, phages may well become an integrated solution in livestock production especially within the remit of controlling significant pathogens such as *Salmonella*. While some products have made it to market, current legislation needs further development prior to widespread acceptance of phage therapeutics in animals and on retail products. The next generation of phage research is set to take advantage of developments in the fields of machine-based learning and other computationally oriented approaches. Such exciting techniques may offer a more refined approach towards the application of phages for elimination of *Salmonella* from pig and poultry production.

Acknowledgements

Dr. Steve Hooton and Dr. Adriano M. Gigante are funded by the Biotechnology and Biological Sciences Research Council (BBSRC – BB/T006196/1) and we thank BBSRC for providing the funding for this work.

Conflict of interest

The authors declare no conflict of interest.

Author details

Anisha M. Thanki[1], Steve Hooton[1], Adriano M. Gigante[2], Robert J. Atterbury[2] and Martha R.J. Clokie[1*]

1 Department of Genetics and Genome Biology, University of Leicester, Leicester, UK

2 School of Veterinary Medicine and Science, University of Nottingham, Leicestershire, UK

*Address all correspondence to: mrjc1@leicester.ac.uk

References

[1] Neill J. Tackling drug-resistant infections globally: final report and recommendations. 2016. Avaliable from: https://amr-review.org/sites/default/files/160518_Final%20paper_with%20cover.pdf

[2] Walsh TR. A one-health approach to antimicrobial resistance. Nature Microbiology. 2018;3:8:854-855. DOI:10.1038/s41564-018-0208-5

[3] Hernando-Amado S, Coque TM, Baquero F, and Martínez JL. Defining and combating antibiotic resistance from One Health and Global Health perspectives. Nature Microbiology. 2019;4:1432-1442. DOI:10.1038/s41564-019-0503-9

[4] Robinson TP, Bu DP, Carrique-Mas J, Fevre, EM, Gilbert M, Grace D, Hay SI, Jiwakanon J, Makkar M, Kariuki S, Laxminarayan R, Lubroth J, Magnusson U, Thi Ngoc P, Van Boeckel TP, Woolhouse MEJ. Antibiotic resistance: Mitigation opportunities in livestock sector development. Animal. 2017;11:1-3. DOI: 10.1017/S1751731116001828

[5] Patel SJ, Wellington M, Shah RM, Ferreira MJ. Antibiotic stewardship in food-producing animals: challenges, progress, and opportunities. Clinical Theraupectics. 2020;42:1649-1658. DOI: 10.1016/j.clinthera.2020.07.004.

[6] Diana A, Boyle LA, Leomard FC, Carroll C, Sheehan E, Murphy D, Manzanilla EG. Removing prophylactic antibiotics from pig feed: how does it affect their performance and health? BMC Veterinary Research. 2019;15,:1-8. DOI: 10.1186/s12917-019-1808-x

[7] Helm ET, Curry S, Trachsel JM, Schroyen M, and Gabler NK. Evaluating nursery pig responses to in-feed sub-therapeutic antibiotics. PLoS One. 2019;14:1-16. DOI: 10.1371/journal.pone.0216070

[8] Emond-Rheault JG, Hamel J, Jeukens J, Freschi L, Kukavica-Ibrulj I, Boyle B, Tamber S, Malo D, FRanz E, Burnett E, Daigle F, Arya G, Sanderson K, Wiedmann M, Slawson RM, Weadge JT, Stephan R, Bekal S, Gruenheid S, Goodridge LD, Levesque RC. The *Salmonella enterica* plasmidome as a reservoir of antibiotic resistance. Microorganisms. 2020;8:1-18. DOI: 10.3390/microorganisms8071016

[9] Lima T, Domingues S, and Da Silva GJ. Plasmid-mediated colistin resistance in *Salmonella enterica*: a review. Microorganisms. 2019;7:1-17. DOI: 10.3390/microorganisms7020055

[10] Hooton SPT, Timms AR, Moreton J, Wilson R, Connerton IF. Complete genome sequence of *Salmonella enterica* serovar Typhimurium U288. Genome Announcoument. 2013;1:2010-2011. DOI: 10.1128/genomeA.00467-13

