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Efficacy of phage therapy in pigs: systematic review and meta-analysis

Karina Desiree,[1] Sabrina Mosimann,[1] and Paul Ebner[2]

Department of Animal Sciences, Purdue University, West Lafayette, IN 47907, USA

[1]These authors contributed equally to this work.

[2]Corresponding author: pebner@purdue.edu

Abstract

Limits on the use and efficacy of various antibiotics coupled with negative consumer perception of the practice have together spurred substantial research into compounds that could reduce the use antibiotics to control bacterial diseases in pigs. Bacteriophages are often among such potential compounds, and various groups have examined the efficacy of bacteriophages or bacteriophage products in limiting transmission or colonization of targeted bacteria. The study presented here provides a systematic review of such studies followed by a meta-analysis of aggregated data produced by each study. The data set was limited to inputs (n = 19; 576 total observations) from studies where: 1) live pigs were inoculated with a known quantity of challenge bacteria; 2) challenged animals were treated with a known quantity of phages; 3) concentrations of the challenge bacteria were measured in different tissues/fluids following phage treatment; and 4) SD (or SE to allow calculation of SD) was reported. Concentrations of challenge bacteria were significantly lower in phage-treated pigs versus challenged but untreated pigs ($P < 0.0001$; effect size = –1.06 $1\log_{10}$ colony-forming units [**CFU**]/g). The effect size of phage treatment was significantly greater ($P < 0.05$) in samples collected 48 to 96 h following phage treatment versus those collected ≤ 24 h following phage treatment. Likewise, effect size of phage treatment was significantly greater in piglets versus market-weight pigs. Across observations, phage treatment effect sizes were greatest ($P < 0.01$) in fecal samples versus ileal or cecal samples. Taken together, these data indicate that phage treatment can significantly reduce the concentrations of targeted bacteria in pigs; scenarios exist, however, where phage treatment could predictably be more or less effective.

Key words: meta-analysis, phage, systematic review, swine

Introduction

The control of bacterial diseases in both veterinary and human medicine is central to the One Health concept (CDC, 2021). Effective antibiotics have been and continue to be a cornerstone of bacterial disease prevention and management programs. By most metrics, however, the development of antibiotic resistance outpaces the discovery and availability of new antibiotic drugs (Årdal et al., 2020). At the same time, numerous countries including the United States, China, and EU member states, among many others, have implemented policies that effectively curtail the use of many antibiotics in livestock for performance (e.g., improved growth efficiency; European Commission, 2018; Hu and Cowling, 2020; US FDA, 2021). The prospect of limitations on both efficacy and availability of existing antibiotics for treating or preventing emerging bacterial pathogens has spurred research into the antibacterial properties of nonantibiotic compounds for the treatment or prevention of bacterial infections (Ghosh et al., 2019).

One potential alternative to antibiotics is bacteriophages. Bacteriophages, or simply "phages," are viruses specific to bacteria. The traditional use of bacteriophages as antibacterials, termed "phage therapy," takes advantage of the infectious cycle

Abbreviations

CFU	colony-forming units
DNA	deoxyribonucleic acid
I^2	inconsistency index or percentage of heterogeneity
MOI	multiplicity of infection
PBS	phosphate-buffered saline
PFU	plaque-forming units

of lytic phages which results in lysis or death of the bacterial host. Recent phage research, however, has also investigated the therapeutic potential of isolated phage lysins (Fischetti, 2018; Vázquez et al., 2018) and lysogenic phages or temperate phages where infection leads to integration of phage DNA into the bacterial chromosome rather than immediate lysis (Yosef et al., 2015; Park et al., 2017; Monteiro et al., 2019). The use of bacteriophages as antibacterials predates the widespread introduction of antibiotics in both veterinary and human medicine (Sulakvelidze et al., 2001; Kutter et al., 2010), but the practice has regained attention in recent decades as a possible nonantibiotic means of bacterial disease control.

There is a growing body of research examining the efficacy of using phages as antibacterials specifically in food animal production. The majority of this research has involved monogastric animals, and this manuscript presents a systematic review of studies focused on the application of phage treatments to swine. The systematic review is accompanied by a meta-analysis aimed at aggregating results across studies to determine whether administering bacteriophages reduces concentrations of challenge bacteria in swine. The data used for meta-analysis were then disaggregated in an attempt to identify scenarios (e.g., phages used for prevention vs. treatment) or factors (e.g., effect of target pathogen, age of pigs, or sampling time, etc.) that may influence the impact of phage treatment on targeted bacterial concentrations.

Methods

A primary literature search for peer-reviewed published research assessing the efficacy of bacteriophage application in reducing or preventing bacterial colonization in pigs was conducted in the summer of 2020. The search utilized PubMed and Google Scholar databases and was restricted to research published between 1990 and present and identified using search terms including "swine + phage," "pig + phage," "swine + bacteriophage," and "pig + bacteriophage." The resulting articles were screened to remove review articles, non-peer-reviewed articles (e.g., theses/dissertations), other gray literature, articles utilizing species other than swine, and articles using ex vivo, in vitro, or in vivo models. This initial screen produced a pool of 25 articles describing experiments in which phages were delivered to live pigs to assess the antibacterial capacity of the phage treatment. References cited in remaining articles were screened to determine if other articles existed that fit selection criteria.

