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# Bacteriophages to treat infections with multidrug resistant pathogens

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# Bacteriophages to treat infections with multidrug resistant pathogens

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# Editorial: Bacteriophages to treat infections with multidrug resistant pathogens

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KEYWORDS

**bacteriophage (phage), phage therapy, antimicrobial resistance (AMR), bacteria, infections**

Editorial on the Research Topic
Bacteriophages to treat infections with multidrug resistant pathogens

This Research Topic was designed by us in response to the growing threat of antibiotic resistance of bacterial infections worldwide. The use of bacteriophages (phages) as alternatives or adjunct agents to treat infections is gaining wider use. Indeed, phage therapy has been successfully used to treat patients across a broad range of pathologies, with substantial clinical improvement and bacterial eradication (1). However, we noted that only a few clinical requests for phage therapy have been fulfilled successfully due to a lack of understanding of the biology of phage-host interactions and resistance development (2), the human immune response to phage particles (3), the right phage dosage and pharmacokinetics (4). Bacterial defenses such as, restriction-modification and CRISPR-Cas system, also contribute to abortive infections by modifying phage DNA (5).

At the conclusion of the Research Topic, 12 articles have been published that address critical and interesting issues. There were six original or brief research reports, five reviews or mini reviews and one case report. There were reviews of phage therapy; studies on phages active against *Shigella* spp., *Salmonella* strains, *Escherichia coli*, and *Clostridioides difficile*; use of phage endolysin or tail proteins; the effect of mucous or dopamine on phages; a salutary case report of phage therapy failure; use of machine learning and simulations to test phage therapy; and a review of regulatory hurdles for the use of phage.

Phages are versatile agents that can be used for various purposes, from food safety to human health. However, lack of comprehensive guidelines and regulations in many countries hinders their widespread applications. Karn et al. reviewed the use of phages in a variety of situations such as veterinary science, agriculture, food preservation and of course human health (for example bacteremia, gastrointestinal tract infections and respiratory tract infections) due to their high specificity, low toxicity and ability to adapt to bacterial mutations. However, their use has challenges such as their pharmacokinetics and pharmacodynamics and delivery. This paper also includes information on FDA-approved bacteriophage-based product, commercial phage product, and global list of companies using phages for therapeutic purposes. Whilst Karn et al.

touched upon the regulatory challenges of phage therapy, this was discussed more in the review of Yang et al.. In Russia and Georgia, phage therapies can be purchased without prescription partly because they have been used frequently and for a long time in these countries. Phage therapy was classified as a medicinal product by European Medicines Agency, but different European countries regulate its use in different ways, with Belgium having "an established, innovative, and distinctive regulatory framework." China, as many other countries, is developing regulatory systems as the importance of phage therapy is being recognized as a rapidly growing and important technology.

Two papers reviewed the potential and challenges of phage therapy for chronic airways disease, a condition characterized by persistent bacterial infections and neutrophilic inflammation. Phage therapy may help phagocytes of our innate immune system control chronic airways disease, although the Laucirica et al. concluded that the exact mechanisms underlining this require further investigation. As well, phages can adhere to the mucous, helping them remain in lungs, although whether this helps or hinders productive infection was considered by Ling et al. to be relatively understudied.

Phage therapy is a promising, but two original research papers highlighted the challenges it encountered. The study by Zhang et al. demonstrated that dopamine could change the structure of phages preventing them from infecting their host cells. This may have important implications especially as dopamine is being used to bind various moieties including antimicrobials to medical devices (6). Whilst these antimicrobial surfaces are designed to reduce medical device-associated infections, should infections occur, albeit more rarely, phage therapy for infections of such devices could fail. This deserves further research. The case report of Blasco et al. provided a reminder that phage therapy may not always progress as hoped. A patient with a recurring prosthetic vascular graft infection caused by *Pseudomonas aeruginosa* was treated with a phage cocktail initially alone and subsequently in combination with ceftazidime-avibactam. Another blood stream infection occurred after the phage therapy, although interestingly the *P. aeruginosa* isolated at that time had reverted to being susceptible to ß-lactams and quinolones, highlighting the beneficial trade-off effects of phage therapy, which could increase the susceptibility of bacteria to antibiotics (7).

Plunder et al. describes how *in silico* simulations may help tailor future therapeutic choices. The paper developed and tested a novel method that uses machine learning and multi-criterial optimization to find the optimal viral dose and administration time for phage therapy against bacterial infections.

Three Research Topic demonstrated the characteristics and applications of different phages and phage products for bacterial infections. Ahamed et al. characterized two lytic phages that targeted *Shigella flexneri, Shigella dysenteriae,* and *Shigella sonnei*. The phages individually or as a cocktail could reduce the numbers *Shigella* on raw chicken, indicating their potential use in the food industry, and as therapeutics for *Shigella* infection. However, there are many regulations, dose and stability issues that need to be addressed before phage therapy can be widely applied in the food industry. Jo et al. characterized a new jumbo phage that was active against antimicrobial resistant strains of *E. coli*. The phage, EJP2, was active against resistant and pathogenic strains of *E. coli,* as well as biofilms and had synergistic activity with cefotaxime (Jo et al.). Not all bacterial infections are immediately amenable to phage therapy. For example, Umansky and Fortier describe phages of *C. difficile* as usually being not lytic and so need to be engineered. Studies on the mechanism of action of phage products demonstrated that the tailspike proteins of epsilon 34 phage disrupted the membrane of the *Salmonella* Typhimurium and *Salmonella* Newington as well as causing reductions in the bacteria's dehydrogenase activity (Ibrahim et al.). Stevens et al. demonstrated that phage endolysin helped to treat a chronic *Enterococcus faecalis* prostate infection that was causing severe chronic pelvic pain syndrome associated with bacterial prostatitis.

While phages exhibit promising potential as alternatives for treating drug-resistant bacterial infections and have shown success in various pathologies, the limited fulfillment of clinical requests underscores challenges in comprehending phage biology, host interactions, and the necessity for standardized regulations to fully exploit the benefits of phage therapy.

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# References

1. Uyttebroek S, Chen B, Onsea J, Ruythooren F, Debaveye Y, Devolder D, et al. Safety and efficacy of phage therapy in difficult-to-treat infections: a systematic review. *Lancet Infect Dis.* (2022) 22:e208–20. doi: 10.1016/S1473-3099(21)00612-5

2. Stone E, Campbell K, Grant I, McAuliffe O. Understanding and exploiting phage–host interactions. *Viruses.* (2019) 11:567. doi: 10.3390/v11060567

3. Van Belleghem JD, Dabrowska K, Vaneechoutte M, Barr JJ, Bollyky PL. Interactions between bacteriophage, bacteria, and the mammalian immune system. *Viruses.* (2018) 11:10. doi: 10.3390/v11010010

4. Nang SC, Lin YW, Petrovic Fabijan A, Chang RYK, Rao GG, Iredell J, et al. Pharmacokinetics/pharmacodynamics of phage therapy: a major hurdle to clinical translation. *Clin Microbiol Infect.* (2023) 29:702–9. doi: 10.1016/j.cmi.2023.01.021

5. Abedon ST. Bacterial 'immunity' against bacteriophages. *Bacteriophage.* (2012) 2:50–4. doi: 10.4161/bact.18609

6. Browne K, Kuppusamy R, Chen R, Willcox MDP, Walsh WR, Black DSC, et al. Bioinspired polydopamine coatings facilitate attachment of antimicrobial peptidomimetics with broad-spectrum antibacterial activity. *Int J Mol Sci.* (2022) 23:2952. doi: 10.3390/ijms23062952

7. Gordillo Altamirano F, Forsyth JH, Patwa R, Kostoulias X, Trim M, Subedi D, et al. Bacteriophage-resistant *Acinetobacter baumannii* are resensitized to antimicrobials. *Nat Microbiol.* (2021) 6:157–61. doi: 10.1038/s41564-020-00830-7

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# Determination of phage load and administration time in simulated occurrences of antibacterial treatments

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The use of phages as antibacterials is becoming more and more common in Western countries. However, a successful phage-derived antibacterial treatment needs to account for additional features such as the loss of infective virions and the multiplication of the hosts. The parameters critical inoculation size ($V_F$) and failure threshold time ($T_F$) have been introduced to assure that the viral dose ($V_\phi$) and administration time ($T_\phi$) would lead to the extinction of the targeted bacteria. The problem with the definition of $V_F$ and $T_F$ is that they are non-linear equations with two unknowns; thus, obtaining their explicit values is cumbersome and not unique. The current study used machine learning to determine $V_F$ and $T_F$ for an effective antibacterial treatment. Within these ranges, a Pareto optimal solution of a multi-criterial optimization problem (MCOP) provided a pair of $V_\phi$ and $T_\phi$ to facilitate the user's work. The algorithm was tested on a series of *in silico* microbial consortia that described the outgrowth of a species at high cell density by another species initially present at low concentration. The results demonstrated that the MCOP-derived pairs of $V_\phi$ and $T_\phi$ could effectively wipe out the bacterial target within the context of the simulation. The present study also introduced the concept of mediated phage therapy, where targeting booster bacteria might decrease the virulence of a pathogen immune to phagial infection and highlighted the importance of microbial competition in attaining a successful antibacterial treatment. In summary, the present work developed a novel method for investigating phage/bacteria interactions that can help increase the effectiveness of the application of phages as antibacterials and ease the work of microbiologists.

KEYWORDS

**microbial ecology models, phage therapy, machine learning, Pareto optimization, antibacterial treatment**

## Introduction

First employed in the medical field about a century ago, bacteriophages (phages) are currently experiencing a renewed clinical and veterinary interest particularly for their potential to contain antibiotic-resistant bacteria (1, 2). Phages are employed, albeit still in an experimental way, to treat clinical bacterial infections (3) including those due to antibiotic resistant species (4, 5), resolve caries (6, 7), preserve food, and decontaminate livestock (8). Although phages will likely be used in conjunction with antibiotics, at least in clinical settings (9, 10), their broad range of applications necessitates a deep understanding of their behavior to predict the efficacy of the treatment. Because phages are not static entities but rather replicate in proportion to their hosts' density, failing to account for this characteristic may result in therapeutic failure (11). To establish a self-sustaining infectious chain, there is the need for a minimum concentration of hosts for the phages known as "proliferation threshold" (12, 13):

$$X_P \approx \frac{\lambda\,(\eta - \mu)}{\delta\beta\eta} \tag{1}$$

where $\mu$ is the growth rate of the bacterial host, and the other parameters are the life-history tracts of the phages ($\lambda$ = decay rate; $\eta$ = reciprocal of the latency time $\tau$; $\delta$ = adsorption rate; $\beta$ = burst size). $X_P$ is reached at a time known as "proliferation onset time":

$$T_P \approx \frac{1}{\mu} ln\left(\frac{\lambda\,(\eta - \mu)}{\delta\eta\beta N}\right) \tag{2}$$

where $N$ is the total bacterial population. These parameters depend on each pair of bacteria and phages and provide a guidance on the possible outcome of the phage application. Consequently, if phages are given before $T_P$, they will not replicate successfully. However, if the viral load administered ($V_\phi$) is high enough, phages will massively lyse their hosts even in the absence of replication, and the treatment (known as "passive") will resemble antibiotic features where the drug does not amplify once administered. Conversely, if the administration time ($T_\phi$) is occurring after $T_P$, the phage-derived antibacterial treatment is defined as "active" because the virus will actively replicate establishing a self-sustained infectious cycle.

The parameter "critical inoculation size" ($V_F$) was introduced to provide a guide to the minimum amount of phages that $V_\phi$ should be administered to achieve an effective therapy (12). The critical inoculation size is defined as:

$$V_F = \varepsilon exp\left(\omega\left(T_P - T_\phi\right) + \frac{\omega}{\mu} e^{-\mu\left(T_P - T_\phi\right)} - 1\right) \tag{3}$$

where $\varepsilon$ is the dilution factor to obtain one phage in the system and $\omega$ is the decay or wash-out of the microbes. Similarly, the "failure threshold time" ($T_F$) provides a guide for the inoculation time:

$$T_F = T_P - \frac{1}{\omega} ln\left(\frac{V_\phi}{\varepsilon}\right) - \frac{1}{\mu} \tag{4}$$

Another feature to consider in phage therapy is that the interaction with other species influences bacterial behavior. For instance, it has been shown that the pathogenic *Escherichia coli* strain O157:H7 can adhere to substrates more easily when in the presence of *Pseudomonas aeruginosa* (14). In addition, it has been demonstrated experimentally that certain microorganisms inhibit the growth of other microbial species. For example, the commensal *Lactobacillus crispatus* slows the growth rate of the pathogens *Gardnerella viginalis* and *Neisseria gonorrhoeae* (15), whereas *Lactobacillus brevis* inhibits *Chlamydia trachomatis* (16). The opposite occurrence is also possible, with microorganisms experiencing increased growth rates when co-cultured with boosting species. For instance, the pathogens *Aggregatibacter actinomycetemcomitans* and *Candida albicans* increased the growth rate of *Streptococcus mutans*, a bacterium ubiquitous in the oral flora (17, 18). Moreover, it has been shown that phages might be able to reduce the density of a target species only in the presence of a competing microbe. For instance, phages T7 and T5 could induce the extinction *E. coli* in a culture only when *Salmonella enterica* was present (19). Microbes can, therefore, influence each other's fitness including phagial virulence. *In vivo*, the situation is even more complicated because it is necessary to account for the immune response against both bacteria and phages (20). Within this context, the case might arise of a phage-resistant pathogen whose booster species is instead sensible to phage infection. In that case, targeting the booster species might reduce the virulence of the pathogen and hereby help the clearance of the infection.

Both Eqs. 3 and 4 were defined to account for these biological characteristics to improve the effectiveness of phage-derived antibacterial treatments. However, the issue with the definitions of $V_F$ and $T_F$ is that Eqs. 3 and 4 are *a posteriori* approximations which depend on the sought-after unknown quantities $T_\phi$ and $V_\phi$ required for effective therapy. Since both Eqs. 3 and 4 are non-linear equations, resolving this dependency requires solution of a system of non-linear inequalities, which is cumbersome and, without further conditions, not unique.

The aim of the present work was to use a numerical approach to identify $T_F$, $V_F$, $T_\phi$, and $V_\phi$. A decision tree algorithm was developed to explore the different outcomes of microbial consortia undergoing phage treatment and to identify the best pairs of $V_\phi$ and $T_\phi$ for achieving either active or passive treatment. The identification of a $V_\phi/T_\phi$ pair will facilitate the microbiologist's work in implementing an effective therapy. The algorithm was tested on a series of microbial consortia: (*i*) the scenario described by Payne and Jansen in their study on phage therapy; (*ii* and *iii*) dual bacteria combinations; (*iv*) two species boosting each other's fitness.

# Materials and methods

## Microbial growth models

The focus of the present analysis was on what can be described as "allochthonous invasion," based on the definition of *autochthonous* species (a permanent component of a specific micro-environment) and *allochthonous* (introduced anew into such a niche) species (21). At the beginning of the simulation ($t_0$), the initial density of autochthonous species was considered higher than that of the allochthonous species, but the latter outgrew the former at a later time *t*.

Bacterial growth was implemented using logistic functions and the phage expansion was linked to the bacterial host by the following ordinary differential equations (ODEs):

$$\dot{X} = \mu X\left(1-\frac{N}{\kappa}\right) - \delta XP - \omega X - H(t)\,X \tag{5}$$

$$\dot{I} = \delta XP - \eta I - \omega I - H(t)\,I \tag{6}$$

$$\dot{R} = \xi R\left(1-\frac{N}{\kappa}\right) - \omega R - H(t)\,R \tag{7}$$

$$\dot{P} = \eta\beta I - \delta XP - \lambda P - \omega P - h(t)\,P \tag{8}$$

*X* and *I* indicate the population of susceptible and infected bacteria, respectively, whereas *R* is the population of bacteria resistant to phage (*P*) infection, that is a competitive species. The terms $\mu$ and $\xi$ indicated the growth rate of the susceptible/infected and resistant bacteria. The logistic terms were expressed as the ratio of the total bacterial population *N* to the carrying capacity $\kappa$. The phagial life-history traits were: $\beta$, burst size; $\delta$, adsorption rate; and $\lambda$, decay rate (22). In addition, $\eta$ represented the reciprocal of the latency time $\tau$. An additional parameter $\omega$ was included for a possible wash-out of microbes; this was set to 0.15 ml/h in all models. The terms *H(t)* and *h(t)* represent the immune response against bacteria and phages, respectively. These terms were dependent on time because the immune response is not immediate (12). Since the present study focused on *in vitro* applications of phages, both *H(t)* and *h(t)* were set to zero. A list of the parameters used in the present study is reported in **Table 1**.