[11] Hooton SPT, Timms AR, Cummings NJ, Moreton J, Wilson R, Connerton IF. The complete plasmid sequences of *Salmonella enterica* serovar Typhimurium U288. Plasmid. 2014;76:32-39. DOI: 10.1016/j.plasmid.2014.08.002

[12] Gosling RJ, Mueller-Doblies D, MartelliF, Nunez-GarciaJ, KellN, RabieA, Wales AD, Davies RH. Observations on the distribution and persistence of monophasic *Salmonella* Typhimurium on infected pig and cattle farms. Veterinary Microbiology. 2018;227:90-96. DOI: 10.1016/j.vetmic.2018.10.032

[13] APHA. *Salmonella* in livestock production in GB. Animal Plant Health Agency. 3-240. 2020

[14] Bonardi S. *Salmonella* in the pork production chain and its impact

on human health in the European Union. Epidemiology & Infection. 2017;145:1513-1526. DOI: https://doi.org/10.1017/S095026881700036X

[15] Broadway PR, Brooks JC, Mollenkopf DF, Calle MA, Loneragen GH, Miller MF, Carroll JA, Sanchez NCB, Wittum TE. Prevalence and antimicrobial susceptibility of *Salmonella* serovars isolated from U.S. retail ground pork. Foodborne Pathogens and Disease. 2021. DOI: 10.1089/fpd.2020.2853

[16] Koutsoumanis K, Allende A, Alvarez-Ordonez A, Bolton D, Bover-Cid S, Chemaly M, De Cesare A, Herman L, Hilbert F, Lindqvist R, Nauta M, Peixe L, Ru G, Simmons M, Skandamis P, Suffredini E, Dewulf J, Hald T, Michel V, Niskanen T, Ricci A, Snary E, Boelaert F, Messens W, Davies R. *Salmonella* control in poultry flocks and its public health impact. EFSA Journal. 2019. Avaliable at: https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2019.5596

[17] Velasquez CG, Macklin KS, Kumar S, Bailey M, Ebmer PE, Oliver HF, Martin-Gonzalez FS, Singh M. Prevalence and antimicrobial resistance patterns of *Salmonella* isolated from poultry farms in southeastern United States. Poultry Science. 2018;97:2144-2152. DOI: 10.3382/ps/pex449

[18] Yang X, Huang J, Zhang Y, Liu S, Chen L, Xiao C, Zeng H, Wei X, Gu Q, Li Y, Wang J, Ding Y, Zhang J, Wu Q. Prevalence, abundance, serovars and antimicrobial resistance of *Salmonella* isolated from retail raw poultry meat in China. Science of Total Environment. 2020;713:136385. DOI: 10.1016/j.scitotenv.2019.136385

[19] Hussain MA, Liu H, Wang Q, Zhong F, Guo Q, Balamurugan S. Use of encapsulated bacteriophages to enhance farm to fork food safety. Critical Reviews in Food Science and Nutrition. 2017;57:2801-2810. DOI: 10.1080/10408398.2015.1069729

[20] Sillankorva SM, Oliveira H, Azeredo J. Bacteriophages and their role in food safety. International Journal of Microbiology. 2012;2012. DOI: 10.1155/2012/863945

[21] Atterbury RJ, Van Bergen MAP, Ortiz F, Lovell A, Harris JA, De Boer A, Wagenaar JA, Allen VM, Barrow PA. Bacteriophage therapy to reduce *Salmonella* colonization of broiler chickens. Applied and Environmental Microbiology. 2007;73:4543-4549. DOI: 10.1128/AEM.00049-07

[22] Wall SK, Zhang J, Rostagno MH, Ebner PD. Phage therapy to reduce preprocessing *Salmonella* infections in market-weight swine. Applied and Environmental Microbiology. 2010;76:48-53. DOI: 10.1128/AEM.00785-09

[23] Hooton SPT, Atterbury RJ, Connerton IF. Application of a bacteriophage cocktail to reduce *Salmonella* Typhimurium U288 contamination on pig skin. International Journal of Food Microbiology. 2011;151:157-163. DOI: 10.1016/j.ijfoodmicro.2011.08.015