Descriptive statistics including the study author, year published, a brief results summary, study location, targeted bacteria, a description of experimental animal characteristics, and whether or not an animal trial was performed were recorded for each study. A qualitative assessment of each study was then conducted using the self-developed rubric described in Supplementary Table 1. Based on a review of this qualitative assessment, it was hypothesized that further meta-analysis with acceptable heterogeneity across studies could be conducted using studies that: 1) challenged live pigs with a known quantity of challenge bacteria (e.g., 10^8 colony-forming units [**CFU**] of *Salmonella enterica* Typhimurium per pig); 2) treated challenged animals with a known quantity of phages (e.g., 10^8 plaque-forming units [**PFU**] of *Salmonella* Phage FFH1); 3) measured concentrations of the challenge bacteria in different tissues/fluids following phage treatment; and 4) reported either SD or SE to allow calculation of SD. Thus, studies involving natural challenges or challenges with unknown quantities of bacteria, studies reporting only qualitative or semi-qualitative data (e.g., frequency of bacterial shedding), studies reporting total bacteria concentrations post-treatment but not challenge bacteria concentrations post-treatment specifically, or studies not reporting SD (or SE) were excluded. As a result, our final data set included 19 inputs (i.e., 19 distinct phage treatments/experiments) across five published studies with concentration of challenge bacteria following phage treatment as the principal outcome variable.

Statistical methods and packages (dmetar; Harrer et al., 2019a) were used following previously published guidelines (Harrer et al., 2019b) and processed using RStudio (RStudio Team, 2020). To construct the main data set for meta-analysis, the following data were extracted from each input ($n = 19$): 1) number of animals in treatment group; 2) number of animals in control group (i.e., animals receiving the challenge bacteria but not phage or any other antibacterial treatment); 3) mean concentration of the challenge bacteria in phage-treated animals with SD; and 4) mean concentration of challenge bacteria in control animals with SD. When concentration means, SD, or SE values were not included in the text, these values were estimated based on graphical data. These estimations are noted as such in the text of the systematic review below when they occurred. When multiple, independent experiments or treatments were reported within single research articles, each independent experiment or treatment was included as a separate input unless treatments only differed in terms of the titer of phage inocula, in which case input means were pooled. When a single experiment contained one control group but multiple phage treatment groups or inputs, the number of animals in the control group was divided equally across the number of treatment groups to avoid over-representation of individual studies in the data set. As a result, our data set included 360 treatment observations (challenge bacteria concentrations in challenged pigs treated with phages lytic against the challenge bacteria) and 240 control observations (challenge bacteria concentrations in challenged pigs not receiving phage or other antibacterial treatment).

Data were analyzed using the dmetar package of R (Harrer et al., 2019a) and the full data set was first screened for the presence of publication bias and "P-hacking". Subsequently, data were analyzed as a random-effects model to account for variation between studies (i.e., differences in breed and age of pigs, differences in study methods and operators, etc.; Harrer et al., 2019b). Variance of distribution of effect size was estimated using Sidik-Jonkman and the random-effects model was adjusted using the Hartung-Knapp method (Harrer et al., 2019b). Experimental designs of those studies producing observations identified in R as outliers were re-examined for evidence justifying removal of the observations. Data were also disaggregated by sampling time post-phage treatment, sample site (ileum, cecum, feces, or rectum), delivery or application method (e.g., gavage vs. feed), delivery time (e.g., phage given before or after bacterial challenge), age of pigs, type of treatment (e.g., single vs. poly-phage), species of challenge bacteria, frequency of phage application, and phage preparation (e.g.,

microencapsulation or other form of protection). Heterogeneity across studies was measured using the inconsistency index, or percentage of heterogeneity (I^2; Higgins and Thompson, 2002). Heterogeneity was considered low when I^2 values were between 0% and 25%, moderate when I^2 values were between 26% and 50%, considerable when I^2 values were between 51% and 75%, and significant when I^2 values were between 76% and 100%. As the identification of subgroups for data disaggregation was not random, comparisons between subgroups were made using a mixed-effects model that utilized a random-effects model within subgroups and a fixed-effects model between subgroups (Harrer et al., 2019a). Post-hoc power statistics (%) were calculated for the overall data set and for each disaggregated data set. Differences were considered statistically significant at $P < 0.05$.

Results and Discussion

Systematic review

Screening of the articles retrieved in the original primary literature search resulted in the identification of five articles for systematic review (Table 1) that: 1) challenged live animals with a known quantity of challenge bacteria (e.g., 10^8 CFU of *Salmonella enterica* Typhimurium per pig); 2) treated challenged animals with a known quantity of phages (e.g., 10^8 PFU of *Salmonella* Phage FFH1 per pig); 3) measured concentrations of the challenge bacteria in different tissues/fluids following phage treatment; and 4) reported a SD or SE. It is of note that the majority of studies included in this review measured other effects in addition to the principal outcome variable, such as performance (e.g., average daily gain, feed intake, etc.) and pathology (e.g., temperature, fecal scores, etc.) among others; however, those effects were outside the scope of this review and not discussed.

Saez et al. (2011) administered a microencapsulated phage cocktail by gavage to young pigs challenged with *Salmonella* Typhimurium at 2, 4, and 6 h post-challenge. No significant differences ($P > 0.05$) in the incidence of the challenge organism in fecal samples were observed between phage-treated and untreated pigs at any sampling point (2, 4, and 6 h post-challenge). At necropsy (6 h post-challenge), cecal concentrations of *Salmonella* Typhimurium were not significantly different ($P > 0.05$) in phage-treated versus untreated pigs. However, ileal concentrations of *Salmonella* Typhimurium were significantly lower ($P < 0.05$) in phage-treated (1.0 $\log_{10}$ CFU/mL) versus untreated pigs (3.0 $\log_{10}$ CFU/mL). In addition, ileal concentrations of *Salmonella* Typhimurium were below the detectable limit (< 10^2 CFU/mL) in samples from phage-treated pigs (undetectable in 71.4% of ileal samples) significantly more often ($P < 0.05$) than in samples from untreated pigs (undetectable in 19.0% of ileal samples).