The examples used in the present work were derived either from batch (closed vessel) or continuous (chemostat) culture. In the former case, the growth was converted from an explicit consumption of a limiting nutritive resource to implicit consumption under the assumption that the limiting resource would have remained constant. In particular, the specific growth rates were calculated from the maximum growth rates using the Monod term:

$$\mu = \frac{\mu_{max} S}{K_S + S} \tag{9}$$

with *S* being the concentration of the limiting nutrient, and $K_S$ being the half-saturation constant (23, 24).

## Estimation of growth rates

The microbes' life traits were based on information retrieved from the literature. When not provided by the experimental settings of the studies considered herein, the growth rates were calculated as a function of the bacterial population at time $t_0$ ($N_0$) and at time *t* ($N_t$) with the formula (24):

$$\mu = \frac{log_{10}(N_t) - log_{10}(N_0)}{log_{10}(2)\,(t - t_0)} \tag{10}$$

The growth rate was numerically computed as the slope of a linear model based on the bacterial densities displaying a linear distribution.

Since the model for case 4 included occurrences where the growth of a given microbe was influenced by that of another species, we addressed the use of dynamic growth rates, modifying the ODE system as follows. The growth rate of a microbe *X* cultivated alone was indicated with $\mu_\varepsilon$ (from the Greek ἐρημία: erēmíā, *loneliness*), whereas $\mu_o$ (from the Greek ὁμαρτῆ: homarte, *at the same time and place*) indicated its growth rate in presence of another microbe *Y* (booster) capable of enhancing the bacterial growth. Similar to *X*, *Y*'s growth rates could be indicated by $\nu_\varepsilon$ and $\nu_o$. A consortium of a bacterium and a booster required $\mu$ terms that could shift between $\mu_\varepsilon$ and $\mu_o$. Since the species in the model started mixed together, the baseline growth rate was $\mu_o$, but a loneliness term $\varepsilon$ was added to shift $\mu_o$ toward $\mu_\varepsilon$ with decreasing amounts of the booster species. The loneliness term was defined as: $\varepsilon = \Delta\vartheta$, with $\Delta = (\mu_o - \mu_\varepsilon)$. The $\vartheta$ was a "quorum term" obtained by adapting the Hill function (25):

$$\vartheta = \frac{\rho^n}{\rho^n + \varrho^n} \tag{11}$$

with $\rho$ being the density of the affected species, $\varrho$ the density of the booster species, and $n = 1$. The property of $\varepsilon$ was that it ranged between $\Delta$ in absence of booster species ($\vartheta = 1$) and $\Delta/2$ when the bacterial densities were equal ($\vartheta = 1/2$). Thus, the constant growth rate $\mu$ in Eq. 5 was substituted by a function M defined as:

$$\begin{aligned} M &= f(\mu_\varepsilon, \mu_o, \rho, \varrho) = \mu_o - \varepsilon = \mu_o - \Delta\vartheta = \mu_o \\ &- (\mu_o - \mu_\varepsilon)\,\vartheta = \mu_o - (\mu_o - \mu_\varepsilon)\,\frac{\rho}{\rho + \varrho} \end{aligned} \tag{12}$$

obtaining $\dot{X} = f(\mu_\varepsilon, \mu_o, \rho, \varrho)X(1 - N/\kappa) - \delta XP - \omega X$ (replacing Eq. 5) and the dynamic of the booster species *Y* is given by $\dot{Y} = f(\nu_\varepsilon, \nu_o, \rho, \varrho)Y(1 - N/\kappa) - \omega Y$.

## Ensemble simulations

The computation of $V_F$ and $T_F$ (Eqs. 3 and 4) is in general difficult due to their non-linearity. To study how $V_\phi$ and $T_\phi$ affected the treatment outcome, an ensemble simulation

TABLE 1 Variables and parameters used in the present study.

| Parameter | Symbol | Units | Case 1 | Case 2 | Case 3 | Case 4 |
|---|---|---|---|---|---|---|
| Growth rate targeted species | $\mu$ | $h^{-1}$ | 0.500 | 0.79 | 0.32 | 0.23–0.49 |
| Growth rate competitor species | $\xi$ | $h^{-1}$ | – | 0.22 | 0.20 | 0.18 |
| Growth rate booster species | $\nu$ | $h^{-1}$ | – | – | – | 0.24–0.42 |
| Carrying capacity | $\kappa$ | CFU × $ml^{-1}$ | $6.5 \times 10^6$ | $6.5 \times 10^6$ | $5.0 \times 10^9$ | $5.0 \times 10^9$ |
| Adsorption rate | $\delta$ | ml × $min^{-1}$ | $1.66 \times 10^{-9}$ | $5.0 \times 10^{-10}$ | $5.0 \times 10^{-10}$ | $4.5 \times 10^{-10}$ |
| Decay rate | $\lambda$ | PFU × $h^{-1}$ | 5.000 | 0.068 | 0.068 | 0.072 |
| Burst size | $\beta$ | PFU | 100 | 150 | 150 | 115 |
| Latency time | $t$ | min | – | 23 | 23 | 42 |
| Reciprocal of latency time | $\eta$ | $h^{-1}$ | 5.0 | 2.61 | 2.61 | 1.4 |
| Wash out rate | $\omega$ | ml × $h^{-1}$ | 0.15 | 0.15 | 0.15 | 0.15 |
| Simulation time | $t$ | h | 20 | 60 | 67 | 100 |

with 16 384 repetitions was performed. For each iteration, the viral amounts $V_\phi$ and administration times $T_\phi$ varied. The values for viral density were randomly selected between $10^2$ and $10^{12}$ plaque forming units (PFU/ml), with logarithmic scaling. The range for the viral amount was chosen on the assumption that, while it is possible to make virus dilutions at any desired concentration, administering less than 100 particles per milliliter would have been both impractical and ineffective. Overly concentrated viral suspensions, on the other hand, could produce virion aggregation, reducing the efficiency of the preparation. A topic review of the literature carried out for the present work showed that virtually all phage therapies administer between $10^4$ and $10^9$ PFU/ml. Thus, the range was deemed broad enough to cover virtually all phage therapy situations. The administration times were equidistant from 0 h to the end of the simulation's time frame.

For each iteration, the trajectory of the phage was analyzed to determine the treatment's outcome, following the classification suggested by Payne et al. (12). Host density above $10^2$ PFU/ml at the end of the simulation marked a "failed" treatment. The therapy was considered "passive" when the phage density never exceeded 105% of the initial administered amount ($V_\phi$). The therapy was considered "delayed" if the peak in phage density was obtained after more than 4 h and when it was at least 105% of $V_\phi$. The therapy was considered "active" if the phage density increased immediately over 105% of $V_\phi$.

## Decision tree algorithm

To compute ranges of viral load and administration times for each type of therapy, a decision tree algorithm (26, 27) was applied to the output of the ensemble simulation. The decision tree provided a partition of the set of therapy pairs which classified each pair by their expected therapy outcome and the estimated accuracy of the prediction. The resulting ranges gave a simplified representation of the regions of "active," "delayed," "passive," and "failed" outcomes. The boundary of these ranges fulfilled a similar role as the critical values $V_F$ and $T_F$ introduced by Payne et al. (12). In comparison to Eqs. 3 and 4, the output of the decision tree did not depend on any asymptotic assumptions on the dynamics of the concentrations. The ranges provide an *a priori* approximation of $V_F$ and $T_F$; therefore, these values can be used as a decision criterium for suitable therapy parameters $V_\phi$ and $T_\phi$. However, they were not as general in the sense that the ranges were only valid for fixed model parameters.

## Pareto optimal therapy pair

The decision tree-driven classification was not sufficient to select optimal therapy pairs for a specific treatment. For example, therapy pairs at the boundary of the computed ranges are very sensitive to perturbations, resulting in undesirable outcomes for the final user. Thus, the present study solved a multi-criteria optimization problem (MCOP) (28) to provide the user with a pair of phage load ($V_\phi$) and administration time ($V_\phi$) that always resulted in the chosen outcome ("active," "delayed," "passive," and "failed"). MCOP is widely used to guide the decision of treatment parameters (29). The criteria employed to achieve an effective therapy was a maximal insensitivity to perturbations combined with the shortest possible administration time. For a given therapeutic pair ($V_\phi$, $T_\phi$), the measure of insensitivity was the largest radius $R$ of an ellipse such that all perturbed pairs $\tilde{V}_\phi$, $\tilde{T}_\phi$ which satisfied the inequality $(T_\phi - \tilde{T}_\phi)^2 + w_\phi{}^2(\log(V_\phi) - \log(\tilde{V}_\phi))^2 < R^2$ also yielded the desired therapy outcome (**Figure 1**). The scaling constant $w_\phi$ determined the shape of the ellipse of perturbations. For all cases in this article, the value $w_\phi = 2$ was used. The data from the ensemble simulation provided a fast way to approximate $R(V_\phi, T_\phi)$. The weighted sum method (28) in conjuncture with the particle swarm method (30) was used to compute Pareto optimal solutions. The approach used was prototypical in the sense that, depending on the specific application, other criteria could be chosen instead.

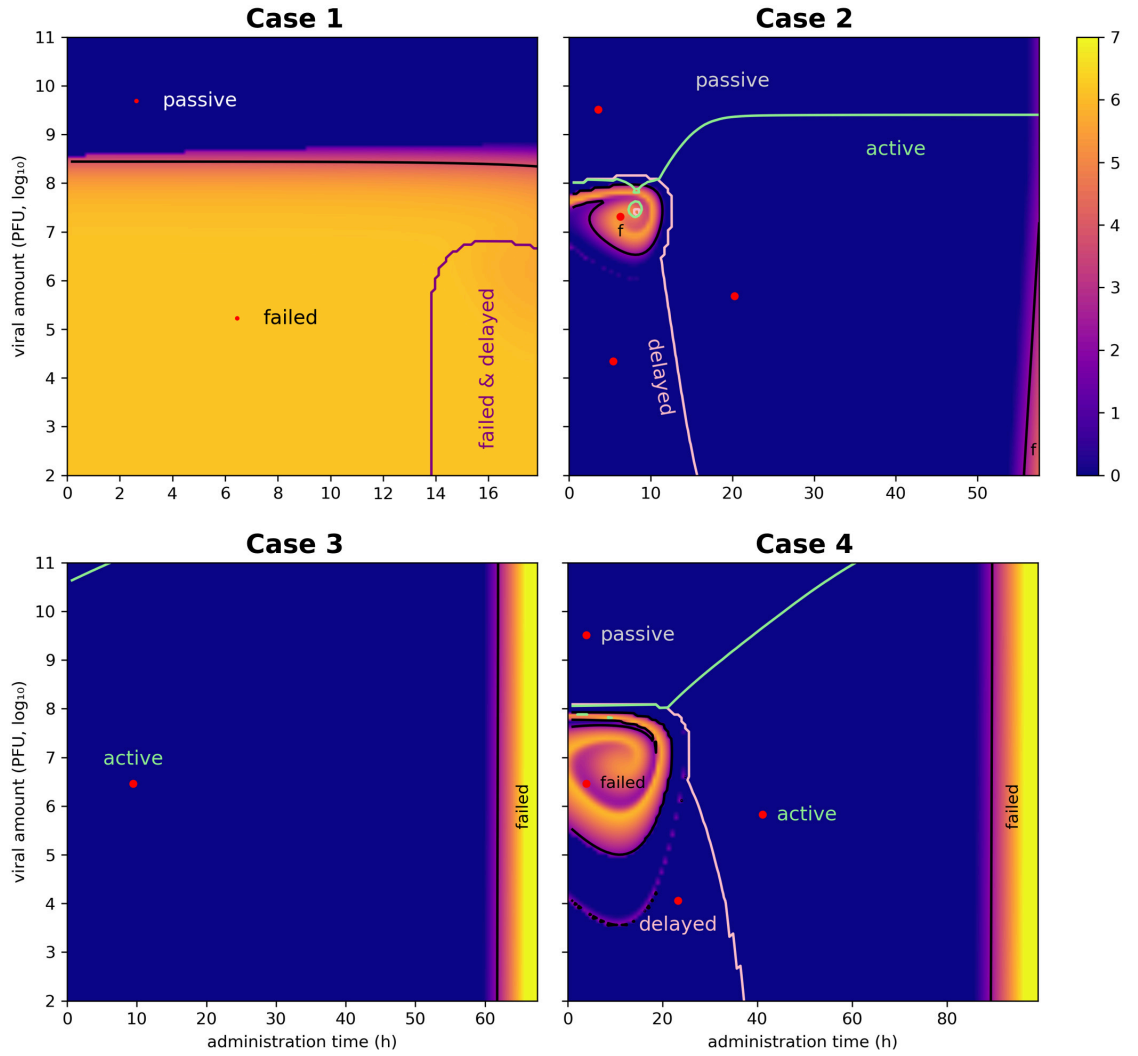


FIGURE 1
Heat maps for the selection of the most effective pair of $V_\phi$ and $T_\phi$. The ensemble simulation generates a space of viral dose and administration times whose employment lead to a different outcome. Each pixel of the plot represents the outcome of the simulation, color-coded according to the natural logarithm of the host's density at the end of the simulation (bar on the right of the plots). There are 128 intervals in both the *x*-axis (administration time $T_\phi$) and the *y*-axis (viral load $V_\phi$), determining 16 328 simulations. The curves indicate the boundaries of the different outcomes (active, delayed, passive, and failed therapies), representing critical values equivalent to $V_F$ and $T_F$. The selection of optimal pairs of viral load and administration times (equivalent to $V_\phi$ and $T_\phi$) was obtained with a Pareto approach implemented with as a multi-criteria optimization problem (MCOP). These values are visualized by red dots.

## Implementation

Computations were carried out in *Julia* 1.7 (31) and implemented with the packages: *DifferentialEquations* (solution of differential equations) (32); *LsqFit, Dierckx*, and *Roots* (regression); *DecisionTrees* (classification); *Optim* (optimization) (30); and *PyPlot* (plotting). Data estimation from the original plots was obtained using *WebPlotDigitizer* 4.5.[1] Bacterial growth rates were computed using a custom function *growthRate*, built-in *R* 4.1, that selected the points of bacterial density over time most describing a continuous line and then generated a linear model on those points. The slope of the model was used as the growth rate value. Retrieval of phages species for a given bacterium was obtained by inquiring the *Virus-Host Database* during the year 2021 (33).

## Results

In the following sections, the ensemble simulations were performed for selected cases describing allochthonous

1 https://automeris.io/WebPlotDigitizer/

TABLE 2 Summary of the phage therapy outcomes obtained by decision tree approach for the cases presented in the present study.

| Case | Microbial consortium | Phage | Outcome (efficacy) | $V_\phi$ range† (PFU × ml$^{-1}$) | $T_\phi$ range‡ (h) |
|---|---|---|---|---|---|
| 1 | Hypothetical* | Hypothetical | Passive (100%) | $\geq 2.6 \times 10^8$ | ≥0 |
| 2 | *Escherichia coli** + *Pseudomonas aeruginosa* | T4 | Active (99.7%) | $\leq 2.6 \times 10^9$ | 15.5–56 |
| | | | Delayed (100%) | $\leq 3.7 \times 10^6$ | ≤13.26 |
| | | | Passive (100%) | $\geq 2.6 \times 10^9$ | ≥0 |
| 3 | *Escherichia coli** + *Azotobacter vinelandii* | T4 | Active (100%) | $\leq 4.1 \times 10^{10}$ | ≤61.3 |
| 4 | *Streptococcus mutans** + *Candida albicans* + *Lactobacillus reuteri* | λ | Active (100%) | $\leq 2.2 \times 10^9$ | 36.8–89.5 |
| | | | Delayed (98.7%) | $\leq 1.2 \times 10^5$ | ≤30.6 |
| | | | Passive (100%) | $\geq 9.6 \times 10^8$ | ≤33 |

*Targeted bacterial species.
†The upper end of the range is $10^{11}$ PFU/ml.
‡The upper end of the range is the end of the simulation's time.

invasions. The decision trees defined the limits for each type of phage-derived antibacterial treatment ("passive," "delayed," "active," or "failed"), providing values equivalent to $T_F$ and $V_F$ (Table 2). Moreover, a pair of viral load and administration time, equivalent to the parameters $V_\phi$ and $T_\phi$, was determined by a multi-criteria optimization problem to provide the user with convenient values for implementing the chosen treatment. The cases reported below represented *in vitro* applications of phages to eliminate a target bacterium; thus, the cases did not involve the immune system. Moreover, the cases were based on the application of lytic phages; the presence of prophages in the host bacteria was not considered. The antibiotic resistance capability of the hosts and their potential virulence factors were also excluded from the modeling.

## Case 1: Hypothetical bacterium and phage

Payne and Jansen described the growth of a hypothetical bacterium and the administration of its phage, highlighting four main treatment outcomes: "failed," "passive," "active," and "delayed" (11). In the present study, the failed outcome was used as a base to implement an effective passive therapy. The parameters of the simulation, derived from the Payne and Jansen's study, were as follows. Initial concentration of bacteria ($X_0$): 1,000 colonies forming units per milliliter (CFU/ml); $V_\phi$: $10^8$ plaque forming units per milliliter (PFU/ml); $T_\phi$: 2.5 h; μ: 0.5 h$^{-1}$; δ: $1.66 \times 10^{-9}$ ml/min; η: 5 h$^{-1}$; β: 100 PFU; λ: 5 PFU/h. The bacterial growth was adapted to account for a logistic growth with κ = $6.5 \times 10^6$ CFU/ml and ω = 0.15 ml/h. The simulation time-frame was 20 h (Figure 2A).