[24] Gouvêa DM, Mendonça RCS, Soto ML, Cruz RS. Acetate cellulose film with bacteriophages for potential antimicrobial use in food packaging. LWT - Food Science and Technology. 2015;63:85-91. DOI: https://doi.org/10.1016/j.lwt.2015.03.014

[25] Gouvêa DM, Mendonça RCS, Lopez MES, Batalha LS. Absorbent food pads containing bacteriophages for potential antimicrobial use in refrigerated food products. LWT - Food Science and Technology. 2016;67:159-166. DOI: https://doi.org/10.1016/j.lwt.2015.11.043

[26] Clokie MRJ, Millard AD, Letarov AV, Heaphy S. Phages in nature. Bacteriophage. 2011;1:31-45. DOI: 10.4161/bact.1.1.14942

[27] Duckworth DH. Who discovered bacteriophage? Bacteriology Reviews. 1976;40:793-802. Avaliable at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC413985/

[28] Xu Y, Yu X, Gu Y, Huang X, Liu G, Liu X. Characterization and genomic study of phage vB_EcoS-B2 infecting multidrug-resistant *Escherichia coli*. Frontiers in Microbiology. 2018;9:793. DOI: 10.3389/fmicb.2018.00793

[29] Thanki AM, Brown N, Millard AD, Clokie MRJ. Genomic characterization of jumbo *Salmonella* phages that effectively target United Kingdom *Salmonella* serotypes. 2019;10:1-14. DOI: 10.3389/fmicb.2019.01491

[30] Abedon ST, Kuhl SJ, Blasdel BG, Kutter EM. Phage treatment of human infections. Bacteriophage. 2011;1:66-85. DOI: 10.4161/bact.1.2.15845

[31] Gigante A, Atterbury RJ. Veterinary use of bacteriophage therapy in intensively-reared livestock. Virology Journal. 2019;16:155. DOI: https://doi.org/10.1186/s12985-019-1260-3

[32] Endersen L, O'Mahony J, Hill C, Ross RP, McAuliffe O, Coffey A. Phage therapy in the food industry. Annual Review of Food Science and Technology. 2014;327-349. DOI: 10.1146/annurev-food-030713-092415

[33] Adriaenssens EM, Rodney Brister J. How to name and classify your phage: An informal guide. Viruses. 2017;9:1-9. DOI: 10.3390/v9040070

[34] Ackermann HW. Phage classification and characterization. In Bacteriophages: Methods and protocols. 2009;1:127-140. DOI: 10.1007/978-1-60327-164-6_13

[35] Clokie MRJ, Kropinski AMB. Bacteriophages: Methods and Protocols. Molecular and applied aspects. Volume 1. Humana Press. 2009. Avaliable at: https://www.springer.com/gp/book/9781588296825

[36] Żbikowska K, Michalczuk M, Dolka B. The use of bacteriophages in the poultry industry. Animals. 2020:10;5. DOI: https://doi.org/10.3390/ani10050872

[37] McLaughlin MR, King RA. Characterization of *Salmonella* bacteriophages isolated from swine lagoon effluent. Current Microbiology. 2008;56;208-213. DOI: 10.1007/s00284-007-9057-9

[38] Goode D, Allen VM, Barrow PA. Reduction of experimental *Salmonella* and *Campylobacter* contamination of chicken skin by application of lytic bacteriophages. Applied and Environmental Microbiology. 2003;69:5032-5036. DOI: 10.1128/AEM.69.8.5032-5036.2003

[39] De Jonge PA, Nobrega FL, Brouns SJJ, Dutilh BE. Molecular and evolutionary determinants of bacteriophage host range. Trends in Microbiology. 2019;27:51-63. DOI: https://doi.org/10.1016/j.tim.2018.08.006

[40] Chan BK, Abedon ST, Loc-carrillo C. Phage cocktails and the future of phage therapy. Future Medicine. 2013;8:769-783. DOI: 10.2217/fmb.13.47