In the same paper, the authors administered the same phage cocktail via feed to pigs using a similar challenge and sampling model. In this experiment, phage-treated pigs had a significantly lower ($P < 0.05$) incidence of *Salmonella* Typhimurium in fecal samples at 2 (38.1% positive) and 4 h (42.9% positive) post-challenge compared to untreated pigs (2 h: 71.4% positive; 4 h: 85.7% positive). At 6 h post-challenge, all fecal samples were *Salmonella* Typhimurium-positive regardless of treatment. At necropsy, a numerical difference ($P < 0.10$) was observed in the cecal concentrations of *Salmonella* Typhimurium in phage-treated (2.7 $\log_{10}$ CFU/mL) versus untreated pigs (3.7 $\log_{10}$ CFU/mL). As in the previous experiment, ileal concentrations of challenge bacteria at necropsy were significantly lower ($P < 0.05$) in phage-treated (2.0 $\log_{10}$ CFU/mL) versus untreated pigs (3.0 $\log_{10}$ CFU/mL). It is not clear why ileal concentrations of the challenge bacteria significantly differed between phage-treated and untreated pigs in both trials while cecal concentrations did not. Average *Salmonella* Typhimurium concentrations were similar across cecal and ileal samples from untreated pigs, suggesting that phage-bacterium interactions and bacterial lysis were possible in both organs. As before, *Salmonella* Typhimurium concentrations in ileal samples from phage-treated pigs were more frequently ($P < 0.05$) below detectable levels (undetectable in 42.8% of ileal samples) than in samples from untreated pigs (undetectable in 19.0% of ileal samples). The authors noted that phage concentrations in ileal and cecal contents of pigs given phages via feed were 2 to 3 $\log_{10}$ PFU/mL higher at necropsy than those in ilea and ceca of pigs given phages via oral gavage, suggesting better rates of phage survival when phages were administered in feed.

Employing similar experimental methods, Wall et al. (2010) conducted two experiments in which a microencapsulated phage cocktail was administered to *Salmonella* Typhimurium-challenged young pigs. At necropsy (6 h post-challenge), phage-treated pigs had numerically lower (not statistically compared) concentrations of *Salmonella* Typhimurium in ileal (0.6 $\log_{10}$ CFU/mL), cecal (0.4 $\log_{10}$ CFU/mL), and cecal tonsil samples (0.4 $\log_{10}$ CFU/mL) versus untreated pigs (ileal: 2.6 $\log_{10}$ CFU/mL, cecal: 3.6 $\log_{10}$ CFU/mL; cecal tonsil: 3.6 $\log_{10}$ CFU/mL). Four of six lymph node samples from untreated pigs and three of six lymph nodes samples from phage-treated pigs were positive for the challenge organism; all fecal samples were positive for the challenge organism by 6 h post-challenge. Among phage-treated pigs, five of six ileal samples, five of six cecal samples, and five of six cecal tonsil samples had levels of *Salmonella* Typhimurium below the detectable limit (< 10^2 CFU/mL). In contrast, only three of five ileal samples, two of five cecal samples, and one of five cecal tonsil samples from untreated pigs had undetectable levels of *Salmonella* Typhimurium. These values, however, were not statistically compared.

In the second experiment of the same study, the authors inoculated market weight pigs with *Salmonella* Typhimurium, subsequently placing the pigs in a pen and allowing manure to accrue for 48 h. After 48 h, unchallenged pigs were administered the microencapsulated phage cocktail and comingled with the challenged pigs (mock treated pigs were included as controls). At necropsy (6 h post-mingling), cecal concentrations of *Salmonella* Typhimurium were significantly lower ($P < 0.05$) in phage-treated (1.5 $\log_{10}$ CFU/mL) versus mock-treated pigs (2.9 $\log_{10}$ CFU/mL). There was a statistical trend ($P = 0.06$) for lower ileal concentrations of *Salmonella* Typhimurium in phage-treated (1.7 $\log_{10}$ CFU/mL) versus mock-treated pigs (2.7 $\log_{10}$ CFU/mL). As the authors note, differences in concentrations of the challenge organism in phage-treated pigs in the first and second trials may have been due to differences in the challenge model and response to the bacterial challenge and phage treatment between older and younger pigs.

The incidence of *Salmonella* Typhimurium in lymph node and fecal samples was not significantly different ($P > 0.05$) between phage-treated and mock-treated pigs at any sampling point. All fecal samples were positive for the challenge organism at 6 h post-mingling regardless of treatment. The authors report that in both studies fecal and lymph node samples were enriched twice due to low initial counts of the challenge organism. For this reason, and because concentrations of the challenge organism were not enumerated for these sample types, the

Table 1. Descriptions of studies measuring challenge bacteria concentrations following application of phage in pigs included in systematic review and meta-analysis

Study code	Reference	Phage description	Challenge organism	Phage preparation	Pig characteristics	Delivery method	Delivery schedule	Sample sites	Number of inputs	Total number of observations
Swine_4	Albino et al., 2014	Six phages isolated with *Salmonella enterica* Typhimurium	*Salmonella enterica* Typhimurium	Suspended in SM buffer [1]	90–100 kg	Oral	Phages delivered after bacterial challenge	I[2], C[3], F[4]	4	90
Swine_14	Wall et al., 2010	15 phages isolated with *Salmonella enterica* Typhimurium	*Salmonella enterica* Typhimurium	Microencapsulated	15 kg (Exp. 1); 90 kg (Exp. 2)	Oral gavage	Phages delivered before bacterial challenge	I, C, F	4	120
Swine_15	Callaway et al., 2011	Two phages isolated with *Salmonella enterica* Typhimurium	*Salmonella enterica* Typhimurium	Suspended in phosphate-buffered saline (PBS)[5]	10 kg	Oral gavage	Phages delivered after bacterial challenge	C, F	4	192
Swine_16	Saez et al., 2011	15 phages isolated with *Salmonella enterica* Typhimurium	*Salmonella enterica* Typhimurium	Microencapsulated	15 kg	Oral gavage (Exp. 1); In-feed (Exp. 2)	Phages delivered before bacterial challenge	I, C, F	4	126
Swine_22	Han et al., 2016	Multiple (number not stated) phages isolated with *E. coli* K88 or *E. coli* K99	*E. coli* K88 (Exp. 1) or *E. coli* K99 (Exp. 2)	Suspended in SM buffer and freeze-dried 0.2 M Tris buffer (pH 7.5) containing 0.1 M NaCl, 1 mM MgSO4, and 0.01% gelatin, and freeze-dried	~28 d of age	In-feed	Phages delivered after bacterial challenge	F	4	48