The decision tree algorithm developed herein reported only one effective outcome: passive. The Pareto optimal pair of viral load and administration time was identified as $4.5 \times 10^9$ PFU/ml and 2.8 h. The Pareto optimal pair of viral load and administration time was identified as $1.99 \times 10^{10}$ and 3.4 h. The results of the therapy clearly illustrated the characteristics of an effective passive approach: there was no increase in phage density with respect to the initial input and there were no bacteria left in the environment at the end of the simulation, indicating that the infection had been cleared as required (Figure 2B).

The outcome of the therapy was dependent on the time scale of the application. While a range of 20 h allowed only for passive therapy, a longer scale (for instance, 48 h) provided also active and delayed outcomes which reduced the host below $10^2$ PFU/ml (Supplementary Figure 2). A dynamic plot was implemented to actively explore the role of the different parameters in modeling phage therapy (Supplementary File 1). The figure shows that the outcome of the phagial administration is strongly dependent on the parameters used in the computation, highlighting the fact that phage therapy is case-specific.

Remarkably, an oscillation in population density was serendipitously obtained with $V_\phi = 1.6 \times 10^5$ PFU/ml and $T_\phi$ = 15.9 h. The model showed a first wave of phage expansion followed by bacterial decrease and a second wave of phage expansion that caused the collapse of the host population (Supplementary Figure 3A).

## Case 2: *Escherichia coli* vs. *Pseudomonas aeruginosa*

The growth of *Escherichia coli* C-8 and *P. aeruginosa* PAO283 was described by Hansen and Hubbell in 1980 using batch cultures (34). The life-history traits reported by this study for these bacteria were as follows. *E. coli*: yield ($Y$) $2.5 \times 10^{10}$ cells per gram (cell/g) of limiting substance; half saturation constant ($K_S$) $3.0 \times 10^{-6}$ grams per liter (g/L) of limiting substance; $\mu_{max} = 0.81$ h$^{-1}$. *P. aeruginosa*: $Y = 3.8 \times 10^{10}$ cell/g; $K_S = 3.0 \times 10^{-6}$ g/L; $\xi_{max} = 0.91$ h$^{-1}$. The bacteria were growth in 100 ml flasks containing minimal medium with tryptophan as limiting nutrient, provided at an initial concentration of $1.0 \times 10^{-4}$ g/L. The growth rates were calculated according to

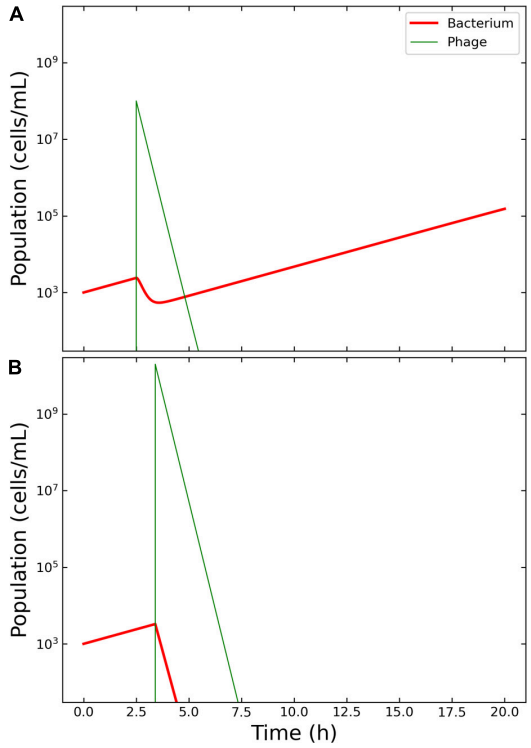


FIGURE 2
Model of the competition between hypothetical bacteria and phages. Outcome for case 1. **(A)** Failed therapy. The simulation shows a passive therapy, since there is no amplification of the phages, where the virions are depleted from the system before the bacterium could be wiped out. To note the decrease in bacterial concentration after the application of $V_\phi = 10^8$ phages at $T_\phi = 2.5$ h and the increase in density of the escaped bacteria. **(B)** Effective therapy. The only effective therapy possible was passive therapy, with ample margins of administration. The Pareto-derived pair for passive therapy was: $4.5 \times 10^9$ PFU/ml and 2.8 h.

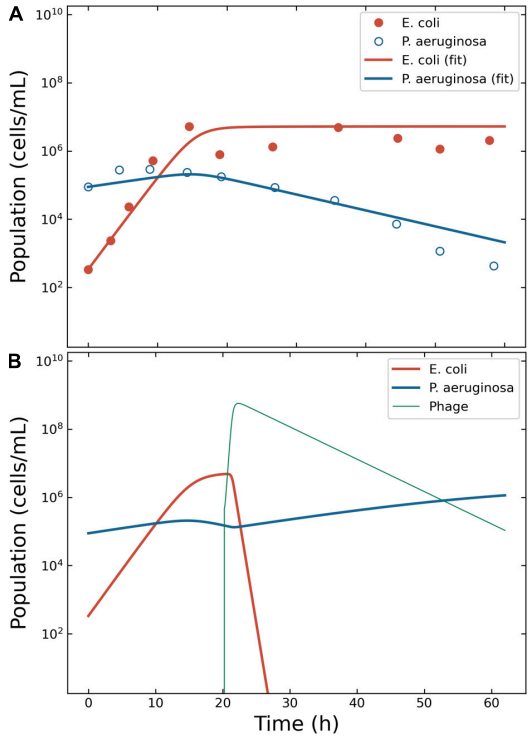


FIGURE 3
Model of the competition between *Escherichia coli* and *Pseudomonas aeruginosa*. Outcome for case 2. **(A)** Bacterial competition in absence of phages. The data estimated from the original plots for *E. coli* and *P. aeruginosa* is represented together with the fitting obtained using ODE models for *E. coli* and *P. aeruginosa*. **(B)** Bacterial competition in presence of phages. The Pareto-derived pair for active therapy was: $2.0 \times 10^5$ PFU/ml and 17.7 h, leading to the extinction of the invading bacterium *E. coli* and the recovery of the resident species *P. aeruginosa*.

Eq. 9: $\mu = 0.79\ h^{-1}$ for *E. coli* and $\xi = 0.22\ h^{-1}$ for *P. aeruginosa*. The carrying capacity $\kappa$ was estimated from the original graph at $6.5 \times 10^6$ cells/ml. The initial seed of bacteria was extracted from the original graphs: *E. coli*, 334 cells/ml; *P. aeruginosa*, 88 516 cells/ml. These quantities gave a *P. aeruginosa*/*E. coli* ratio of 265.4, in line with the reported 200:1 for the initial densities of these bacteria. *Escherichia coli* outgrew *P. aeruginosa* about 9.2 h after the beginning of the experiment and the latter was wiped out in about 60 h. $X_P$ was calculated to 10 556 cells and $T_P$ at 4.4 h after the beginning of the experiment (Figure 3A).

To simulate the phage therapy, the life-history traits of the coliphage T4 were retrieved from the literature (35): $\delta = 5.0 \times 10^{-10}$ ml/min; $\tau = 23$ min (resulting in $\eta = 2.61\ h^{-1}$); $\lambda = 0.068$ PFU/h; $\beta = 150$ PFU. The simulation time-frame was 60 h with $\omega = 0.15$ ml/h and $\kappa = 6.5 \times 10^6$ CFU/ml. The decision tree identified three possible effective outcomes: "passive," "active," and "delayed active." The best pair of viral load and administration time for active therapy were identified in $4.8 \times 10^5$ PFU/ml and 20.2 h (Figure 3B). The best pair of viral load and administration time for delayed treatment were identified in $2.2 \times 10^4$ PFU/ml and 5.4 h (data not shown). The best pair of viral load and administration time for passive treatment were identified in $3.2 \times 10^9$ PFU/ml and 3.6 h (data not shown).

As for case 1, an oscillation in population density was serendipitously obtained with $V_\phi = 1.0 \times 10^6$ PFU/ml and $T_\phi = 10.0$ h. The model showed a first wave of phage expansion followed by bacterial decrease and a second wave of phage expansion that caused the collapse of the host population (Supplementary Figure 3B).

## Case 3: *Escherichia coli* vs. *Azotobacter vinelandii*

The growth of the bacteria *E. coli* B/r and *A. vinelandii* OP was described by Jost and collaborators in 1973 using

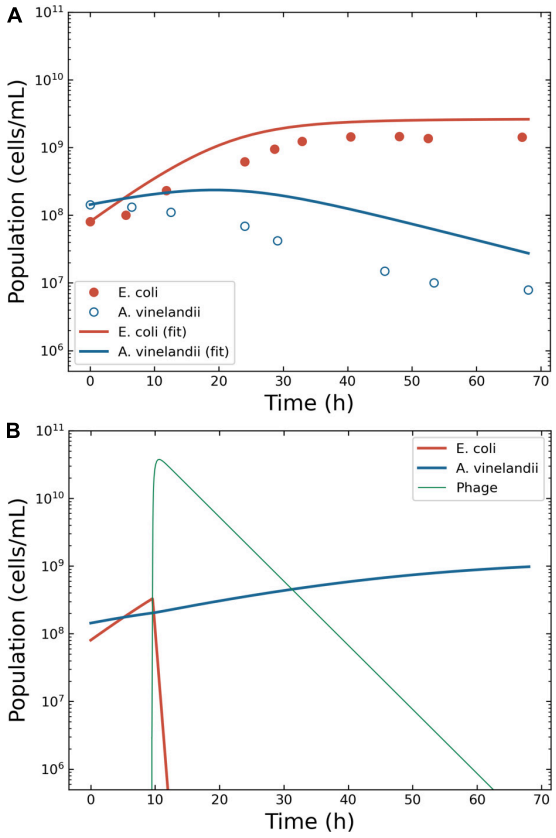


FIGURE 4
Model of the competition between *Escherichia coli* and *Azotobacter vinelandii*. Outcome for case 3. **(A)** Bacterial competition in absence of phages. The dots represent the data estimated from the original plots for *E. coli* and *A. vinelandii*, the lines the conversion to a logistic model. **(B)** Bacterial competition in presence of phages. The Pareto-derived pair for active therapy was: $9.0 \times 10^9$ PFU/ml and 66.8 h, leading to the extinction of the invading bacterium *E. coli* and the recovery of the resident species *A. vinelandii*.

continuous culture (36). The authors reported specific growth rates of 0.32 and 0.23 $h^{-1}$ for *E. coli* and *A. vinelandii*, with $K_S$ of $1.0 \times 10^{-7}$ and $1.2 \times 10^{-2}$, respectively. The concentration of glucose in the reactor was 0.005 mg/ml, providing maximum growth rates of 0.32 and 0.07 $h^{-1}$ for *E. coli* and *A. vinelandii*. The carrying capacity κ was estimated from the original graph at $5.0 \times 10^9$ CFU/ml. The calculated growth rate of *A. vinelandii* matched what reported in the public domain (37) but did not allow the building of a fitting model (**Supplementary Figure 4**). A value of $\xi = 0.20 \pm 0.01$ was reported in the literature (38) and allowed for a better description of the data (**Figure 4A**). The data for the simulation were extracted from the original figure of Jost et al., providing $X_0$ of 80 251 179 CFU/ml for *E. coli* and 143 462 884 CFU/ml for *A. vinelandii*.

The phage therapy was assumed to use coliphage T4; thus, the life traits were the same as in case 2. The simulation time-frame was 67 h with $\omega = 0.15$ ml/h. The decision tree identified two effective therapeutic outcomes: "passive" and "active." The Pareto optimal pair of viral load and administration time for active therapy were identified in $2.9 \times 10^6$ PFU/ml and 9.5 h (**Figure 4B**). The Pareto optimal pair of viral load and administration time for passive therapy were identified in $1.6 \times 10^6$ PFU/ml and 8.4 h (data not shown).

## Case 4: *Candida albicans*, *Streptococcus mutans*, and *Lactobacillus reuteri*

The present case investigated the effect of phage therapy on mutually synergic microbial species. *C. albicans* is an opportunistic fungus that can cause infections in multiple organs and associated to increased risk of oncogenesis (39, 40). In particular, the presence of several virulence factors allows this fungus to invade and thrive in several tissues and it can develop biofilms that protect it from antibiotic treatments (41). Being a protist, *C. albicans* is immune to phagial infection. However, experimental evidence reported that this pathogen's growth rate is increased by booster bacteria, namely *S. mutans* (17). Consequently, targeting the booster species will provide, in theory, a "mediated phage therapy" that could reduce the pathogen's virulence. As a proof-of-concept, we defined a hypothetical microbial consortium composed by *C. albicans* as the phage-resistant pathogen, *S. mutans* as the boosting species susceptible to phage infection, and *L. reuteri* as the commensal bacterium.

The details of the simulation were as follows. Even if not a bacterium, the growth of *C. albicans* has been modeled using logistic models (42). Thus, Eqs. 5–8 were deemed suitable to model the growth of this fungus. The growth rates of *C. albicans* and *S. mutans* were estimated from the original figures (17, 18) (**Supplementary Figure 5**). The density of *S. mutans* in the initial phases of growth in the presence of *C. albicans* was $8.4 \pm 6.2 \times 10^7$ CFU/ml; conversely, the mean density of *C. albicans* in the presence of *S. mutans* was $1.9 \pm 1.1 \times 10^6$ CFU/ml. Thus, the ratio *S. mutans*/*C. albicans* was 44.6. However, these measurements were taken from two different series of experiments, making it difficult to determine an accurate value of $\mu_o$ for a single consortium. The growth rate of *S. mutans* was computed at 0.23 $h^{-1}$ when cultivated alone, and at 0.49 $h^{-1}$ when cultivated together with *C. albicans*. Conversely, the growth rate of *C. albicans* was computed at 0.24 $h^{-1}$ when alone and 0.42 $h^{-1}$ when in presence of *S. mutans*. The *L. reuteri* growth rate was derived from the public domain: 0.18 $h^{-1}$ (43) and was considered constant. The model considered an initial seed of $1 \times 10^4$ CFU/ml for both *S. mutans* and *C. albicans*, and $1 \times 10^8$ CFU/ml for *L. reuteri*. The model

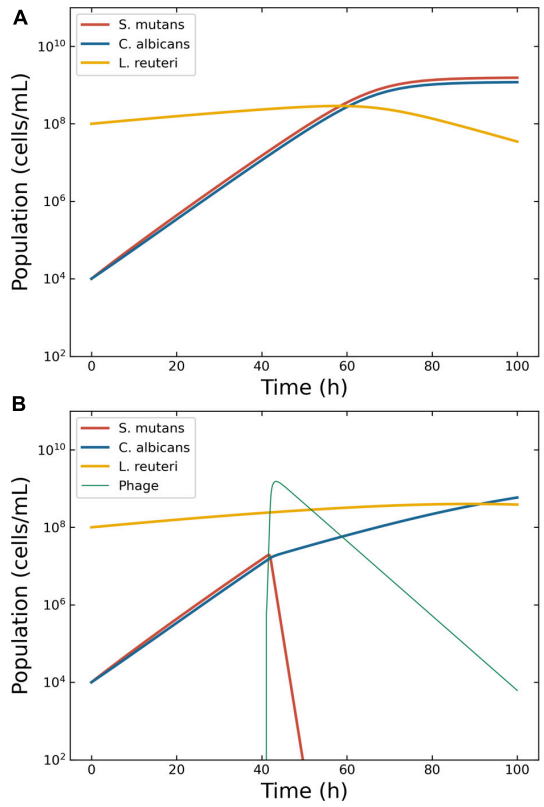


FIGURE 5
Model of the competition between *Streptococcus mutans*, *Candida albicans* and *Lactobacillus reuteri*. Outcome for case 4. **(A)** Bacterial competition in absence of phages. Models generated for a hypothetical consortium of two bacteria (*S. mutans* and *L. reuteri*) and one protist (*C. albicans*). The boosting species *S. mutans* (solid line) and the pathogen *C. albicans* increase each other growth rate causing a depletion in the commensal *L. reuteri*. **(B)** Bacterial competition in presence of phages. The Pareto-derived pair for active therapy was: $1.0 \times 10^7$ PFU/ml and 42.6 h, leading to the extinction of the boosting bacterium *S. mutans* and consequently causing a reduction in the density of the pathogen *C. albicans* and the recovery of the commensal *L. reuteri*.

showed that both *S. mutans* and *C. albicans* grew with similar dynamics and overgrew *L. reuteri* within 60 h after the beginning of the simulation (**Figure 5A**). Specifically, at the end of the simulation, *C. albicans* and *L. reuteri* had densities of $1.2 \times 10^9$ and $3.5 \times 10^7$ CFU/ml, respectively.