[41] Stern A, Sorek R. The phage-host arms race: shaping the evolution of microbes. Bioessays. 2011;33:43-51. DOI: 10.1002/bies.201000071

[42] Labrie SJ, Samson JE, Moineau S. Bacteriophage resistance mechanisms. Nature Reviews Microbiology. 2010;8:317-27. DOI: https://doi.org/10.1038/nrmicro2315

[43] Berchieri A, Lovell MA, Barrow PA. The activity in the chicken alimentary tract of bacteriophages lytic for *Salmonella* Typhimurium. Research in Microbiology. 1991;142:541-549. DOI: 10.1016/0923-2508(91)90187-f

[44] Gonçalves GAM, Donato TC, Baptista AAS, de Oliveira Corrêa IM, Garcia KCOD, Filho RLA. Bacteriophage-induced reduction in *Salmonella* Enteritidis counts in the crop of broiler chickens undergoing preslaughter feed withdrawal. Poultry Science. 2014;93:216-220. DOI: 10.3382/ps.2013-03360

[45] Toro H, Price SB, Mckee AS, Hoerr FJ, Krehling J, Perdue M, Bauermeister L. Use of bacteriophages in combination with competitive exclusion to reduce *Salmonella* from infected chickens. Avian Diseases. 2005;49:118-124. DOI: 10.1637/7286-100404R

[46] Clavijo V, Baquero D, Hernandez S, Farfan JC, Arias J, Arevalo A, Donado-Godoy P, Vives-Flores M. Phage cocktail SalmoFREE® reduces *Salmonella* on a commercial broiler farm. Poultry Science. 2019;98:5054-5063. DOI: 10.3382/ps/pez251

[47] Clavijo V, Morales LT, Flores MJV, Reyes A. *Salmonella* phage cocktail, its effects and benefits on the gut of chickens in a commercial farm. Research square pre-print. 2020. DOI: 10.21203/rs.3.rs-29114/v1

[48] Sklar IB, Joerger RD. Attempts to utilize bacteriophage to combat *Salmonella enterica* serovar Enteritidis infection in chickens. Journal of Food Safety. 2001;21:15-29. DOI: https://doi.org/10.1111/j.1745-4565.2001.tb00305.x

[49] Atterbury RJ, Gigante AM, Lozano MSR, Medina RDM, Robinson G, Alloush H, Barrow PA, Allen VM. Reduction of *Salmonella* contamination on the surface of chicken skin using bacteriophage. Virology Journal. 2020;17:1-8. DOI: 10.1186/s12985-020-01368-0

[50] Hungaro HM, Mendonça RCS, Gouvêa DM, Vanetti MCD, de O. Pinto CL. Use of bacteriophages to reduce *Salmonella* in chicken skin in comparison with chemical agents. Food Research International. 2013;52:75-81. DOI: https://doi.org/10.1016/j.foodres.2013.02.032

[51] Sukumaran AT, Nannapaneni R, Kiess A, Sharma CS. Reduction of *Salmonella* on chicken meat and chicken skin by combined or sequential application of lytic bacteriophage with chemical antimicrobials. International Journal of Food Microbiology. 2015;207:8-15. DOI: 10.1016/j.ijfoodmicro.2015.04.025

[52] Higgins JP, Higgins SE, Guenther KL, Huff W, Donoghue AM, Donoghue DJ, Hargis BM. Use of a specific bacteriophage treatment to reduce *Salmonella* in poultry products. Poultry Science. 2005;84:1141-1145. DOI: https://doi.org/10.1093/ps/84.7.1141

[53] Duc HM, Son HM, Honjoh KI, Miyamoto T. Isolation and application of bacteriophages to reduce *Salmonella* contamination in raw chicken meat, LWT - Food Science and Technology. 2018;91:353-360. DOI: https://doi.org/10.1016/j.lwt.2018.01.072

[54] Yan T, Liang L, Yin P, Zhou Y, Sharoba AM, Lu Q, Dong X, Liu K, Connerton IF, Li J. Application of a novel phage LPSEYT for biological control of *Salmonella* in foods. Microorganisms. 2020;8:247. DOI: 10.3390/microorganisms8030400