[1]SM buffer, 0.2 M Tris buffer (pH 7.5) containing 0.1 M NaCl, 1 mM $MgSO_4$, and 0.01% gelatin; [2]I, ileal contents; [3]C, cecal contents; [4]F, feces (or rectal contents); [5]PBS, 0.137M NaCl, 2.7mM KCl, 0.01M Na_2HPO_4, 1.8mM KH_2PO_4.

authors suggest that the quantitative data on cecal and ileal concentrations of *Salmonella* Typhimurium may be more meaningful than fecal and lymph node qualitative data when evaluating phage treatment efficacy.

Albino et al. (2014) administered a phage cocktail (at four different concentrations) suspended in sodium bicarbonate to *Salmonella* Typhimurium-challenged market-weight pigs (90 to 100 kg). At necropsy (18 ± 2 h post-treatment), concentrations of *Salmonella* Typhimurium in cecal and ileal samples were not significantly different ($P > 0.05$) between phage-treated and untreated pigs. The largest numerical differences in *Salmonella* Typhimurium concentrations between groups were observed between pigs treated with 10^{10} PFU/pig (ileal contents: ~4.2 $\log_{10}$ CFU/mL; cecal contents: ~5.1 $\log_{10}$ CFU/mL) and untreated pigs (ileal contents: ~5.5 $\log_{10}$ CFU/mL; cecal contents: ~6.3 $\log_{10}$ CFU/mL). Similarly, the incidence of *Salmonella* Typhimurium in fecal samples collected at necropsy was not significantly different ($P > 0.05$) in phage-treated versus untreated pigs, though numerical differences in incidence were observed (10^4 PFU/pig treatment group: 83.3% positive; 10^6 PFU/pig treatment group: 50% positive; 10^8 PFU/pig treatment group: 33.3% positive; 10^{10} PFU/pig treatment group: 33.3% positive; untreated pigs: 100% positive).

Callaway et al. (2011) orally administered a two-phage cocktail to weaned pigs (~10 kg) at 24 and 48 h after a *Salmonella* Typhimurium challenge. No significant differences ($P > 0.05$) in fecal concentrations of *Salmonella* Typhimurium were observed between phage-treated and untreated pigs at any sampling point (24, 48, 72, and 96 h post-challenge) during the experiment, though numerically lower fecal concentrations of the challenge organism were recorded for phage-treated pigs at 48 (phage-treated: ~1.25 $\log_{10}$ CFU/mL; untreated: ~2.25 $\log_{10}$ CFU/mL), 72 (phage-treated: ~0.70 $\log_{10}$ CFU/mL; untreated: ~1.70 $\log_{10}$ CFU/mL), and 96 h (phage-treated: ~0.38 $\log_{10}$ CFU/mL; untreated: ~1.25 $\log_{10}$ CFU/mL) post-challenge. At necropsy, cecal and rectal concentrations of *Salmonella* Typhimurium did not significantly differ ($P > 0.05$) between treatment groups, though concentrations were numerically lower in phage-treated pigs (cecal contents: ~1.40 $\log_{10}$ CFU/mL; rectal contents: ~0.38 $\log_{10}$ CFU/mL) versus untreated pigs (cecal contents: ~2.75 $\log_{10}$ CFU/mL; rectal contents: ~1.3 $\log_{10}$ CFU/mL). The incidence of *Salmonella* Typhimurium was significantly lower ($P < 0.05$) in rectal samples from phage-treated pigs (25% positive) compared to those of untreated pigs (~83.3% positive). No significant differences ($P > 0.05$) in the incidence of the challenge organism in ileal or lymph node samples were observed between any groups. To note, all concentrations and percentages stated here are approximations based on the authors' graphical data.

Han et al. (2016) administered a phage cocktail via feed to 28-d-old pigs challenged with Escherichia *coli* K88 and K99. Phage treatment began on the day of challenge and continued until the conclusion of the study (7 d post-challenge). Over the course of sampling (1, 3, and 7 d post-challenge), fecal concentrations of *E. coli* K99 did not significantly differ ($P > 0.05$) between phage-treated and untreated pigs. However, overall fecal concentrations of *E. coli* K88 were significantly lower ($P < 0.01$) in phage-treated versus untreated pigs (quantitative data for overall fecal concentrations not shown). Adhesion of *E. coli* K88 to the ileum and cecum of phage-treated pigs (ileum: 5.57 ± 0.263 $\log_{10}$ CFU/g; cecum: 3.92 ± 0.800 $\log_{10}$ CFU/g) was significantly lower ($P < 0.05$) than adhesion to the same tissue types in untreated pigs (ileum: 7.24 ± 0.460 $\log_{10}$ CFU/g; cecum: 6.32 ± 0.504 $\log_{10}$ CFU/g). Adhesion of *E. coli* K88 to the duodenum, jejunum, colon, and mesenteric lymph node did not significantly differ ($P > 0.05$) between treatment groups. Adhesion of *E. coli* K99 did not significantly differ ($P > 0.05$) between phage-treated and untreated pigs for any sample type.

Meta-analysis

Data from the studies described in the above systematic review were aggregated, producing 19 inputs for meta-analysis and 576 total observations. Funnel plots did not show definite signs of asymmetry indicating no presence of publication bias (data not shown). Similarly, results from an Egger's test were not significant ($P = 0.34$), indicating a low likelihood of small sample bias. Finally, *P*-curve analysis indicated the dataset had evidential value or effect size (Right Skewness Test: $P < 0.05$) and adequate power (Flatness Test: $P = 0.99$) with no evidence of "*P*-hacking".