The Virus-Host Database reported three phages for *S. mutans*: Streptococcus phage φAPCM01, M102, and M102AD. These phages, all belonging to the family *Siphoviridae*, were highly genetically related: M102 and M102AD shared about 91% similarity at the nucleotide level (44), and φAPCM01 shared 85% nucleotide identity with them (6). Apart for the M102AD's adsorption rate ($\delta = 1.5 \times 10^{-10}$ min$^{-1}$ (44)), no other life traits were available in the public domain. Hence, the parameters for the present simulation were derived from another member of the *Siphoviridae* family: phage λ (35). Thus, $\delta = 4.5 \times 10^{-10}$ ml/min; $\tau = 42$ min; $\eta = 1.4$ h$^{-1}$; $\lambda = 0.072$ PFU/h; $\beta = 115$ PFU. The carrying capacity $\kappa$ was set at $5.0 \times 10^9$ CFU/ml; $\omega = 0.15$ ml/h$^{-1}$; the simulation time-frame was 100 h.

The decision tree identified two possible therapeutic outcomes: "passive" and "active". The Pareto optimal pair of viral load and administration time for active therapy was identified in $6.7 \times 10^5$ PFU/ml and 41.0 h (**Figure 5B**). The best pair of viral load and administration time for passive therapy was identified in $3.2 \times 10^9$ PFU/ml and 3.9 h (data not shown).

As for cases 1 and 2, an oscillation in population density was serendipitously obtained with $V_\phi = 2.9 \times 10^6$ PFU/ml and $T_\phi = 3.9$ h (**Supplementary Figure 3C**). The model showed a first wave of phage expansion followed by bacterial decrease and a second wave of phage expansion that caused the collapse of the host population.

## Discussion

In the present study, a machine learning approach was implemented to quickly analyze the possible outcomes of phage-derived antimicrobial treatments and provide the user with a pair of viral load and administration time that can result in effective antibacterial interventions. These values, equivalent to the parameters $V_\phi$ and $T_\phi$ introduced by Payne et al. (12), were extracted from a mathematical space (administration time vs. viral load) that accounted for different types of treatment ("active," "delayed," "passive," and "failed"). The boundaries between these regions were equivalent to the parameters $V_F$ and $T_F$ defined by Payne et al. (12). The *in silico* applications presented herein (cases 1–4) did not include the immune response in the model because represented *in vitro* applications.

Nonetheless, more and more studies are reporting the role of the immune system in the effectiveness of phage therapy due to what has been called "immunephage synergy" (45–47). Notably, immunity was excluded, albeit considered, in the work by Payne and Jansen (11), Payne et al. (12). Such an assumption can be accepted considering the treatment fast enough to be completed before an immune response to both the bacterial pathogen and the phages could be instantiated. The first clinical applications of phagotherapy reported bacterial clearance as extremely rapid (48). For instance, in 1919, three young brothers were admitted to the Hôpital des Enfants-Malades, Paris, with acute dysentery. Félix d'Herelle, the first to use phages in clinical settings administered phages to them children. The children recovered in 24 h. However, contemporary clinical applications of phages last for at least 1 week (49, 50); thus, the immune response becomes a critical aspect of the therapy. Nonetheless, the immune response to phages varies among treated people. Antiphage activity of sera (AAS) was observed in about half of the patients after the tenth day of oral administration of phages (51). AAS may even be present in patients before phage therapy is initiated: phage administration resulted in a 37% increase in

the baseline response in phage-naive patients; 23% of patients undergoing phagotherapy showed AAS; and it has been reported that about 80% of healthy people carry anti-phage antibodies (51, 52).

Because of the current worldwide spread of multi-drug resistant bacteria, the use of phages to clear bacterial infections is experiencing a resurgence of interest in Western countries (53). Nonetheless, to be effective, the application of phages as antibacterials should consider several factors aside from the immune response, including the host replication and rate of phage decay (12). The development of bacteria resistant to specific viral infection is also a fundamental feature to consider to obtain an effective phage treatment (54). Several models account for the bacterial development of resistance to phagial infection (55, 56). However, in the absence of experimental data, including this feature would have increased the model's complexity without providing any real benefit to the present study. However, the increasing application of phages for eco-restoration (57), food safety (58, 59), and sterilization of surfaces (60–64) implies that phage-derived antibacterial treatments need to work even in the absence of a complementary immune response.

The present study aimed to help microbiologists involved in the medical field choosing the right amount of phages and the most effective administration time to clear an infection. While it may be tempting to administer as many phages as possible as soon as possible, Payne and Jansen's research highlighted that doing so does not ensures the treatment's effectiveness. Moreover, applying very high amounts of phages would trigger passive therapy, nullifying the dynamic feature that bacterial viruses have over antibiotics. The model we have introduced herein was intended to provide microbiologists involved in ecological studies with a means to assess the interactions between bacteria and phages quickly.

Case 1 was based on the aforementioned work by Payne et al. (12). The authors described (a) a failed therapy with the combination $V_\phi = 10^8$ PFU/ml, $T_\phi = 2.5$ h, and (b) an effective passive therapy with $V_\phi = 1.0 \times 10^9$ PFU/ml, $T_\phi = 2.5$ h. The results obtained herein confirmed that, within a time frame of 20 h, only passive therapy could effectively clear the infection, and the obtained margins included the values used by Payne and Jansen to achieve effective passive therapy.

In case 2, all types of therapeutic outcome were possible. The present paper focused on active therapies, and even in this case the target bacterium (*E. coli*) was eradicated from the simulation within the allotted time. Nonetheless, the heath maps described a zone at low dispensation time (below 15 h) and intermediate viral load (around $10^7$ PFU/ml) where the treatment produced a failed outcome (**Figure 1**). Such a result highlighted the need to assess the outcomes of the treatment to improve its effectiveness.

Case 3 confirmed that the outcome of phage therapy is dependent on the peculiar condition of the microbial consortium. In this case, only active and passive therapy were possible. The target bacterium (*E. coli*) was removed from the *in silico* environment allowing the recovery of *A. vinelandii* as required. The analysis of this consortium was unambiguous. However, the lack of empirical data precluded the selection of the most fitting model for the growth of the bacteria. While we chose, for simplicity, the logistic growth model (Eqs. 5–8) to describe the replication of naïve bacteria, the shape of the data extracted from published experiments (36) suggested that other functions providing more sigmoid profiles, such as Holling type IV, might be viable alternatives. The precise definition of the underlying growth function was deemed of little value in the absence of experimental data.

Case 4 introduced the concept of "indirect phage therapy," that is the targeting of a booster bacterium to decrease the pathogenesis of a phage-resistant microbe, in this case *C. albicans*. Given the morbidity and mortality associated with this fungus, driven primarily by its capacity to generate biofilms that can be colonized by a variety of microbes that facilitate horizontal gene transfer (65), methods to eradicate this opportunistic pathogen would be clinically beneficial. Since the growth rate of the microbes in the simulation was not constant but was related to the density of the partner species, we defined a dynamic growth rate for the interacting species. In the literature, there is a paucity of cases of mutually interactive microbes and their growth models. We retrieved a growth rate as a function of bacterial density in the theoretical description of cross feeding (66). Such a model required a term $c_x$ to avoid the problem of infinity when the selected species's density was equal to zero.

$$\dot{X} = X\left(\mu + b_{xy}\frac{Y}{X + c_x}\right)\left(1 - \frac{X + Y}{\kappa}\right) \tag{13}$$

In Eq. 13, $b_{xy}$ indicates the benefit of the species $Y$ over the growth of $X$, but $c_x$ does not represent a biological capacity. The function we introduced to adjust the growth rate according to the bacterial densities (Eq. 12) avoided division by zero by adapting the Hill function $aX^n(X^n + Y^n)^{-1}$, with $a = 1$ and $n = 1$, to the microbial densities, dispensing the need for a $c_x$ term. Even in case 4, as in cases 1 and 2, there was a zone a failure at low administration times and intermediate viral loads.

Within the present framework, as in Payne et al.'s study (12), the effectiveness of the therapy was based on the complete removal of the target bacterium and assumed the absence of an immune response. While such an assumption is feasible for *in silico* systems like those included in the present study, recent models indicated that actual phage therapy, in combination or not with antibiotics, would fail without a complementary immune response (20, 67). Thus, *in vivo*, the complete removal of the target species is probably not essential to achieve remission from infection. Effective therapy will consist of phage-driven reductions in the density of the host below a threshold where the immune system can wipe out the target. Recent research has shown that phage administration

activates the innate immune response and reduces harmful pro-inflammatory pathways (10, 68), but their role in the treatment outcome is still unknown. To date, the role of immunity in phage therapy remains under active investigation.

It has been shown *in vitro* that microbial competition can act synergistically with phages to reduce the density of *E. coli* (69). The *P. aeruginosa* PAO1 density decreased significantly more upon phage administration in the presence of additional species (*Staphylococcus aureus* and *S. macrophilia*, either independently or in combination) than in the absence of competitors (70). As a result, there may be a parallel between the role of the immune response *in vivo* and microbial competition *in vitro*. In both cases, phagial infection may not be enough to eliminate a specific bacterium from the environment. However, the increased selective pressure imposed by viral infection may cause a shift in microbial competition against the targeted bacterium. The role of competition in phage therapy could have significant implications for treatments that do not involve the immune response, such as in environmental applications. These data suggest a scenario where phages alone are not sufficient to eradicate a targeted bacterial host from a given micro-environment. Conversely, the simultaneous action of phages and other features (immune response, microbial competition, or antibiotics) assure the elimination of the targeted bacteria.

Moreover, the development of host-side resistance to infection has significant implications for the phage-derived antibacterial treatment. Phage-resistant mutants will counteract the phage treatment, allowing the targeted species to survive in the micro-environment (55, 71). In the present work, as in the mentioned study of Payne and Jansen, mutation was not accounted for. More refined modeling will require to include such a feature. In the absence of experimental data, the inclusion of mutation would have increased the complexity of the model without any real gain.

Consequently, the adaptation of *in silico* models to *in vivo* contexts is not trivial due to the still poorly understood additional factors involved in phage therapy. Thus, a successful *in silico* treatment does not assure the success of *in vitro* or *in vivo* implementations (72). Nonetheless, modeling is an essential part of the experimental investigation because models allow to predict results, provide explanation of empirical data, and streamline wet lab experiments (73). The method presented in the present study was devised toward microbial modeling to improve the efficacy of phage therapy by making it simple to determine the phage load and administration time ranges to be tested in the experimental settings. Our model should be regarded as a preliminary framework that can be expanded to include additional features to improve its ability to fit experimental data. For example, the current model considered the "sur-mesure" approach to phage treatment (50). In other words, a single specific phage is administered after careful characterization of a chosen pathogen. In many real-world applications, however, the most common phage therapy approach is the so-called "pret-a-porter," where a cocktail of different phages is administered simultaneously. To account for such a phagial variability, the model would have to increase the number of phage instances to accommodate multiple life-history traits, as previously proposed (71, 74, 75). The resulting model would be much more complex than the one presented herein but, in the absence of experimental data, it would not provide any additional benefit. Similarly, the model did not consider the presence of integrated lysogenic phages in the hosts that might become activated upon infection with lytic phages to avoid unneeded complexity.

We observed indications for oscillations in population density. The peaks in host density preceded that of the phages, in accordance with the Lotka-Volterra model, namely a peak in prey density occurring before the decline in host density (76). Recent data highlighted that, in some instances of phage therapy, an invading bacterium can coexist with the resident flora, resulting in a new equilibrium (77). It is known that bacteria and phages can establish an equilibrium in the presence of specific life traits and densities (56). It has been shown that oscillatory conditions between phages and bacteria might occur when the infection rate $\eta$ is within a range whose lower end ($\eta_c$) is defined as:

$$\eta_c = \frac{\omega\,(\delta K + \omega)}{\delta K\,(\beta - 1) - \omega} \tag{14}$$

where $K = \kappa(1 - \omega\mu^{-1})$ (56). The value for $\eta_c$ could be calculated in 0.036, 0.001, and 0.001 $h^{-1}$ for cases 2, 3, and 4, respectively. These critical thresholds were indeed below the values of 2.6 and 1.4 $h^{-1}$ used in the models for cases 2–4, respectively. There is, therefore, a real risk that non-optimal viral loads might determine not the eradication of the targeted bacterium but the establishment of an unforeseen new microbial environment. The equilibrium between the target bacterium (namely a pathogen) and the dispensed lytic phage might stabilize a harmful species at low density, which might subsequently expand when the right conditions present themselves. While, *in vivo*, such species can be considered a "pathobiont" (78), at the environmental level they can still cause damage, for instance spoiling milk during cheese production. The present work will help toward the avoidance of such occurrences and increase the effectiveness of phage therapy.

The present study had some limitations. The results presented herein were only theoretical and will require empirical validation. In particular, the precision of Eq. 12 could not be determined. In the absence of experimental data, such an effort would be of very little gain; thus, the present study must be considered a proof-of-concept for further analysis. Similarly, the role of the immune response in the outcome of the treatment could not be implemented. It can be expected that expanding models modeling the interaction between bacteria and phage to include the immune response will be challenging because AAS varies depending on administration method, formulation (monotherapy vs. phage cocktails), and recipient immune

status. Another major limitation of the present study was the paucity of growth rates and life-history traits. In particular, the literature on the experimental use of phages to eradicate bacteria rarely reports the exact growth rates and life-history traits of the microbes used in the experiments. The current study had to rely on a variety of information sources, which could have resulted in a distortions in the computation. Because the current model is an *in silico* approach, it is critical to empirically improve the description of bacterial and viral interactions to provide more and more accurate parameters that can increase the model's accuracy. The increasing use of phages as antibacterial agents will necessitate a greater availability of the pool of life-history traits available to researchers and practitioners, a goal that can only be achieved through a multi-center effort. There were also relevant computational limitations. The use of a decision tree algorithm provided a tool to compute ranges for each therapy. One trade-off was that the decision tree has to approximate the domains for each therapy by rectangles. If these domains are curved the algorithm provides multiple smaller ranges to approximate the behavior around the curves. Other machine learning tools such as state vector machines are more suitable in such scenarios, but their output does not provide ranges but more complicated representations. Another computational limitation was that for the ensemble simulation the ODE system needs to be solved for many different therapy pairs. This made it challenging to configure the ODE solver optimal, since too higher tolerances lead to instability issues and but solving for all therapy pairs with low tolerances leads to very time consuming computations. Depending on the model such instability issues can cause a major problem.

In conclusion, the present study applied machine learning, in the form of a decision tree algorithm, to determine ranges for the phagial dose and administration times needed to achieve passive, active, or delayed antibacterial treatment. A multi-criteria optimization problem provided Pareto optimal treatment parameters. The procedure used herein simplified the workflow to achieve effective phage therapy. The present study also introduced the concept of mediated phage therapy, where targeting a booster bacteria might decrease the virulence of a pathogen immune to phagial infection.

## Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## Author contributions

SP and LM conceived the ideas, designed methodology, and analyzed the data. LM collected the data and led the writing of the manuscript. SV supervised the project and granted the funds. All authors contributed critically to the drafts and gave final approval for publication.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## Supplementary material

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# References


1. Hatfull GF. Actinobacteriophages: genomics, dynamics, and applications. *Annu Rev Virol.* (2020) 7:37–61. doi: 10.1146/annurev-virology-122019-070009

2. Hatfull GF, Dedrick RM, Schooley RT. Phage therapy for antibiotic-resistant bacterial infections. *Annu Rev Med.* (2022) 73:197–211. doi: 10.1146/annurev-med-080219-122208

3. Brives C, Pourraz J. Phage therapy as a potential solution in the fight against AMR: obstacles and possible futures. *Palgr Commun.* (2020) 6:100. doi: 10.1057/s41599-020-0478-4

4. Aslam S, Lampley E, Wooten D, Karris M, Benson C, Strathdee S, et al. Lessons learned from the first 10 consecutive cases of intravenous bacteriophage therapy to treat multidrug-resistant bacterial infections at a single center in the United States. *Open Forum Infect Dis.* (2020) 7:ofaa389. doi: 10.1093/ofid/ofaa389

5. Terwilliger A, Clark J, Karris M, Hernandez-Santos H, Green S, Aslam S, et al. Phage therapy related microbial succession associated with successful clinical outcome for a recurrent urinary tract infection. *Viruses.* (2021) 13:2049. doi: 10.3390/v13102049

6. Dalmasso M, de Haas E, Neve H, Strain R, Cousin FJ, Stockdale SR, et al. Isolation of a novel phage with activity against *Streptococcus mutans* biofilms. *PLoS One.* (2015) 10:e0138651. doi: 10.1371/journal.pone.0138651

7. Khalifa L, Brosh Y, Gelman D, Coppenhagen-Glazer S, Beyth S, Poradosu-Cohen R, et al. Targeting Enterococcus faecalis biofilms with phage therapy. *Appl Environ Microbiol.* (2015) 81:2696–705. doi: 10.1128/AEM.00096-15

8. Harada LK, Silva EC, Campos WF, Del Fiol FS, Vila M, Dąbrowska K, et al. Biotechnological applications of bacteriophages: state of the art. *Microbiol Res.* (2018) 212–213:38–58. doi: 10.1016/j.micres.2018.04.007

9. Petrovic Fabijan A, Khalid A, Maddocks S, Ho J, Gilbey T, Sandaradura I, et al. Phage therapy for severe bacterial infections: a narrative review. *Med J Aust.* (2020) 212:279–85. doi: 10.5694/mja2.50355

10. Petrovic Fabijan A, Lin RCY, Ho J, Maddocks S, Ben Zakour NL, Iredell JR. Safety of bacteriophage therapy in severe *Staphylococcus aureus* infection. *Nat Microbiol.* (2020) 5:465–72. doi: 10.1038/s41564-019-0634-z

11. Payne RJ, Jansen VA. Understanding bacteriophage therapy as a density-dependent kinetic process. *J Theor Biol.* (2001) 208:37–48. doi: 10.1006/jtbi.2000.2198

12. Payne RJ, Phil D, Jansen VA. Phage therapy: the peculiar kinetics of self-replicating pharmaceuticals. *Clin Pharmacol Ther.* (2000) 68:225–30. doi: 10.1067/mcp.2000.109520

13. Payne RJH, Jansen VAA. Pharmacokinetic principles of bacteriophage therapy. *Clin Pharmacokinet.* (2003) 42:315–25. doi: 10.2165/00003088-200342040-00002

14. Klayman BJ, Volden PA, Stewart PS, Camper AK. *Escherichia coli* O157:H7 requires colonizing partner to adhere and persist in a capillary flow cell. *Environ Sci Technol.* (2009) 43:2105–11.