[55] Saez AC, Zhang J, Rostagno MH, Ebner PD. Direct feeding of microencapsulated bacteriophages to reduce *Salmonella* colonization in pigs. Foodborne Pathogens and Disease. 2011;8:1269-1274. DOI: 10.1089/fpd.2011.0905

[56] Gebru E, Lee JS, Son JC, Yang SY, Shin SA, Kim B, Kim MK, Park SC. Effect of probiotic-, bacteriophage-, or organic acid-supplemented feeds or fermented soybean meal on the growth performance, acute-phase response, and bacterial shedding of grower pigs challenged with *Salmonella enterica* serotype Typhimurium. Journal of Animal Science. 2010;88:3880-3886. DOI: 10.2527/jas.2010-2939

[57] Wójcik EA, Stanczyk M, Wojtasik A, Kowalska JD, Nowakowska M, Lukasiak M, Bartnicka M, Kazimierczak J, Dastych J. Comprehensive evaluation of the safety and efficacy of BAFASAL® bacteriophage preparation for the reduction of *Salmonella* in the food chain. Viruses. 2020;12:7. DOI: 10.3390/v12070742

[58] Moye ZD, Woolston J, Sulakvelidze A. Bacteriophage applications for food production and processing. Viruses. 2018;10:1-22. DOI: 10.3390/v10040205

[59] Torres-Barceló C, Hochberg ME. Evolutionary rationale for phages as complements of antibiotics. Trends in Microbiology. 2016;24:249-256. DOI: 10.1016/j.tim.2015.12.011

[60] Tagliaferri TL, Jansen M, Horz HP. Fighting pathogenic bacteria on two fronts: phages and antibiotics as combined strategy. Frontiers in Cellular and Infection Microbiology. 2019;9:22. DOI: 10.3389/fcimb.2019.00022

[61] Jeon G, Ahn J. Assessment of phage-mediated inhibition of *Salmonella* Typhimurium treated with sublethal concentrations of ceftriaxone and ciprofloxacin. FEMS Microbiology Letters. 2020;367:19. DOI: https://doi.org/10.1093/femsle/fnaa159

[62] Jeon G, Ahn J. Evaluation of phage adsorption to *Salmonella* Typhimurium exposed to different levels of pH and antibiotic. Microbial Pathogensis. 2021;150:104726. DOI: https://doi.org/10.1016/j.micpath.2020.104726

[63] Bielke LR, Higgins SE, Donoghue AM, Donoghue DJ, Hargis BM, Tellez G. Use of wide-host-range bacteriophages to reduce *Salmonella* on poultry products. International Journal of Poultry Science. 2007;6:754-757. DOI: 10.3923/ijps.2007.754.757

[64] Santos SB, Fernandes E, Carvalho CM, Sillankorva S, Krylov VN, Pleteneva EA, Shaburova OV, Nicolau A, Ferreira EC, Azeredo J. Selection and characterization of a multivalent *Salmonella* phage and its production in a nonpathogenic *Escherichia coli* strain. Applied and Environmental Microbiology. 2010;76:7338-7342. DOI: 10.1128/AEM.00922-10

[65] Dąbrowska K, Abedon ST. Pharmacologically aware phage therapy: pharmacodynamic and pharmacokinetic obstacles to phage antibacterial action in animal and human bodies," Microbiology and Molecular Biology Reviews. 2019;83:1-25, 2019. DOI: 10.1128/MMBR.00012-19

[66] Danis-Wlodarczyk K, Dąbrowska K, Abedon ST. Phage therapy: the pharmacology of antibacterial viruses. Current Issues in Molecular Biology. 2020;40:81-163. DOI: 10.21775/cimb.040.081

[67] Casey E, Sinderen DV, Mahony J. *In Vitro* characteristics of phages to guide 'real life' phage therapy suitability. Viruses. 2018;30:163. DOI: 10.3390/v10040163

[68] Yongsheng M, Pacan JC, Wang Q, Xu Y, Huang X, Korenevsky A, Sabour PM. Microencapsulation of bacteriophage felix 01 into chitosan-alginate microspheres for oral delivery. Applied and Environmental Microbiology. 2008;74:4799-4805. DOI: 10.1128/AEM.00246-08

[69] Colom J, Cano-Sarabia M, Otero J, Cortés P, Maspoch D, Llagostera M.