Comparisons of effects sizes across all groups and subgroups are presented in Tables 2 and 3. When all observations ($n = 576$) were analyzed together, concentrations of challenge bacteria were significantly lower ($P < 0.0001$) in pigs treated with phages compared to untreated pigs ($P < 0.0001$; effect size = –1.06 $\log_{10}$ CFU/g). While heterogeneity across all observations was considerable ($I^2 = 64.2\%$), post-hoc analysis of power indicated a 100% probability of detecting statistical differences in the data set.

Phages are biologicals and their viability and subsequent lytic capacity can be affected by environmental conditions including heat, extreme pH, multiplicities of infection, and the presence of proteinases, among other factors (Iriarte et al., 2007; Huff et al., 2010; Knezevic et al., 2011; Hodyra-Stefaniak et al., 2015; Zhang et al., 2015; Colom et al., 2017; El-Dougdoug et al., 2019). Thus, our data set was disaggregated based on different factors in an effort to identify conditions under which effect size (i.e., reductions in challenge bacteria) was greatest.

As phages are self-replicating and lysis is often influenced by multiplicities of infection (Delbrück, 1940; Payne and Jansen, 2001; Kasman et al., 2002; Payne and Jansen, 2003; Huff et al., 2006; Callaway et al., 2008), we hypothesized that assessments of whether or not phage treatment reduced challenge bacteria could be influenced by sampling time, i.e., how soon bacterial concentrations were measured following phage application. To test this hypothesis, observations were disaggregated into two subgroups: 1) observations of bacterial concentrations measured ≤ 24 after the first application of bacteriophages; and 2) observations of bacterial concentrations measured 48 to 96 h after the first application of bacteriophages. Concentrations of challenge bacteria in phage-treated pigs were lower than in untreated pigs whether samples were collected ≤ 24 h following phage application ($P < 0.001$; effect size = –0.82 $\log_{10}$ CFU/mL; $I^2 = 57.4\%$) or 48 to 96 h following application of phages ($P < 0.03$; effect size = –1.78 $\log_{10}$ CFU/mL; $I^2 = 36.6\%$). Effect size, however, was larger ($P < 0.05$) with less heterogeneity in samples collected 48 to 96 h following application of phages. Thus, while phage application can significantly reduce concentrations of targeted bacteria within 24 h, greater reductions may be observed 2 to 4 d following phage application. Such results could have implications for the timing of phage administration for maximum bacterial reductions at desired time points.

Younger pigs are often more susceptible to infections than older pigs. This is true regarding gastrointestinal pathogens and, as our data set only included studies utilizing *Salmonella* or *E. coli* challenge models (Fairbrother et al., 2005; Wales et al., 2011), it was of interest to disaggregate the data set based on pigs' age (i.e., piglets vs. market weight pigs). Treating piglets or market weight pigs with phages significantly ($P < 0.05$)

Table 2. Meta-analysis (effect sizes) of studies measuring challenge bacteria concentrations following application of phage in pigs

Parameter	I^2, %	Effect size	Effect significance, *P*-value	Power, %	No. observations, Treatment	No. observations, Control	Total observations
All observations together	64.2	−1.06	<0.0001	100	336	240	576
Sampling Time							
Samples collected ≤24 h post-phage treatment	57.4	−0.82	0.0007	96.96	252	156	408
Samples collected 48–96 h post-phage treatment	36.6	−1.78	0.022	99.72	84	84	168
Age of Pig							
Piglets	62.8	−1.31	0.0001	100	216	174	390
Piglets, samples collected ≤24 h post-phage treatment	65.8	−1.075	0.0037	99.97	132	90	222
Piglets, samples collected 48–96 h post-phage treatment	36.6	−1.78	0.022	99.72	84	84	168
Market Weight Pigs, samples collected ≤24 h post-phage treatment	0	−0.50	0.0138	99.76	120	66	186
Targeted Bacteria							
Salmonella	64.2	−0.95	<0.0001	100	312	216	528
Salmonella, samples collected ≤24 h post-phage treatment	59.3	−0.77	0.002	100	240	144	384
Salmonella, samples collected 48–96 h post-phage treatment[1]	0	−1.53	0.002	99.22	72	72	144
E. coli	69.1	−1.89	0.1084	71.46	24	24	48
E. coli, samples collected ≤24 h post-phage treatment[1]	66.5	−1.29	0.3743	43.15	12	12	24
E. coli, samples collected 48–96 h post-phage treatment	82.7	−2.68	0.3622	431.5	12	12	24
Frequency of Phage Application							
Single Dose[1]	0.0	−0.25	0.0202	80.67	72	18	90
Multiple Doses	63.4	−1.21	<0.0001	100	264	222	486
Multiple Doses, samples collected ≤24 h post-phage treatment	60.9	−0.98	0.0011	100	180	138	318
Multiple Doses, samples collected 48–96 h post-phage treatment	36.6	−1.78	0.022	99.72	84	84	168
Prophylaxis vs. Treatment							
Prophylaxis	0.0	−0.72	0.0017	99.99	144	102	270
Prophylaxis, samples collected ≤24 h post-phage treatment	0.0	−0.24	0.0017	99.99	144	102	246
Treatment	71.0	−1.31	0.0025	100	192	138	330
Treatment, samples collected ≤24 h post-phage treatment	78.4	−0.93	0.0748	99.28	108	54	162
Treatment, samples collected 48–96 h post-phage treatment	36.6	−1.78	0.022	99.72	84	84	168
Sample Site							
Ileum[2]	30.4	−0.40	0.0008	99.77	96	78	174
Cecum	50.6	−0.92	0.0133	99.97	120	102	222
Cecum, samples collected ≤24 h post-phage treatment	79.2	−0.60	0.2709	99.77	96	78	174
Feces	69.4	−1.58	0.0062	99.97	120	102	222
Feces, samples collected ≤24 h post-phage treatment	81.4	−1.28	0.1078	95.28	60	42	102
Feces, samples collected 48–96 h post-phage treatment	50.6	−1.94	0.0617	97.91	60	60	120
Administration Route							
Gavage	68.9	−1.00	0.0002	100	270	216	486
Gavage, samples collected ≤24 h post-phage treatment	67.6	−0.82	0.0061	100	198	144	342
Gavage, samples collected 48–96 h post-phage treatment[1]	0	−1.53	0.002	99.22	72	72	144
Feed	62.7	−1.35	0.0631	98.72	66	66	132
Feed, Gavage, samples collected ≤24 h post-phage treatment	25.5	−0.81	0.0966	96.64	54	54	108
Feed, samples collected 48–96 h post-phage treatment[1]	82.7	−2.68	0.3622	43.15	12	12	24