15. Breshears LM, Edwards VL, Ravel J, Peterson ML. *Lactobacillus crispatus* inhibits growth of *Gardnerella vaginalis* and *Neisseria gonorrhoeae* on a porcine vaginal mucosa model. *BMC Microbiol.* (2015) 15:276. doi: 10.1186/s12866-015-0608-0

16. Mastromarino P, Di Pietro M, Schiavoni G, Nardis C, Gentile M, Sessa R. Effects of vaginal lactobacilli in Chlamydia trachomatis infection. *Int J Med Microbiol.* (2014) 304:654–61. doi: 10.1016/j.ijmm.2014.04.006

17. Sztajer H, Szafranski SP, Tomasch J, Reck M, Nimtz M, Rohde M, et al. Cross-feeding and interkingdom communication in dual-species biofilms of *Streptococcus mutans* and *Candida albicans*. *ISME J.* (2014) 8:2256–71. doi: 10.1038/ismej.2014.73

18. Szafrański SP, Deng Z-L, Tomasch J, Jarek M, Bhuju S, Rohde M, et al. Quorum sensing of *Streptococcus mutans* is activated by *Aggregatibacter actinomycetemcomitans* and by the periodontal microbiome. *BMC Genomics.* (2017) 18:238. doi: 10.1186/s12864-017-3618-5

19. Harcombe WR, Bull JJ. Impact of phages on two-species bacterial communities. *Appl Environ Microbiol.* (2005) 71:5254–9. doi: 10.1128/AEM.71.9.5254-5259.2005

20. Leung CYJ, Weitz JS. Modeling the synergistic elimination of bacteria by phage and the innate immune system. *J Theor Biol.* (2017) 429:241–52. doi: 10.1016/j.jtbi.2017.06.037

21. Stolp H. *Microbial ecology: Organisms, Habitats, Activities*. Cambridge, MA: Cambridge University Press (1988).

22. García R, Latz S, Romero J, Higuera G, García K, Bastías R. Bacteriophage production models: an overview. *Front Microbiol.* (2019) 10:1187. doi: 10.3389/fmicb.2019.01187

23. Harder W, Veldkamp H. Competition of marine psychrophilic bacteria at low temperatures. *Antonie Van Leeuwenhoek.* (1971) 37:51–63. doi: 10.1007/BF02218466

24. Prescott L, Harley J, Klein D. *Microbiology*. 3rd ed. Dubuque: Wm. C. Brown Publishers (1996).

25. Hill AV. The possible effects of the aggregation of the molecules of haemoglobin on its dissociation curves. *J Physiol.* (1910) 40:i–vii. doi: 10.1113/jphysiol.1910.sp001386

26. Wasserman L. *All of Statistics: A Concise Course in Statistical Inference*. Berlin: Springer (2004).

27. Hastie T, Tibshirani R, Friedman J. *The Elements of Statistical Learning: Data Mining, Inference, and Prediction*. Berlin: Springer (2009).

28. Ehrgott M. *Multicriteria Optimization*. Berlin: Springer (2005).

29. Broekhuizen H, Groothuis-Oudshoorn CGM, van Til JA, Hummel JM, IJzerman MJ. A review and classification of approaches for dealing with uncertainty in multi-criteria decision analysis for healthcare decisions. *Pharmacoeconomics.* (2015) 33:445–55. doi: 10.1007/s40273-014-0251-x

30. Zhan Z-H, Zhang J, Li Y, Chung HS-H. Adaptive particle swarm optimization. *IEEE Trans Syst Man Cybern Part B.* (2009) 39:1362–81. doi: 10.1109/TSMCB.2009.2015956

31. Perkel JM. Julia: come for the syntax, stay for the speed. *Nature.* (2019) 572:141–2. doi: 10.1038/d41586-019-02310-3

32. Rackauckas, C, Nie Q. Differentialequations.jl–a performant and feature-rich ecosystem for solving differential equations in Julia. *J Open Res Softw.* (2017) 5:15.

33. Mihara T, Nishimura Y, Shimizu Y, Nishiyama H, Yoshikawa G, Uehara H, et al. Linking virus genomes with host taxonomy. *Viruses.* (2016) 8:66. doi: 10.3390/v8030066

34. Hansen SR, Hubbell SP. Single-nutrient microbial competition: qualitative agreement between experimental and theoretically forecast outcomes. *Science.* (1980) 207:1491–3. doi: 10.1126/science.6767274

35. De Paepe M, Leclerc M, Tinsley CR, Petit M-A. Bacteriophages: an underestimated role in human and animal health? *Front Cell Infect Microbiol.* (2014) 4:39. doi: 10.3389/fcimb.2014.00039

36. Jost JL, Drake JF, Fredrickson AG, Tsuchiya HM. Interactions of *Tetrahymena pyriformis*, *Escherichia coli*, *Azotobacter vinelandii*, and glucose in a minimal medium. *J Bacteriol.* (1973) 113:834–40. doi: 10.1128/jb.113.2.834-840.1973

37. Tsai JC, Aladegbami SL, Vela GR. Phosphate-limited culture of *Azotobacter vinelandii*. *J Bacteriol.* (1979) 139:639–45. doi: 10.1128/jb.139.2.639-645.1979

38. Millán C, Peña C, Flores C, Espín G, Galindo E, Castillo T. Improving glucose and xylose assimilation in *Azotobacter vinelandii* by adaptive laboratory evolution. *World J Microbiol Biotechnol.* (2020) 36:46. doi: 10.1007/s11274-020-02822-5

39. Nobile CJ, Johnson AD. *Candida albicans* biofilms and human disease. *Annu Rev Microbiol.* (2015) 69:71–92. doi: 10.1146/annurev-micro-091014-104330

40. Engku Nasrullah Satiman EAF, Ahmad H, Ramzi AB, Abdul Wahab R, Kaderi MA, Wan Harun WHA, et al. The role of *Candida albicans* candidalysin ECE1 gene in oral carcinogenesis. *J Oral Pathol Med.* (2020) 49:835–41. doi: 10.1111/jop.13014

41. Pereira R, Dos Santos Fontenelle RO, de Brito EHS, de Morais SM. Biofilm of *Candida albicans*: formation, regulation and resistance. *J Appl Microbiol.* (2021) 131:11–22. doi: 10.1111/jam.14949

42. Brusca MI, Irastorza RM, Cattoni DI, Ozu M, Chara O. Mechanisms of interaction between *Candida albicans* and *Streptococcus mutans*: an experimental and mathematical modelling study. *Acta Odontol Scand.* (2013) 71:416–23. doi: 10.3109/00016357.2012.690530

43. Pancheniak E, de FR, Maziero MT, Rodriguez-León JA, Parada JL, Spier MR, et al. Molecular characterisation and biomass and metabolite production of *Lactobacillus reuteri* LPB P01-001: a potential probiotic. *Braz J Microbiol.* (2012) 43:135–47. doi: 10.1590/S1517-83822012000100015

44. Delisle AL, Guo M, Chalmers NI, Barcak GJ, Rousseau GM, Moineau S. Biology and genome sequence of *Streptococcus mutans* phage M102AD. *Appl Environ Microbiol.* (2012) 78:2264–71. doi: 10.1128/AEM.07726-11

45. Tiwari BR, Kim S, Rahman M, Kim J. Antibacterial efficacy of lytic *Pseudomonas* bacteriophage in normal and neutropenic mice models. *J Microbiol.* (2011) 49:994–9. doi: 10.1007/s12275-011-1512-4

46. Roach DR, Leung CY, Henry M, Morello E, Singh D, Di Santo JP, et al. Synergy between the host immune system and bacteriophage is essential for successful phage therapy against an acute respiratory pathogen. *Cell Host Microbe.* (2017) 22:38e–47.e4. doi: 10.1016/j.chom.2017.06.018

47. Van Belleghem JD, Dąbrowska K, Vaneechoutte M, Barr JJ, Bollyky PL. Interactions between bacteriophage, bacteria, and the mammalian immune system. *Viruses.* (2018) 11:10. doi: 10.3390/v11010010

48. Kuchment A. *The Forgotten Cure: the Past and Present of Phage Therapy*. Berlin: Springer (2014).

49. Onsea J, Soentjens P, Djebara S, Merabishvili M, Depypere M, Sriet I, et al. Bacteriophage applications for difficult-to-treat musculoskeletal infections: development of a standardized multidisciplinary treatment protocol. *Viruses.* (2019) 11:891. doi: 10.3390/v11100891

50. Marongiu, L, Burkard M, Lauer UM, Hoelzle LE, Venturelli S. Reassessment of historical clinical trials supports the effectiveness of phage therapy. *Clin Microbiol Rev.* (2022). doi: 10.1128/cmr.00062-22 [Epub ahead of print].

51. Żaczek M, Łusiak-Szelachowska M, Jończyk-Matysiak E, Weber-Dąbrowska B, Międzybrodzki R, Owczarek B, et al. Antibody production in response to staphylococcal MS-1 phage cocktail in patients undergoing phage therapy. *Front Microbiol.* (2016) 7:1681. doi: 10.3389/fmicb.2016.01681

52. Dabrowska K, Miernikiewicz P, Piotrowicz A, Hodyra K, Owczarek B, Lecion D, et al. Immunogenicity studies of proteins forming the T4 phage head surface. *J Virol.* (2014) 88:12551–7. doi: 10.1128/JVI.02043-14

53. Sannathimmappa MB, Nambiar V, Aravindakshan R. Antibiotics at the crossroads – do we have any therapeutic alternatives to control the emergence and spread of antimicrobial resistance? *J Educ Health Promot.* (2021) 10:438. doi: 10.4103/jehp.jehp_557_21

54. Dedrick, RM, Smith BE, Cristinziano M, Freeman KG, Jacobs-Sera D, Belessis Y, et al. Phage therapy of mycobacterium infections: compassionate-use of phages in twenty patients with drug-resistant mycobacterial disease. *Clin Infect Dis.* (2022) ciac453. doi: 10.1093/cid/ciac453

55. Lenski RE, Levin BR. Constraints on the coevolution of bacteria and virulent phage: a model, some experiments, and predictions for natural communities. *Am Nat.* (1985) 125:585–602. doi: 10.1086/284364

56. Weitz JS. *Quantitative Viral Ecology: Dynamics of Viruses and Their Microbial Hosts*. Princeton, NJ: Princeton University Press (2016).

57. Sharma RS, Karmakar S, Kumar P, Mishra V. Application of filamentous phages in environment: a tectonic shift in the science and practice of ecorestoration. *Ecol Evol.* (2019) 9:2263–304. doi: 10.1002/ece3.4743

58. Olson EG, Micciche AC, Rothrock MJJ, Yang Y, Ricke SC. Application of bacteriophages to limit campylobacter in poultry production. *Front Microbiol.* (2021) 12:458721. doi: 10.3389/fmicb.2021.458721

59. Wang Z, Zhao X. The application and research progress of bacteriophages in food safety. *J Appl Microbiol.* (2022) 133:2137–47. doi: 10.1111/jam.15555

60. Liu Y, Mi Z, Niu W, An X, Yuan X, Liu H, et al. Potential of a lytic bacteriophage to disrupt *Acinetobacter baumannii* biofilms in vitro. *Future Microbiol.* (2016) 11:1383–93. doi: 10.2217/fmb-2016-0104

61. D'Accolti M, Soffritti I, Lanzoni L, Bisi M, Volta A, Mazzacane S, et al. Effective elimination of Staphylococcal contamination from hospital surfaces by a bacteriophage-probiotic sanitation strategy: a monocentric study. *Microb Biotechnol.* (2019) 12:742–51. doi: 10.1111/1751-7915.13415

62. Pinto G, Almeida C, Azeredo J. Bacteriophages to control Shiga toxin-producing *E. coli* – safety and regulatory challenges. *Crit Rev Biotechnol.* (2020) 40:1081–97. doi: 10.1080/07388551.2020.1805719

63. Stachler E, Kull A, Julian TR. Bacteriophage treatment before chemical disinfection can enhance removal of plastic-surface-associated *Pseudomonas aeruginosa*. *Appl Environ Microbiol.* (2021) 87:e0098021. doi: 10.1128/AEM.00980-21

64. Liao Y-T, Zhang Y, Salvador A, Harden LA, Wu VCH. Characterization of a T4-like bacteriophage vB_EcoM-Sa45lw as a potential biocontrol agent for shiga toxin-producing *Escherichia coli* O45 contaminated on mung bean seeds. *Microbiol Spectr.* (2022) 10:e0222021. doi: 10.1128/spectrum.02220-21

65. Ponde NO, Lortal L, Ramage G, Naglik JR, Richardson JP. Candida albicans biofilms and polymicrobial interactions. *Crit Rev Microbiol.* (2021) 47:91–111. doi: 10.1080/1040841X.2020.1843400

66. Bull JJ, Harcombe WR. Population dynamics constrain the cooperative evolution of cross-feeding. *PLoS One.* (2009) 4:e4115. doi: 10.1371/journal.pone.0004115

67. Rodriguez-Gonzalez RA, Leung CY, Chan BK, Turner PE, Weitz JS. Quantitative models of phage-antibiotic combination therapy. *mSystems.* (2020) 5:e00756-19. doi: 10.1128/mSystems.00756-19

68. Khatami A, Lin RCY, Petrovic-Fabijan A, Alkalay-Oren S, Almuzam S, Britton PN, et al. Bacterial lysis, autophagy and innate immune responses during adjunctive phage therapy in a child. *EMBO Mol Med.* (2021) 13:e13936. doi: 10.15252/emmm.202113936

69. Laird T, Abraham R, Sahibzada S, Abraham S, O'Dea M. In Vitro demonstration of targeted phage therapy and competitive exclusion as a novel strategy for decolonization of extended-spectrum-cephalosporin-resistant *Escherichia coli*. *Appl Environ Microbiol.* (2022) 88:e0227621. doi: 10.1128/aem.02276-21

70. Mumford R, Friman V-P. Bacterial competition and quorum-sensing signalling shape the eco-evolutionary outcomes of model in vitro phage therapy. *Evol Appl.* (2017) 10:161–9. doi: 10.1111/eva.12435

71. Bull JJ, Vegge CS, Schmerer M, Chaudhry WN, Levin BR. Phenotypic resistance and the dynamics of bacterial escape from phage control. *PLoS One.* (2014) 9:e94690. doi: 10.1371/journal.pone.0094690

72. Melo LDR, Oliveira H, Pires DP, Dabrowska K, Azeredo J. Phage therapy efficacy: a review of the last 10 years of preclinical studies. *Crit Rev Microbiol.* (2020) 46:78–99. doi: 10.1080/1040841X.2020.1729695

73. Styles KM, Brown AT, Sagona AP. A Review of using mathematical modeling to improve our understanding of bacteriophage, bacteria, and eukaryotic interactions. *Front Microbiol.* (2021) 12:724767. doi: 10.3389/fmicb.2021.724767

74. Cortez MH, Weitz JS. Coevolution can reverse predator-prey cycles. *Proc Natl Acad Sci U.S.A.* (2014) 111:7486–91. doi: 10.1073/pnas.1317693111

75. Chaudhry WN, Pleška M, Shah NN, Weiss H, McCall IC, Meyer JR, et al. Leaky resistance and the conditions for the existence of lytic bacteriophage. *PLoS Biol.* (2018) 16:e2005971. doi: 10.1371/journal.pbio.2005971

76. Bulmer MG. Phase relations in the ten-year cycle. *J Anim Ecol.* (1975) 44:609–21. doi: 10.2307/3614

77. Javaudin F, Latour C, Debarbieux L, Lamy-Besnier Q. Intestinal bacteriophage therapy: looking for optimal efficacy. *Clin Microbiol Rev.* (2021) 34:e0013621. doi: 10.1128/CMR.00136-21

78. Jochum L, Stecher B. Label or concept – what is a pathobiont? *Trends Microbiol.* (2020) 28:789–92. doi: 10.1016/j.tim.2020.04.011

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# Bacteriophage: A new therapeutic player to combat neutrophilic inflammation in chronic airway diseases

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Persistent respiratory bacterial infections are a clinical burden in several chronic inflammatory airway diseases and are often associated with neutrophil infiltration into the lungs. Following recruitment, dysregulated neutrophil effector functions such as increased granule release and formation of neutrophil extracellular traps (NETs) result in damage to airway tissue, contributing to the progression of lung disease. Bacterial pathogens are a major driver of airway neutrophilic inflammation, but traditional management of infections with antibiotic therapy is becoming less effective as rates of antimicrobial resistance rise. Bacteriophages (phages) are now frequently identified as antimicrobial alternatives for antimicrobial resistant (AMR) airway infections. Despite growing recognition of their bactericidal function, less is known about how phages influence activity of neutrophils recruited to sites of bacterial infection in the lungs. In this review, we summarize current *in vitro* and *in vivo* findings on the effects of phage therapy on neutrophils and their inflammatory mediators, as well as mechanisms of phage-neutrophil interactions. Understanding these effects provides further validation of their safe use in humans, but also identifies phages as a targeted neutrophil-modulating therapeutic for inflammatory airway conditions.