Liposome-encapsulated bacteriophages for enhanced oral phage therapy against *Salmonella*. Applied and Environmental Microbiology. 2015;81:4841-4849. DOI: 10.1128/AEM.00812-15

[70] Fister S, Mester P, Witte AK, Sommer J, Schoder D, Rossmanith P. Part of the problem or the solution? Indiscriminate use of bacteriophages in the food industry can reduce their potential and impair growth-based detection methods. Trends in Food Science & Technology. 2019;90:170-174. DOI: https://doi.org/10.1016/j.tifs.2019.02.031

[71] Marco MB, Suarez VB, Quiberoni A, Pujato SA. Inactivation of dairy bacteriophages by thermal and chemical treatments. Viruses. 2019;11:480. DOI: 10.3390/v11050480

[72] Sommer J. Trautner C, Witte AK, Fister S, Schoder D, Rossmanith P, Mester PJ. Don't shut the stable door after the phage has bolted—the importance of bacteriophage inactivation in food environments. Viruses. 2019;11:1-27. DOI: 10.3390/v11050468

[73] Fauconnier A. Phage therapy regulation: From night to dawn. Viruses. 2019:11:352. DOI: 10.3390/v11040352

[74] Brives C, Pourraz J. Phage therapy as a potential solution in the fight against AMR: obstacles and possible futures. Palgrave Communication. 2020;6:1-11. DOI: https://doi.org/10.1057/s41599-020-0478-4

[75] Boeckaerts D, Stock M, Criel B, Gerstmans H, De Baets B, Briers Y. Predicting bacteriophage hosts based on sequences of annotated receptor-binding proteins. Nature Scientific Reports. 2021;11: 1-14. DOI: https://doi.org/10.1038/s41598-021-81063-4

[76] McNair K, Bailey BA, Edwards RA. PHACTS, a computational approach to classifying the lifestyle of phages. Bioinformatics, 2012;28:614-618. DOI: 10.1093/bioinformatics/bts014

[77] Villarroel J, Kleinheinz KA, Jurtz VI, Zschach H, Lund O, Nielsen M, Larsen MV. HostPhinder: A phage host prediction tool. Viruses. 2016;8:116. DOI: 10.3390/v8050116

[78] Ahlgren NA, Ren J, Lu YY, Fuhrman JA, Sun F. Alignment-free d2∗ oligonucleotide frequency dissimilarity measure improves prediction of hosts from metagenomically-derived viral sequences. Nucleic Acids Research. 2017;45:39-53. DOI: 10.1093/nar/gkw1002

[79] Li M, Wang Y, Li F, Zhao Y, Liu M, Zhang S, Bin Y, Smith AI, Webb G, Li J, Song J, Xia J. A Deep Learning-Based Method for Identification of Bacteriophage-Host Interaction," IEEE/ACM Transacations on Computational Biology and Bioinformatics. 2020. DOI: 10.1109/TCBB.2020.3017386

[80] Leite DMC, Brochet X, Resch G, Que YA, Neves A, Peña-Reyes C. Computational prediction of inter-species relationships through omics data analysis and machine learning. BMC Bioinformatics. 2018;19:420. DOI: https://doi.org/10.1186/s12859-018-2388-7

[81] Ren J, Song K, Deng C, Ahlgren NA, Fuhrman JA, Li Y, Xie X, Poplin R, Sun F. Identifying viruses from metagenomic data by deep learning. Quantitative Biology. 2018;8:64-77. DOI: https://doi.org/10.1007/s40484-019-0187-4

[82] Edwards RA, McNair K, Faust K, Raes J, Dutilh BE. Computational approaches to predict bacteriophage-host relationships. FEMS Microbiology Reviews. 2016;40:258-272. DOI: https://doi.org/10.1093/femsre/fuv048