Table 2. Continued

Parameter	I^2, %	Effect size	Effect significance, *P*-value	Power, %	No. observations, Treatment	No. observations, Control	Total observations
Phage Protection							
Protected	43.2	–1.027	0.0029	100	168	126	294
Protected, samples collected ≤24 h post-phage treatment	10	–0.80	0.0011	100	156	114	270
Protected, samples collected ≤24 h post-phage treatment[1]	82.7	–2.68	0.3622	43.15	12	12	24
Unprotected	75.7	–1.13	0.0112	100	144	90	234
Unprotected, samples collected ≤24 h post-phage treatment	85.0	–0.79	0.2307	97.82	96	42	138
Unprotected, samples collected ≤24 h post-phage treatment[1]	0.0	–1.53	0.002	99.22	72	72	144

[1]Only one study; [2]Data same as ileum <24 h.

reduced challenge bacteria concentrations (piglets: $P < 0.0001$; effect size = –1.31 $\log_{10}$ CFU/mL; I^2 = 62.8%; market weight pigs: $P < 0.015$; effect size = –0.50 $\log_{10}$ CFU/mL; I^2 = 0.0%). The piglet subgroup was further disaggregated by sample time. Concentrations of challenge bacteria in samples collected from piglets ≤ 24 h after phage application ($P < 0.004$; effect size = –1.076 $\log_{10}$ CFU/mL; I^2 = 65.8%) or 48 to 96 h after phage application (effect size = –1.78 $\log_{10}$ CFU/mL; I^2 = 36.6%) were both significantly ($P = 0.05$) lower than those of control pigs. As the data set did not contain any observations of samples collected from market weight pigs 48 to 96 h after phage application, the market weight pig subgroup was not further disaggregated by sample time. Taken together, phage treatment appears to be more effective at reducing challenge bacteria concentrations in younger pigs than in older pigs. The increased heterogeneity across studies utilizing market weight pigs and the absence of studies in the data set where challenge bacteria concentrations were measured > 24 h post-phage treatment in market weight pigs should temper these conclusions.

It is reasonable to hypothesize that efficacy of phage treatment depends on the bacterial organism targeted. As previously noted, however, the data set consisted only of studies utilizing either *Salmonella* or *E. coli*, which limited ability to make inferences on phage treatment efficacy to broader groups of bacteria. When the data set was disaggregated by challenge organism (i.e., *Salmonella* or *E. coli*), concentrations of *Salmonella* in phage-treated pigs were significantly lower in phage-treated pigs compared to untreated pig across sample times, but with considerable heterogeneity ($P < 0.0001$; effect size = –0.95$\log_{10}$ CFU/mL; I^2 = 64.2%). As in other comparisons, effect size in studies targeting *Salmonella* was significantly ($P < 0.001$) greater when samples were collected 48 to 96 h (effect size = –1.53 $\log_{10}$ CFU/mL) compared to when samples were collected ≤ 24 h following phage application (effect size: –0.77 $\log_{10}$ CFU/mL). There was a trend for concentrations of *E. coli* in phage-treated pigs ($P = 0.11$; effect size: –1.90 CFU/mL) to be lower than those in untreated pigs. There were no significant differences in concentrations of *E. coli* between phage-treated and untreated pigs when those studies were further disaggregated by sample time. As disaggregation by sample time in this case reduced power statistics to 43.15%, inferences from these results should be tempered.

The studies included in our data set used a variety of dosing regimens. One variable across dosing regimens was the number of times phages were administered to the pigs. Only one study in the data set utilized a single-dose approach; in this instance, no differences in concentrations of the challenge bacteria between phage-treated and untreated pigs were reported. Administration of multiple doses of phages, however, significantly reduced challenge bacteria concentrations between phage-treated and untreated pigs ($P = < 0.0001$; effect size = –1.21 $\log_{10}$ CFU/mL; I^2 = 63.4%). When studies using multiple doses of phage were disaggregated by sample time, there were no differences ($P = 0.13$) in effect size between samples collected ≤ 24 h after phage application (–0.98 CFU/mL) versus those collected 48 to 96 h following phage application (–1.78 CFU/mL).

Phage treatment could be used as a prophylaxis to prevent bacterial colonization and/or as a therapeutic to reduce existing bacterial infections. To assess the potential efficacy of phage treatments in these two scenarios, the data set was disaggregated based on whether phage treatment was applied before (prophylactic scenario) or after (therapeutic scenario) pigs were challenged with bacteria. Concentrations of challenge bacteria in phage-treated pigs were significantly lower in phage-treated pigs compared to control pigs whether the phage treatment was applied in prophylactic ($P < 0.002$; effect size = –0.72 $\log_{10}$ CFU/mL; I^2 = 0.0%) or therapeutic manners ($P < 0.003$; effect size = –1.31 $\log_{10}$ CFU/mL; I^2 = 71.0%). There were no statistical differences in targeted bacteria concentrations between pigs receiving phage prophylactically versus pigs receiving phage therapeutically regardless of sample time. When studies using a therapeutic approach were further disaggregated by sample time, there was a statistical trend ($P = 0.08$) for concentrations of challenge bacteria to be lower in phage-treated versus untreated pigs when samples were collected ≤ 24 h after phage application (effect size = –0.93; I^2 = 78.4%). In contrast, concentrations of challenge bacteria were statistically lower in phage-treated pigs versus untreated pigs in therapeutic scenarios when samples were collected 48 to 96 h following phage application ($P < 0.03$; effect size = –1.78 $\log_{10}$ CFU/mL; I^2 = 36.6) but not when samples were collected < 24 h following phage application. Finally, the efficacy of phages in a therapeutic scenario was not affected by sample time (no significant differences in ≤ 24 h vs. 48 to 96 h post-treatment samples). There were no studies in the data set that measured challenge bacteria concentrations at 48 to 96 h following phage application in a prophylaxis scenario making similar comparisons across sample time impossible.