KEYWORDS

**bacteriophage, phage therapy, chronic airway disease, bacterial infection, neutrophilic inflammation**

## Introduction

Chronic airway diseases are major causes of human mortality (1, 2), represented by conditions such as cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD), bronchiectasis, and asthma. Key features of these diseases are recurrent lower respiratory bacterial infections, which over repeated courses of antibiotic therapy can become antimicrobial resistant (AMR). Across these lung conditions, neutrophilic inflammation sustained by frequent infections can be a major driver of airway damage (3), yet effective anti-inflammatory and neutrophil-targeted therapies are not available.

An emerging tool against AMR infections is the therapeutic use of bacteriophages or phages, viruses that infect bacteria, ubiquitously and abundantly present in the environment (4). First identified in the early twentieth century, phages were initially investigated for use as antimicrobials in humans following observations of bacterial killing *in vitro* (5). Upon the discovery of penicillin and other antimicrobial compounds in the 1940s, interest in phages waned as academia and industry focused on development of these drugs; however, therapeutic phage centers have remained active in certain countries (5). With the emergence of antimicrobial resistance, and four decades since the last antibiotic drug was discovered, there is resurging interest in phages as potential alternatives. Phage therapy works by exploiting the life cycle of lytic phages, which in the process of replication lyse and kill their bacterial host (5). Phages have several characteristics that support their clinical use. They do not infect human cells (5), appear to be safe and well tolerated (6–8), may require less doses compared to conventional antibiotics due to their self-replicating nature (9, 10), and are highly specific to their target bacterial species, meaning unlike antibiotics they do not have broad bactericidal activity against the host microbiome (7, 11). The use of phages as a standard clinical therapeutic is hampered by a still incomplete understanding of phage biology (12), as well as a lack of regulatory manufacturing guidelines for phage products (13, 14) and standardized large-scale clinical trials (15); however, science, medicine, and industry are progressively working to surmount these challenges. In the context of chronic respiratory diseases, phage therapy is now being explored as a treatment for pulmonary infections (16, 17). Intriguingly, emerging evidence suggests that administration of phages may also have significant therapeutic benefits for managing neutrophilic inflammation in the lungs (18–21).

## The role of bacterial infections in chronically diseased airways

The link between bacterial airway infections and chronic inflammatory lung diseases is well established. One significant bacterium in this regard is the opportunistic pathogen *Pseudomonas aeruginosa*, a species which is notably associated with severe and negative health outcomes across multiple chronic airway conditions (22). Perhaps most striking is the early childhood acquisition in the autosomal recessive disorder CF, where *P. aeruginosa* contributes to reduced lung function (23–26), increased airway inflammation (27, 28), permanent airway remodeling (29, 30), and increased mortality in individuals with the disease (31, 32). Treatment strategies initiating eradication therapy in children with CF have reduced *P. aeruginosa* colonization rates from 80 to 50% (33, 34), but acquisition of this bacterium remains a key determinant of long-term CF clinical outcomes (31, 35). Among individuals with COPD, up to 40% will have positive sputum cultures for *P. aeruginosa* (36–39), with over 10% meeting criteria for colonization (39–41). In addition, up to a third of participants in cohorts of non-CF bronchiectasis can be colonized by this pathogen (42–44). The degree to which *P. aeruginosa* colonization in non-CF airway diseases contributes to lung function decline is still not clear (45), but multiple studies in both COPD and bronchiectasis link *P. aeruginosa* to more frequent exacerbations and/or hospitalization (36, 38, 42, 44, 46–48), increased mortality (36, 44, 46, 47, 49, 50), and greater annual lung function decline (40).

The role of bacterial infections in the pathogenesis of asthma is not as well understood as that of respiratory viruses, which are associated with childhood wheezing, compromised epithelial barrier function, asthma development, and exacerbations (51, 52). However, studies have still noted associations between bacterial pathogens and asthma pathologies. For example, in a cohort of 56 asthmatic patients from Royal Brompton Hospital, London, sputum bacterial culture positivity with *P. aeruginosa*, *Haemophilus influenzae,* and *Staphylococcus aureus* was significantly associated with increased asthma duration and frequency of exacerbations in the previous year (53). Other factors including pneumonia, pathogen isolation, as well as sputum production and purulence have also been identified and associated with the development of bronchiectasis in asthma cohorts (54, 55).

A primary concern with treating frequent lung infections in the context of these diseases is the acquisition of antimicrobial resistance, with some pathogens becoming multi-drug resistant (MDR). This makes eradication of these bacterial infections challenging and increases the treatment burden of patients with chronic lung conditions. In 2019, lower respiratory infections globally accounted for over 1.5 million out of 4.95 million estimated deaths associated with antimicrobial resistance, more than any other infectious syndrome (56). Among individuals with chronic lung diseases, acquisition of AMR/MDR pathogens is associated with increased disease severity (57–59), exacerbations (57, 59, 60), and mortality (31, 61). With prevalence of chronic airway conditions increasing by nearly 40% since 1990 (62, 63), novel therapeutics to treat AMR lung infections are desperately needed.

## Neutrophils, drivers of lung damage

As one of the first immune cell types recruited to sites of infection, neutrophils have an important role in the innate immune response to respiratory bacterial infections (64). Historically perceived as functionally rigid and transcriptionally fixed, neutrophils are increasingly described as plastic cells whose function is shaped by their environment (65–67). In the context of chronic lung diseases, studies assessing neutrophils recruited to diseased airways have observed changes in neutrophil antimicrobial functions that result in airway damage and contribute to lung disease progression.

One of the major mechanisms by which neutrophils can damage the airways is through release of neutrophil elastase (NE), a serine protease normally stored within intracellular primary granules. Airway NE is an important marker of inflammation in CF, significantly correlating with severity of lung disease in both children and adults (68, 69). Infection with *P. aeruginosa* is associated with increased sputum NE activity in adults with CF (70), as well as prolonged NE activity in pediatric CF airways (28). Release of NE by neutrophils in CF airways was originally thought to be a consequence of neutrophil death, but studies within the last decade have described how this process occurs from granule exocytosis by viable neutrophil populations in CF lungs (71–73). Work by our group using *in vitro* modeling of the airway infection environment created by *P. aeruginosa* has demonstrated that infection induces neutrophil degranulation (74). We found that neutrophils recruited to infection microenvironments primed by *P. aeruginosa* had significantly increased staining of CD63 and CD66b, neutrophil markers of primary and secondary granule exocytosis, respectively (74). In COPD, airway NE is elevated during exacerbations and can be predictive of bacterially induced exacerbations (75). Studies are also identifying airway NE as a potential biomarker of disease severity in non-CF bronchiectasis. For example, in a cohort of 433 adult patients, Chalmers and colleagues found significant associations between high sputum NE levels and increased dyspnea scores, lung function decline, exacerbation frequency, and radiological scoring of bronchiectasis severity (76). Sputum NE was elevated during exacerbations and reduced in response to antibiotic therapy targeting organisms such as *P. aeruginosa* and *H. influenzae*, highlighting a relationship between release of NE and bacterial respiratory infection (76). Another recent pediatric cross-sectional study of both CF and non-CF related bronchiectasis also found that sputum NE significantly correlated with exacerbation severity and frequency, as well as number of hospitalizations (77). For CF bronchiectasis it was specifically observed that NE correlated with risk of disease progression and increased lung function decline, while for non-CF bronchiectasis, sputum NE positively correlated with airway neutrophil counts and severity of lung disease (77). Asthma is a chronic lung disease with a much broader range of inflammatory phenotypes, and while eosinophil activity is critical to some cohorts, some of the more severe forms of asthma are primarily a result of airway neutrophilic inflammation (78). Past studies of asthma have associated increased airway NE (79–81) and myeloperoxidase (82), another factor released from primary granules, with more severe disease. Studies on allergic asthmatic responses in animal models have also shown reduced airway inflammation (83, 84) and bronchoconstriction (85) following treatment with NE inhibitors.

Neutrophil degranulation and NE release seem to coincide with a reduction in phagocytic ability that, certainly in CF, may contribute to further disease (71–74, 86). There are few reports describing decreased phagocytosis by neutrophils in COPD (87–89), bronchiectasis (90, 91), and asthma (92), and further investigation is required to definitively conclude whether this is a feature of non-CF lung diseases. Impairment of this crucial neutrophil function may contribute to prolonged infection, pathogen colonization, and associated negative health outcomes in chronic airway diseases, and may explain in part why neutrophils in these conditions resort to alternative antimicrobial strategies detrimental to host airways.

In the last 20 years, a novel neutrophil antimicrobial function was discovered and linked to the production of neutrophil extracellular traps (NETs) (93). This was termed NETosis, a process in which neutrophils eject extracellular networks of DNA containing primary granules, NE and other antimicrobial factors, which can trap and neutralize invading pathogens. Also thought to be an event resulting in neutrophil death (94), different NETosis pathways have been described that utilize mitochondrial DNA release rather than nuclear DNA (95), or preserve neutrophil viability after NET formation (96, 97). The degree to which NETs significantly contribute to pathogen clearance is debated (98, 99). The toxic antimicrobial factors harbored within NET complexes may instead contribute to airway damage. Interest in NETs has increased since studies identified them as major sputum components in chronically disease airways (100–102). In CF, NETs influence airway mucus viscosity (100, 103) and are associated with increased airway obstruction (104). CF neutrophils are also inherently predisposed to increased NET formation, delayed apoptosis and increased lifespan as a result of CFTR dysfunction (105). NET formation in COPD sputum has been found to significantly correlate with disease severity, lung function decline, and exacerbation frequency (101, 106). In severe asthma, high extracellular DNA indicative of increased NETosis has been associated with increased corticosteroid use, neutrophilic inflammation, and inflammasome activation (107). An international observational study by Keir et al. performed proteomic analysis of sputum from bronchiectasis patients, finding that NET proteins were abundantly present and strongly associated with increased disease severity, hospital admissions, and mortality (102). A separate study within this report further

showed that low doses of antibiotics over a 12-month period was associated with NET reduction in sputum from individuals with bronchiectasis or asthma (102), underscoring the connection between NETosis and airway bacteria.

Limiting neutrophil migration to the lungs would appear to be a simple solution for preventing damage by aberrant functions of recruited neutrophils. However, reducing airway neutrophil influx can have negative consequences, as was the case in a phase two clinical trial of the leukotriene B4 (LTB4) receptor antagonist BIIL 284 BS (108). This trial was prematurely terminated upon discovering a significant increase in serious adverse events among CF patients receiving the drug (108). A follow up study assessing participant samples and BIIL 284 treatment in *P. aeruginosa* infected mice determined that the drug significantly reduced airway neutrophil counts, leading to increased *P. aeruginosa* in the lung, bacteremia, and increased lung inflammation (109). This suggests that outright reduction of neutrophil numbers in infected lungs is not therapeutically beneficial; a therapy that instead amends neutrophil pathological activity, preserves phagocytic function, and aids in bacterial clearance, may be more effective. In chronic lung diseases, the interplay between bacterial respiratory infections, neutrophilic inflammation, and airway damage highlights an important need for therapies that can treat infection, and the inflammatory processes and neutrophil functions that result in damage to the lungs (**Figure 1**).

## Phage therapy and airway inflammation

Trials of phage therapy for respiratory infections in humans have occurred in a limited number of instances for compassionate use, particularly in cases with MDR pathogens where the traditional spectrum of antibiotics failed to clear infection. Many of the resulting case reports have described positive outcomes, with no adverse effects and infections successfully cleared in treated patients (110–115). However, despite increasing validation for use as antimicrobials in humans, little is known about the innate immune response to respiratory phage therapy, and how airway neutrophils recruited during bacterial infection may respond to treatment. A handful of studies in small animal models have provided some data on inflammation following therapeutic phage administration during experimental airway infection (20, 116–119).

Airway neutrophil recruitment is initiated by detection of chemotactic signals such as interleukin (IL)-8 and LTB4 (120). As neutrophils exit circulation and migrate through tissue, they can encounter additional inflammatory cytokines such as IL-1, IL-6, IL-18, TNFα, and become primed, further increasing their responsiveness and propensity for pathological activation (67, 121). Thus, host cytokines have an important role in modulating neutrophil responses during infection. A study by Pabary et al. assessed the effects of an intranasally administered mixture of individual phages or phage cocktail, before, during, and after *P. aeruginosa* inoculation in mice, measuring inflammatory markers and neutrophil counts in bronchoalveolar lavage fluid (BALF) (116). In experimental infections with *P. aeruginosa* reference strain PAO1, phage treatment at all timepoints significantly reduced viable bacterial numbers, but only the prophylactic administration of phages significantly reduced BALF neutrophil counts compared to untreated animals (116). Simultaneous inoculation with phages and bacteria significantly reduced IL-10 and IL-1β compared to animals infected with bacteria alone, while both delayed and prophylactic administration of phages significantly reduced the neutrophil chemokine keratinocyte chemoattractant (KC) (116). A CF clinical isolate of *P. aeruginosa* was also tested, inoculated simultaneously with lytic phages. Curiously, bacterial clearance of this isolate was not enhanced with phage treatment compared to untreated animals; however, the authors acknowledged differences in bacterial doses and BALF sampling times in experiments with the clinical isolate vs. PAO1 that may account for this (116). Nevertheless, phage treatment in this experiment significantly reduced both neutrophil counts and pro-inflammatory mediators IL-6, IL-10, IL-12p70, KC, and TNFα in BALF of treated animals compared to untreated controls (116). A different study using bioluminescent *P. aeruginosa* was able to image phage-mediated clearance in the lungs of infected mice, with treatment with phages reducing bacterial luminescent signal from the lungs, increasing animal survival, and reducing IL-6 and TNFα in BALF (117). It was also determined that prophylactic phage administration twenty-fours prior to bacterial inoculation had a protective effect against infection (117). Importantly, a study of *Escherichia coli* pneumonia in mice showed that bacterial lysis induced by phage therapy induces similar levels of cytokine release as lysis induced by antibiotics, with phages primarily reducing release of most inflammatory signals (118). This suggests phage-induced bacterial lysis is unlikely to result in more severe inflammation compared to activity of conventional antibiotics, but additional research is needed to verify this.

While by design not a respiratory model, a *CFTR* loss-of-function zebrafish model has been used by Cafora et al. to describe the immunomodulatory potential of phage therapy in CF across two studies. In the first, a phage cocktail administered to zebrafish embryos infected with PAO1 was observed to significantly reduce bacterial load, lethality, and gene expression of IL-1β and TNFα (119). Of note, reduced cytokine gene expression was also observed in embryos exposed to phages alone in the absence of bacteria, suggesting phage anti-inflammatory mechanisms independent of bactericidal activity (119). This was a major focus of the second study, which identified that embryo toll-like receptor (TLR) recognition of phage capsid proteins, and not phage DNA, was necessary to elicit an anti-inflammatory effect (20). The injection of a phage

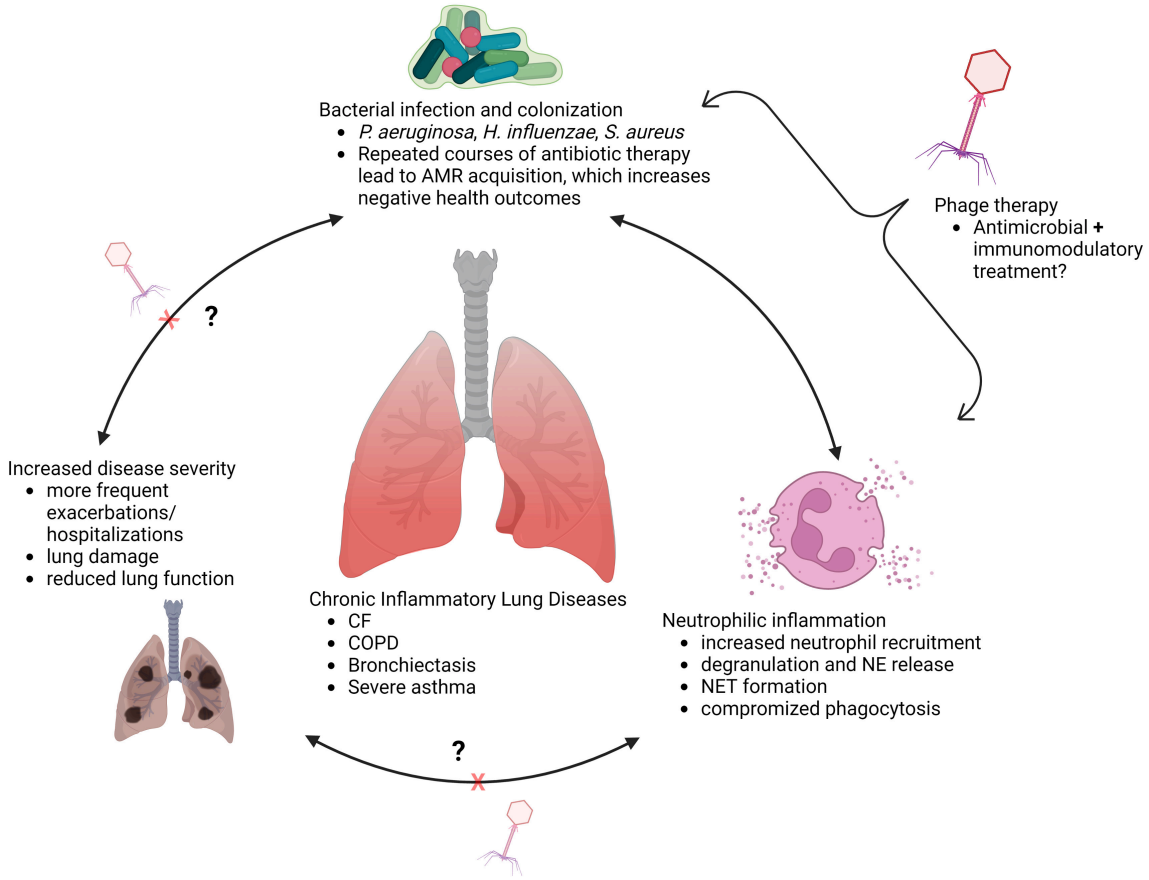


FIGURE 1
Interplay between infection and inflammation in chronically diseased lungs (created with BioRender.com).

cocktail at the site of experimental tail amputation was further observed to reduce neutrophil recruitment to wound sites, further demonstrating phage capacity to influence localized inflammation (20).

As a direct barrier to infection, the airway epithelium is a major source of neutrophil chemotactic signals and inflammatory mediators (122, 123). In perhaps one of the only studies assessing effects of phage therapy on primary airway epithelial cells, Trend and colleagues performed exposures of undifferentiated primary airway epithelial cell cultures to the virulent *P. aeruginosa* phage E79 (124). They observed that E79 did not increase release of pro-inflammatory cytokines IL-1β, IL-6, IL-8, or induce apoptosis, in airway cultures derived from children with and without CF (124), indicating that phages alone are not highly immunostimulatory to human airway cells. Altogether, studies suggest that phage therapy can effectively reduce cytokine signals involved in neutrophil recruitment and activation. This effect is not always a consequence of overt antimicrobial activity, with induction of anti-inflammatory mediators (125), reduced production of reactive oxygen species (126), and LPS binding (127) identified as possible mechanisms. This would make phages an attractive multipurpose therapeutic for managing both airway inflammation and infection in chronic lung diseases. However, further investigation is necessary to understand the specific mechanisms of phage anti-inflammatory activity.

## Phage-neutrophil interactions

The interactions between phages and human phagocytes have been of interest to researchers since the 1920s, when a number of early studies noted increased phagocytosis of bacteria by leukocytes in the presence of phages (128–130). More recent studies in neutropenic mouse models have noted a synergism between phages and neutrophils that is required for successful clearance of bacteria. An investigation by Tiwari et al. found that immunocompetent mice inoculated intranasally with a lethal dose of PAO1 could clear lung infection and maintain an 80–100% survival rate when receiving different doses of lytic phages; however, neutropenic animals failed to clear infection with phage administration alone (131).

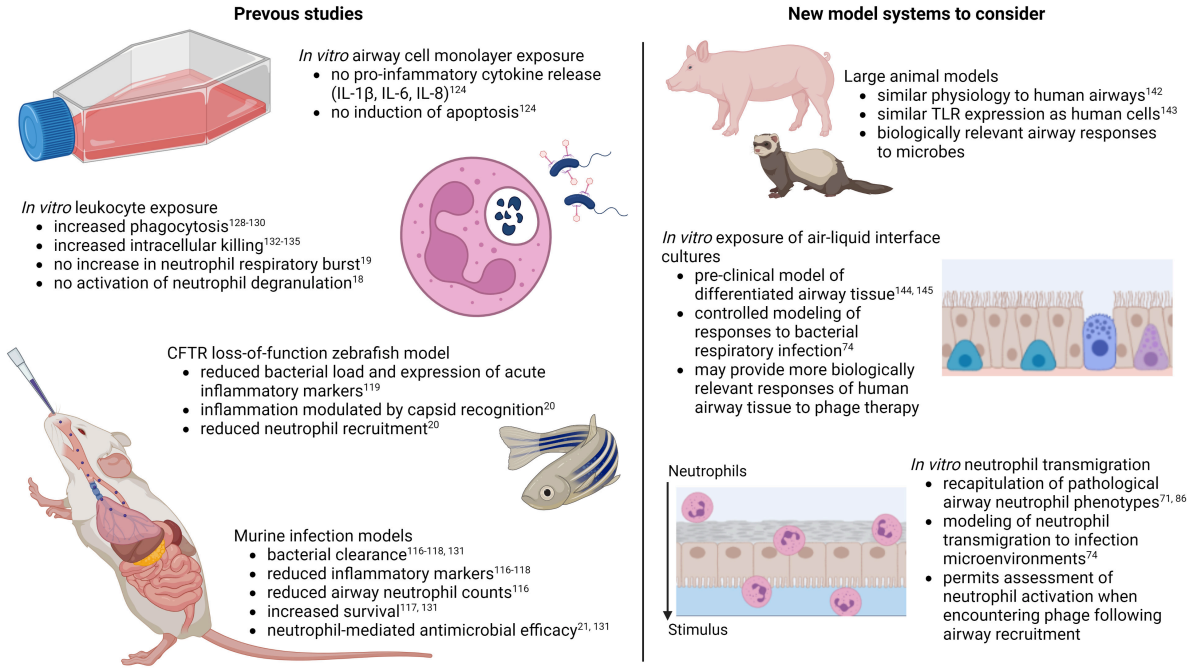


FIGURE 2
Models for assessing innate immune responses to pulmonary phage therapy (created with BioRender.com).

Roach and colleagues took this approach a step further, using *in silico* modeling to identify host innate responses as a necessary feature to overcome emerging phage resistant mutants during respiratory *P. aeruginosa* infection, and neutrophil activity as a key component of successful phage therapy (21). Whether this synergy implies phage-mediated enhancement of neutrophil bacterial killing capacity is an important question for future research. Some studies have linked phages to increased intracellular killing of pathogens within human phagocytes such as *Klebsiella pneumoniae* (132), *Mycobacterium avium* and *tuberculosis* (133), and methicillin-resistant *S. aureus* (134, 135). There are contrasting reports, however, where phages did not significantly influence intracellular killing of pathogenic bacteria (136, 137).

Whether phages contribute to activation of pathological neutrophil functions is critical to ascertain for safe use of this therapy. A study of neutrophil exposure to lytic phages observed little to no respiratory burst activity induced by T4 *E. coli* phage and A3/R *S. aureus* phage preparations, compared to heat inactivated *S. aureus* cells, suggesting that phages alone should not induce oxidative stress when administered in humans (19). Importantly, it has also been shown that A3/R phage and *S. aureus* phage lysate do not elicit neutrophil degranulation, as indicated by low neutrophil expression of CD63 and CD66b (18). This implies that both phages and phage-mediated lysis of bacteria are not likely to activate neutrophil degranulation and consequent NE release during treatment *in vivo*. However, availability of data in this regard is inconsistent across bacterial pathogens and neutrophil activation states, so there remains much work to be done for a definitive understanding. Further exploration of whether phage therapy can restore neutrophil phagocytosis of evasive organisms and ameliorate aberrant functions such as degranulation is warranted, particularly in the context of inflammatory airway diseases.

## Modeling phage therapy and neutrophilic inflammation in the laboratory

Altogether, studies to date suggest potential anti-inflammatory and neutrophil-modulating benefits of phage therapy for respiratory infections. Further research on this topic is justified, as findings could point to novel therapeutic benefits with capacity to improve treatment of multiple chronic inflammatory lung conditions. However, several factors must be taken into account for relevant modeling of human airway immune responses during phage therapy. For the purposes of understanding airway cell and neutrophil responses to pathogen associated molecular patterns during infection, a major limitation of murine models is differential TLR expression. Mice contain a pseudogene for TLR10, an anti-inflammatory TLR shown to detect bacterial and viral ligands, which is normally expressed by human cells (138). Furthermore, mice and rats express three TLRs that are not express in humans,

TLRs 11, 12, and 13, which detect flagellin, fungal profilin, and bacterial ribosomal sequences, respectively (139). In addition, mouse neutrophils may not be activated by certain microbial factors that normally affect human neutrophils, as has been reported with staphylococcal superantigen-like protein 13 (SSL13) (140). These limitations can be overcome by using large animal models of chronic airway disease including pigs and ferrets (**Figure 2**) (141, 142), whose airway physiology and TLR expression more closely resemble that of human airways (143).

A major drawback of large animal models is the high cost, labor, and resources required. This is where cell-based laboratory models have an advantage. Few studies to date have assessed phage safety and efficacy on cultures of differentiated primary airway epithelial cells known as air-liquid interface cultures (**Figure 2**) (144, 145), the gold-standard model for pre-clinical studies in human airways. Further research in this model may provide valuable and biologically relevant insights on innate responses to phage therapy in human lungs. Regarding *in vitro* studies of phage-neutrophil interactions, one of the major caveats of previous studies is the restricted exposure of phages to peripheral blood neutrophils. This may provide insights into how neutrophils in circulation interact with phages delivered by intravenous injection, but it fails to account for the fact that the site of infection and the extravasation process itself contribute to pathological neutrophil activation (66, 67). Granule releasing neutrophils in CF, as an example, are only evident in the airway lumen, as peripheral blood neutrophils in individuals with CF are phenotypically similar to neutrophils from non-CF individuals (71, 73). Furthermore, recapitulation of this neutrophil phenotype in the laboratory can only be achieved following *in vitro* transmigration (71, 86). Existing laboratory models of neutrophil recruitment to the lungs may provide more relevant examples of neutrophil behavior during respiratory phage therapy, as neutrophil responses to phages can be observed following transmigration to the airway infection environment (**Figure 2**).

## Conclusion

In summary, neutrophils are important and necessary for the clearance of bacterial respiratory pathogens. In chronic and inflammatory airway diseases, persistent bacterial infections sustain neutrophil influx into the lungs, wherein exaggerated neutrophil antimicrobial functions can result in host tissue damage. Phage therapy is emerging as novel therapeutic for AMR lung infections resulting from prolonged antibiotic use. A growing body of evidence suggests phage therapy may have important immunomodulatory benefits. Whether this is primarily a consequence of reduced bacterial burden or direct interaction between phages and neutrophils merits further investigation. Various laboratory model systems are available to assess airway innate responses to phage therapy; researchers must ensure that models are representative of these dynamics in human airways.

## Author contributions

DL, LG, and AK conceived the review, conduced the literature search, and wrote the manuscript. SS critically reviewed and edited the manuscript. All authors approved the final version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## References

1. Murphy SLKK, Xu JQ, Arias E. *Mortality in the United States, 2020*. Hyattsville, MD: National Center for Health Statistics (2021).

2. World Health Organisation [WHO]. *The Top 10 Causes of Death*. Geneva: WHO (2020).

3. Jasper AE, McIver WJ, Sapey E, Walton GM. Understanding the role of neutrophils in chronic inflammatory airway disease. *F1000Res.* (2019) 8:F1000FacultyRev–557. doi: 10.12688/f1000research.18411.1

4. Jurczak-Kurek A, Gasior T, Nejman-Falenczyk B, Bloch S, Dydecka A, Topka G, et al. Biodiversity of bacteriophages: morphological and biological properties

of a large group of phages isolated from urban sewage. *Sci Rep.* (2016) 6:34338. doi: 10.1038/srep34338

5. Wittebole X, De Roock S, Opal SM. A historical overview of bacteriophage therapy as an alternative to antibiotics for the treatment of bacterial pathogens. *Virulence.* (2014) 5:226–35. doi: 10.4161/viru.25991

6. Speck P, Smithyman A. Safety and efficacy of phage therapy via the intravenous route. *FEMS Microbiol Lett.* (2016) 363:fnv242. doi: 10.1093/femsle/fnv242

7. Sarker SA, McCallin S, Barretto C, Berger B, Pittet AC, Sultana S, et al. Oral T4-like phage cocktail application to healthy adult volunteers from Bangladesh. *Virology.* (2012) 434:222–32. doi: 10.1016/j.virol.2012.09.002

8. Sarker SA, Sultana S, Reuteler G, Moine D, Descombes P, Charton F, et al. Oral phage therapy of acute bacterial diarrhea with two Coliphage preparations: a randomized trial in children from Bangladesh. *EBioMedicine.* (2016) 4:124–37. doi: 10.1016/j.ebiom.2015.12.023

9. Loc-Carrillo C, Abedon ST. Pros and cons of phage therapy. *Bacteriophage.* (2011) 1:111–4. doi: 10.4161/bact.1.2.14590

10. Principi N, Silvestri E, Esposito S. Advantages and limitations of bacteriophages for the treatment of bacterial infections. *Front Pharmacol.* (2019) 10:513. doi: 10.3389/fphar.2019.00513

11. Chibani-Chennoufi S, Sidoti J, Bruttin A, Kutter E, Sarker S, Brussow H. *In vitro* and *in vivo* bacteriolytic activities of *Escherichia coli* phages: implications for phage therapy. *Antimicrob Agents Chemother.* (2004) 48:2558–69. doi: 10.1128/AAC.48.7.2558-2569.2004

12. Venturini C, Petrovic Fabijan A, Fajardo Lubian A, Barbirz S, Iredell J. Biological foundations of successful bacteriophage therapy. *EMBO Mol Med.* (2022) 14:e12435. doi: 10.15252/emmm.202012435

13. Pirnay JP, Ferry T, Resch G. Recent progress toward the implementation of phage therapy in Western medicine. *FEMS Microbiol Rev.* (2022) 46:fuab040. doi: 10.1093/femsre/fuab040

14. Pires DP, Costa AR, Pinto G, Meneses L, Azeredo J. Current challenges and future opportunities of phage therapy. *FEMS Microbiol Rev.* (2020) 44:684–700. doi: 10.1093/femsre/fuaa017

15. Uyttebroek S, Chen B, Onsea J, Ruythooren F, Debaveye Y, Devolder D, et al. Safety and efficacy of phage therapy in difficult-to-treat infections: a systematic review. *Lancet Infect Dis.* (2022) 22:e208–20. doi: 10.1016/S1473-3099(21)00612-5

16. Chang RYK, Wallin M, Lin Y, Leung SSY, Wang H, Morales S, et al. Phage therapy for respiratory infections. *Adv Drug Deliv Rev.* (2018) 133:76–86. doi: 10.1016/j.addr.2018.08.001

17. Ng RN, Tai AS, Chang BJ, Stick SM, Kicic A. Overcoming challenges to make bacteriophage therapy standard clinical treatment practice for cystic fibrosis. *Front Microbiol.* (2020) 11:593988. doi: 10.3389/fmicb.2020.593988

18. Borysowski J, Miedzybrodzki R, Wierzbicki P, Klosowska D, Korczak-Kowalska G, Weber-Dabrowska B, et al. A3R phage and *Staphylococcus aureus* lysate do not induce neutrophil degranulation. *Viruses.* (2017) 9:36. doi: 10.3390/v9020036