As studies in the data set utilized gastrointestinal pathogens, bacterial concentrations were measured primarily in ileal, cecal, or fecal samples (including rectal samples). When the data set was disaggregated by sample site, significant differences were

Table 3. Comparisons effect sizes across subgroups in studies measuring challenge bacteria concentrations following application of phage in pigs

Parameter Subgroup 1	Effect size	Subgroup 2	Effect size	Subgroup 3	Effect size	P-value
All Observations						
Piglets	−1.31	Market Wt. Pigs	−0.50			0.002
Feed Admin.	−1.35	Gavage Admin.	−1.01			0.57
Poly-phage Treatment	−1.21	Single-phage Treatment	−0.25			<0.001
Target: *E. coli*	−1.89	Target: *Salmonella*	−0.94			0.27
Prophylaxis	−0.72	Therapeutic	−1.31			0.109
Protected Phage	−1.03	Unprotected Phage	−1.13			0.80
Cecal Samples	−1.04	Fecal Samples	−1.58	Ileal Samples	−0.49	0.005
Samples collected ≤24 h post-phage treatment						
Market Wt. Pigs	−0.50	Piglets	−1.08			0.047
Feed Admin	−0.82	Gavage Admin.	−0.82			0.99
Poly-phage Treatment	−0.98	Single-phage Treatment	−0.25			0.0008
Target: *E. coli*	−1.29	Target: *Salmonella*	−0.77			0.56
Therapeutic	−0.93	Prophylaxis	−0.72			0.65
Protected Phage	−0.80	Unprotected Phage	−0.79			0.99
Cecal Samples	−0.92	Fecal Samples	−1.28	Ileal Samples	−0.49	0.16
Samples collected 48–96 h post-phage treatment						
Feed Admin	−2.68	Gavage Admin	−1.53			0.50
Target: *E. coli*	−2.68	Target: *Salmonella*	−1.53			0.50
Protected Phage	−2.68	Unprotected Phage	−1.53			0.50
Cecum	−1.43	Feces	−1.94			0.49
Piglets						
Samples collected ≤24 h post-phage treatment	−1.08	Samples collected 48–96 h post-phage treatment	−1.78			0.20
Feed Administrations						
Samples collected ≤24 h post-phage treatment	−0.81	Samples collected 48–96 h post-phage treatment	−2.68			0.29
Gavage Administrations						
Samples collected ≤24 h post-phage treatment	−0.82	Samples collected 48–96 h post-phage treatment	−1.53			0.0032
Poly-phage Treatment						
Samples collected ≤24 h post-phage treatment	−0.98	Samples collected 48–96 h post-phage treatment	−1.78			0.13
Target: *E. coli*						
Samples collected ≤24 h post-phage treatment	−1.29	Samples collected 48–96 h post-phage treatment	−2.68			0.47
Target: *Salmonella*						
Samples collected ≤24 h post-phage treatment	−0.77	Samples collected 48–96 h post-phage treatment	−1.53			0.00019
Therapeutic Application						
Samples collected ≤24 h post-phage treatment	−0.93	Samples collected 48–96 h post-phage treatment	−1.78			0.18
Protected Phage						
Samples collected ≤24 h post-phage treatment	−0.80	Samples collected 48–96 h post-phage treatment	−2.68			0.27
Unprotected Phage						
Samples collected ≤24 h post-phage treatment	−0.79	Samples collected 48–96 h post-phage treatment	−1.53			0.16
Cecal Samples						
Samples collected ≤24 h post-phage treatment	−0.80	Samples collected 48–96 h post-phage treatment	−1.43			0.14
Fecal Samples						
Samples collected ≤24 h post-phage treatment	−1.28	Samples collected 48–96 h post-phage treatment	−1.94			0.45

found in challenge bacteria concentrations in ileal ($P < 0.001$; effect size: −0.40 CFU/mL; $I^2 = 30.4\%$), cecal ($P < 0.02$; effect size: −0.92 CFU/mL; $I^2 = 50.6\%$) and fecal ($P < 0.01$; effect size: −0.1.58 CFU/mL; $I^2 = 69.4\%$) contents of phage-treated versus untreated pigs. When ileal, cecal, and fecal samples were disaggregated by sample time, no statistical differences in challenge bacteria concentrations between phage-treated and untreated pigs were detected across samples sites whether samples collected ≤ 24 after phage application or 48 to 96 h following phage application.

Phages have been applied orally, intranasally, and in-feed, among other routes (Wall et al., 2010; Saez et al., 2011; Verstappen et al., 2016). The studies included in the data set administered the phage treatments either by gavage (or similar oral application) or in feed. It is likely that the experiments in the data set utilized oral administration routes because the only challenge bacteria included in experiments in the data set, *Salmonella* and *E. coli*, are largely intestinal pathogens. Concentrations of challenge bacteria were significantly lower in phage-treated versus untreated pigs when phages were delivered by gavage ($P < 0.001$; effect size = –1.00 $\log_{10}$ CFU/mL; $I^2 = 68.9\%$) and there was a statistical trend for lower challenge organism concentrations in phage-treated versus untreated pigs when the phage was delivered in feed ($P = 0.063$; effect size = –1.35 $\log_{10}$ CFU/mL; $I^2 = 62.7\%$). The effect size in pigs administered phage by gavage was significantly ($P < 0.004$) greater when samples were collected 48 to 96 h following phage application (effect size: –1.53 CFU/mL) than when samples were collected ≤ 24 h after phage application (effect size: –0.82 $\log_{10}$ CFU/mL). In phage-treated pigs administered phages via feed, there were no statistical differences ($P = 0.29$) in effect size when samples were collected ≤ 24 h post-phage treatment (–0.81 $\log_{10}$ CFU/mL) versus samples collected 48 to 96 h post-phage treatment (–2.68 $\log_{10}$ CFU/mL). The smaller number of observations from studies using feed administration routes likely resulted in reduced statistical power (43.2%) and increased heterogeneity ($I^2 = 82.7\%$), which, when taken together, could have resulted in a scenario where substantially but not statistically different effect sizes were observed.

Numerous groups have shown that bacteriophage viability can be reduced due to digestive enzymes, extreme pH, high temperatures, host immunity, among other environmental factors (Vitiello et al., 2005; Capparelli et al., 2006; Iriarte et al., 2007; Huff et al., 2010; Knezevic et al., 2011; Hodyra-Stefaniak et al., 2015; Zhang et al., 2015; Colom et al., 2017; El-Dougdoug et al., 2019). As such, we disaggregated observations based on whether the phages themselves received any type of preparation in order to protect them from the gastric environment. Whether phages were unprotected or protected (microencapsulation and freeze drying were both used in different studies) prior to application, concentrations of challenge bacteria were significantly lower in phage-treated versus untreated pigs (unprotected: $P < 0.02$; effect size: –1.13 $\log_{10}$ CFU/mL; $I^2 = 75.70\%$; protected: $P < 0.003$; effect size: –1.03 $\log_{10}$ CFU/mL; $I^2 = 43.2\%$). When these data were disaggregated by sample time, concentrations of challenge bacteria in pigs treated with unprotected phages were significantly different from those of untreated pigs at when samples were collected 48 to 96 h following phage application ($P < 0.01$; effect size: –1.53 $\log_{10}$ CFU/mL; $I^2 = 0.0\%$) but not when collected ≤ 24 h after phage application ($P = 0.24$; effect size: –0.79 $\log_{10}$ CFU/mL; $I^2 = 85.0\%$). The opposite was seen among studies using protected phages where concentrations of target bacteria in phage-treated pigs were significantly lower than those of controls when samples were collected ≤ 24 h after phage application ($P = 0.36$; effect size: –2.6838; $I^2 = 82.7\%$). In both cases, the disappearance of statistical significance despite larger effect sizes may be due to the lower number of observations coupled with increased heterogeneity in the disaggregated data set. Likewise, no effect of sample time was detected between pigs treated with unprotected versus protected phages.

There are some potential limits to this study and to extrapolating its results to larger populations. In an effort to limit heterogeneity across inputs, the data set was limited to only studies that challenged pigs with known amounts of bacteria, treated those pigs with known amounts of phage, measured the concentration of the specific challenge bacteria post-phage treatment, and provided standard deviation values (or standard error values allowing standard deviation calculation). While using stringent criteria to select studies produced a data set of very methodologically similar experiments for comparison, doing so also reduced the number of inputs to the data set. Hippel et al. (2015) reported that I^2, the statistic used in this study for gauging heterogeneity, can be biased in meta-analyses by small samples sizes (a case seen in many meta-analyses) as much as inter-study differences. Thus, while heterogeneity in meta-analyses is expected and heterogeneity in our data sets was usually moderate, heterogeneity was substantial in some cases when the data set was further disaggregated to form subgroups. In such cases, I2 values may have been influenced by small-sample bias rather than true heterogeneity. Furthermore, our post-hoc analysis revealed high power and significant effect between phage-treated and untreated group for most groups (Tables 2 and 3).

Additionally, it was of interest to understand the influence of phage concentration or titer on treatment efficacy. Creating sensible subgroups for comparison based on phage concentrations, however, was not possible as: 1) there was not a tremendous amount of variability in phage concentrations employed and few studies included low concentration (e.g., 10^5 PFU/mL) treatments; 2) different studies used different application rates and frequencies presenting confounding factors to accurately calculating the actual amount of phage administered; and 3) of the few studies that measured phages at sites of infection, none discriminated between applied phages and endogenous phages. In the end, subgroups for comparison were made based on the frequency of phage administration over the course of treatment (single vs. multiple applications); however, we recognize that such a comparison provides only limited insight into the impact of phage concentration on phage treatment efficacy.

Nevertheless, the data presented here do indicate that across independent studies, administering phage treatment to pigs results in significantly lower concentrations of the challenge bacteria in tissues of phage treated pigs compared to those of challenged but untreated pigs. As such, these findings support the use of phage therapy as a potential alternative to traditional antibiotics. These data also provide insight regarding scenarios in which phage therapy may be most effective in controlling bacterial transmission in pig production. For example, reductions in targeted bacteria may be time dependent with the greatest effect seen 48 h or more after phage treatment. As in the case of antibiotic treatment, multiple application of phage over the course of the treatments is likely to increase treatment efficacy.

Finally, this meta-analysis considers the impact of phage treatment on concentrations of targeted bacteria. In many cases, antibiotic treatment of animals as a management practice ultimately results in improved growth efficiency or performance. Thus, it will be of interest to conduct similar analyses as presented here to determine whether phage treatment improves key performance indicators such as average daily gain, feed intake, and/or feed:gain conversions.

Supplementary Data

Supplementary data are available at *Journal of Animal Science* online.

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Conflict of interest statement

The authors declare they have no conflicts of interest.

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