19. Borysowski J, Wierzbicki P, Klosowska D, Korczak-Kowalska G, Weber-Dabrowska B, Gorski A. The effects of T4 and A3/R phage preparations on whole-blood monocyte and neutrophil respiratory burst. *Viral Immunol.* (2010) 23:541–4. doi: 10.1089/vim.2010.0001

20. Cafora M, Brix A, Forti F, Loberto N, Aureli M, Briani F, et al. Phages as immunomodulators and their promising use as anti-inflammatory agents in a cftr loss-of-function zebrafish model. *J Cyst Fibros.* (2021) 20:1046–52. doi: 10.1016/j.jcf.2020.11.017

21. Roach DR, Leung CY, Henry M, Morello E, Singh D, Di Santo JP, et al. Synergy between the host immune system and bacteriophage is essential for successful phage therapy against an acute respiratory pathogen. *Cell Host Microbe.* (2017) 22:38–47.e4. doi: 10.1016/j.chom.2017.06.0

22. Garcia-Clemente M, de la Rosa D, Maiz L, Giron R, Blanco M, Olveira C, et al. Impact of *Pseudomonas aeruginosa* infection on patients with chronic inflammatory airway diseases. *J Clin Med.* (2020) 9:3800. doi: 10.3390/jcm9123800

23. Pillarisetti N, Williamson E, Linnane B, Skoric B, Robertson CF, Robinson P, et al. Infection, inflammation, and lung function decline in infants with cystic fibrosis. *Am J Respir Crit Care Med.* (2011) 184:75–81. doi: 10.1164/rccm.201011-1892OC

24. Ramsey KA, Ranganathan S, Park J, Skoric B, Adams AM, Simpson SJ, et al. Early respiratory infection is associated with reduced spirometry in children with cystic fibrosis. *Am J Respir Crit Care Med.* (2014) 190:1111–6. doi: 10.1164/rccm.201407-1277OC

25. John A, Gozdzik-Spychalska J, Durda-Masny M, Czainski W, Pawlowska N, Wlizlo J, et al. *Pseudomonas aeruginosa*, the type of mutation, lung function, and nutritional status in adults with cystic fibrosis. *Nutrition.* (2021) 89:111221. doi: 10.1016/j.nut.2021.111221

26. Kerem E, Viviani L, Zolin A, MacNeill S, Hatziagorou E, Ellemunter H, et al. Factors associated with FEV1 decline in cystic fibrosis: analysis of the ECFS patient registry. *Eur Respir J.* (2014) 43:125–33. doi: 10.1183/09031936.00166412

27. Gangell C, Gard S, Douglas T, Park J, de Klerk N, Keil T, et al. Inflammatory responses to individual microorganisms in the lungs of children with cystic fibrosis. *Clin Infect Dis.* (2011) 53:425–32. doi: 10.1093/cid/cir399

28. Garratt LW, Breuer O, Schofield CJ, McLean SA, Laucirica DR, Tirouvanziam R, et al. Changes in airway inflammation with *Pseudomonas* eradication in early cystic fibrosis. *J Cyst Fibros.* (2021) 20:941–8. doi: 10.1016/j.jcf.2020.12.015

29. Sly PD, Gangell CL, Chen L, Ware RS, Ranganathan S, Mott LS, et al. Risk factors for bronchiectasis in children with cystic fibrosis. *N Engl J Med.* (2013) 368:1963–70. doi: 10.1056/NEJMoa1301725

30. Stick SM, Brennan S, Murray C, Douglas T, von Ungern-Sternberg BS, Garratt LW, et al. Bronchiectasis in infants and preschool children diagnosed with cystic fibrosis after newborn screening. *J Pediatr.* (2009) 155:623–8.e1. doi: 10.1016/j.jpeds.2009.05.005

31. Durda-Masny M, Gozdzik-Spychalska J, John A, Czainski W, Strozewska W, Pawlowska N, et al. The determinants of survival among adults with cystic fibrosis-a cohort study. *J Physiol Anthropol.* (2021) 40:19. doi: 10.1186/s40101-021-00269-7

32. McColley SA, Schechter MS, Morgan WJ, Pasta DJ, Craib ML, Konstan MW. Risk factors for mortality before age 18 years in cystic fibrosis. *Pediatr Pulmonol.* (2017) 52:909–15. doi: 10.1002/ppul.23715

33. Douglas TA, Brennan S, Gard S, Berry L, Gangell C, Stick SM, et al. Acquisition and eradication of *P. aeruginosa* in young children with cystic fibrosis. *Eur Respir J.* (2009) 33:305–11. doi: 10.1183/09031936.00043108

34. Annual Data Report 2021. *Cystic Fibrosis Foundation Patient Registry 2021 Annual Data Report*. Maryland: Bethesda (2022).

35. Pittman JE, Calloway EH, Kiser M, Yeatts J, Davis SD, Drumm ML, et al. Age of *Pseudomonas aeruginosa* acquisition and subsequent severity of cystic fibrosis lung disease. *Pediatr Pulmonol.* (2011) 46:497–504. doi: 10.1002/ppul.21397

36. Jacobs DM, Ochs-Balcom HM, Noyes K, Zhao J, Leung WY, Pu CY, et al. Impact of *Pseudomonas aeruginosa* isolation on mortality and outcomes in an outpatient chronic obstructive pulmonary disease cohort. *Open Forum Infect Dis.* (2020) 7:ofz546. doi: 10.1093/ofid/ofz546

37. Domenech A, Puig C, Marti S, Santos S, Fernandez A, Calatayud L, et al. Infectious etiology of acute exacerbations in severe COPD patients. *J Infect.* (2013) 67:516–23. doi: 10.1016/j.jinf.2013.09.003

38. Rodrigo-Troyano A, Melo V, Marcos PJ, Laserna E, Peiro M, Suarez-Cuartin G, et al. *Pseudomonas aeruginosa* in chronic obstructive pulmonary disease patients with frequent hospitalized exacerbations: a prospective multicentre study. *Respiration.* (2018) 96:417–24. doi: 10.1159/000490190

39. Gallego M, Pomares X, Espasa M, Castaner E, Sole M, Suarez D, et al. *Pseudomonas aeruginosa* isolates in severe chronic obstructive pulmonary disease: characterization and risk factors. *BMC Pulm Med.* (2014) 14:103. doi: 10.1186/1471-2466-14-103

40. Martinez-Garcia MA, Oscullo G, Posadas T, Zaldivar E, Villa C, Dobarganes Y, et al. *Pseudomonas aeruginosa* and lung function decline in patients with bronchiectasis. *Clin Microbiol Infect.* (2021) 27:428–34. doi: 10.1016/j.cmi.2020.04.007

41. Millares L, Ferrari R, Gallego M, Garcia-Nunez M, Perez-Brocal V, Espasa M, et al. Bronchial microbiome of severe COPD patients colonised by *Pseudomonas aeruginosa*. *Eur J Clin Microbiol Infect Dis.* (2014) 33:1101–11. doi: 10.1007/s10096-013-2044-0

42. McDonnell MJ, Jary HR, Perry A, MacFarlane JG, Hester KL, Small T, et al. Non cystic fibrosis bronchiectasis: a longitudinal retrospective observational cohort study of *Pseudomonas* persistence and resistance. *Respir Med.* (2015) 109:716–26. doi: 10.1016/j.rmed.2014.07.021

43. Chalmers JD, Goeminne P, Aliberti S, McDonnell MJ, Lonni S, Davidson J, et al. The bronchiectasis severity index. An international derivation and validation study. *Am J Respir Crit Care Med.* (2014) 189:576–85. doi: 10.1164/rccm.201309-1575OC

44. Araujo D, Shteinberg M, Aliberti S, Goeminne PC, Hill AT, Fardon TC, et al. The independent contribution of *Pseudomonas aeruginosa* infection to long-term clinical outcomes in bronchiectasis. *Eur Respir J.* (2018) 51:1701953. doi: 10.1183/13993003.01953-2017

45. Finch S, McDonnell MJ, Abo-Leyah H, Aliberti S, Chalmers JD. A comprehensive analysis of the impact of *Pseudomonas aeruginosa* colonization on prognosis in adult bronchiectasis. *Ann Am Thorac Soc.* (2015) 12:1602–11. doi: 10.1513/AnnalsATS.201506-333OC

46. Eklof J, Sorensen R, Ingebrigtsen TS, Sivapalan P, Achir I, Boel JB, et al. *Pseudomonas aeruginosa* and risk of death and exacerbations in patients with chronic obstructive pulmonary disease: an observational cohort study of 22 053 patients. *Clin Microbiol Infect.* (2020) 26:227–34. doi: 10.1016/j.cmi.2019.06.011

47. Wang H, Ji XB, Mao B, Li CW, Lu HW, Xu JF. *Pseudomonas aeruginosa* isolation in patients with non-cystic fibrosis bronchiectasis: a retrospective study. *BMJ Open.* (2018) 8:e014613. doi: 10.1136/bmjopen-2016-014613

48. Kwok WC, Ho JCM, Tam TCC, Ip MSM, Lam DCL. Risk factors for *Pseudomonas aeruginosa* colonization in non-cystic fibrosis bronchiectasis and clinical implications. *Respir Res.* (2021) 22:132. doi: 10.1186/s12931-021-01729-5

49. Goeminne PC, Nawrot TS, Ruttens D, Seys S, Dupont LJ. Mortality in non-cystic fibrosis bronchiectasis: a prospective cohort analysis. *Respir Med.* (2014) 108:287–96. doi: 10.1016/j.rmed.2013.12.015

50. Rodrigo-Troyano A, Suarez-Cuartin G, Peiro M, Barril S, Castillo D, Sanchez-Reus F, et al. *Pseudomonas aeruginosa* resistance patterns and clinical outcomes in hospitalized exacerbations of COPD. *Respirology.* (2016) 21:1235–42. doi: 10.1111/resp.12825

51. Busse WW, Lemanske RF Jr., Gern JE. Role of viral respiratory infections in asthma and asthma exacerbations. *Lancet.* (2010) 376:826–34. doi: 10.1016/S0140-6736(10)61380-3

52. Watkinson RL, Looi K, Laing IA, Cianferoni A, Kicic A. Viral induced effects on a vulnerable epithelium; lessons learned from paediatric asthma and eosinophilic oesophagitis. *Front Immunol.* (2021) 12:773600.

53. Zhang Q, Illing R, Hui CK, Downey K, Carr D, Stearn M, et al. Bacteria in sputum of stable severe asthma and increased airway wall thickness. *Respir Res.* (2012) 13:35. doi: 10.1186/1465-9921-13-35

54. Padilla-Galo A, Olveira C, Fernandez de Rota-Garcia L, Marco-Galve I, Plata AJ, Alvarez A, et al. Factors associated with bronchiectasis in patients with uncontrolled asthma; the NOPES score: a study in 398 patients. *Respir Res.* (2018) 19:43. doi: 10.1186/s12931-018-0746-7

55. Dimakou K, Gousiou A, Toumbis M, Kaponi M, Chrysikos S, Thanos L, et al. Investigation of bronchiectasis in severe uncontrolled asthma. *Clin Respir J.* (2018) 12:1212–8. doi: 10.1111/crj.12653

56. GARDP Foundation. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *Lancet.* (2022) 399:629–55. doi: 10.1016/S0140-6736(21)02724-0

57. Fainardi V, Neglia C, Muscara M, Spaggiari C, Tornesello M, Grandinetti R, et al. Multidrug-resistant bacteria in children and adolescents with cystic fibrosis. *Children.* (2022) 9:1330. doi: 10.3390/children9091330

58. Hahn A, Burrell A, Fanous H, Chaney H, Sami I, Perez GF, et al. Antibiotic multidrug resistance in the cystic fibrosis airway microbiome is associated with decreased diversity. *Heliyon.* (2018) 4:e00795. doi: 10.1016/j.heliyon.2018.e00795

59. Gao YH, Guan WJ, Zhu YN, Chen RC, Zhang GJ. Antibiotic-resistant *Pseudomonas aeruginosa* infection in patients with bronchiectasis: prevalence, risk factors and prognostic implications. *Int J Chron Obstruct Pulmon Dis.* (2018) 13:237–46. doi: 10.2147/COPD.S150250

60. Menendez R, Mendez R, Polverino E, Rosales-Mayor E, Amara-Elori I, Reyes S, et al. Risk factors for multidrug-resistant pathogens in bronchiectasis exacerbations. *BMC Infect Dis.* (2017) 17:659. doi: 10.1186/s12879-017-2754-5

61. Montero M, Dominguez M, Orozco-Levi M, Salvado M, Knobel H. Mortality of COPD patients infected with multi-resistant *Pseudomonas aeruginosa*: a case and control study. *Infection.* (2009) 37:16–9. doi: 10.1007/s15010-008-8125-9

62. GBD Chronic Respiratory Disease Collaborators. Prevalence and attributable health burden of chronic respiratory diseases, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet Respir Med.* (2020) 8:585–96. doi: 10.1016/S2213-2600(20)30105-3

63. Xie M, Liu X, Cao X, Guo M, Li X. Trends in prevalence and incidence of chronic respiratory diseases from 1990 to 2017. *Respir Res.* (2020) 21:49. doi: 10.1186/s12931-020-1291-8

64. Craig A, Mai J, Cai S, Jeyaseelan S. Neutrophil recruitment to the lungs during bacterial pneumonia. *Infect Immun.* (2009) 77:568–75. doi: 10.1128/IAI.00832-08

65. Garratt LW. Current understanding of the neutrophil transcriptome in health and disease. *Cells.* (2021) 10:2406. doi: 10.3390/cells10092406

66. Giacalone VD, Margaroli C, Mall MA, Tirouvanziam R. Neutrophil adaptations upon recruitment to the lung: new concepts and implications for homeostasis and disease. *Int J Mol Sci.* (2020) 21:851. doi: 10.3390/ijms21030851

67. Margaroli C, Tirouvanziam R. Neutrophil plasticity enables the development of pathological microenvironments: implications for cystic fibrosis airway disease. *Mol Cell Pediatr.* (2016) 3:38. doi: 10.1186/s40348-016-0066-2

68. Rosenow T, Mok LC, Turkovic L, Berry LJ, Sly PD, Ranganathan S, et al. The cumulative effect of inflammation and infection on structural lung disease in early cystic fibrosis. *Eur Respir J.* (2019) 54:1801771. doi: 10.1183/13993003.01771-2018

69. Dittrich AS, Kuhbandner I, Gehrig S, Rickert-Zacharias V, Twigg M, Wege S, et al. Elastase activity on sputum neutrophils correlates with severity of lung disease in cystic fibrosis. *Eur Respir J.* (2018) 51:1701910. doi: 10.1183/13993003.01910-2017

70. Oriano M, Gramegna A, Terranova L, Sotgiu G, Sulaiman I, Ruggiero L, et al. Sputum neutrophil elastase associates with microbiota and *Pseudomonas aeruginosa* in bronchiectasis. *Eur Respir J.* (2020) 56:2000769. doi: 10.1183/13993003.00769-2020

71. Forrest OA, Ingersoll SA, Preininger MK, Laval J, Limoli DH, Brown MR, et al. Frontline Science: pathological conditioning of human neutrophils recruited to the airway milieu in cystic fibrosis. *J Leukoc Biol.* (2018) 104:665–75. doi: 10.1002/JLB.5HI1117-454RR

72. Margaroli C, Garratt LW, Horati H, Dittrich AS, Rosenow T, Montgomery ST, et al. Elastase exocytosis by airway neutrophils is associated with early lung damage in children with cystic fibrosis. *Am J Respir Crit Care Med.* (2019) 199:873–81. doi: 10.1164/rccm.201803-0442OC

73. Tirouvanziam R, Gernez Y, Conrad CK, Moss RB, Schrijver I, Dunn CE, et al. Profound functional and signaling changes in viable inflammatory neutrophils homing to cystic fibrosis airways. *Proc Natl Acad Sci USA.* (2008) 105:4335–9. doi: 10.1073/pnas.0712386105

74. Laucirica DR, Schofield CJ, McLean SA, Margaroli C, Agudelo-Romero P, Stick SM, et al. *Pseudomonas aeruginosa* modulates neutrophil granule exocytosis in an *in vitro* model of airway infection. *Immunol Cell Biol.* (2022) 100:352–70. doi: 10.1111/imcb.12547

75. Thulborn SJ, Mistry V, Brightling CE, Moffitt KL, Ribeiro D, Bafadhel M. Neutrophil elastase as a biomarker for bacterial infection in COPD. *Respir Res.* (2019) 20:170. doi: 10.1186/s12931-019-1145-4

76. Chalmers JD, Moffitt KL, Suarez-Cuartin G, Sibila O, Finch S, Furrie E, et al. Neutrophil elastase activity is associated with exacerbations and lung function decline in bronchiectasis. *Am J Respir Crit Care Med.* (2017) 195:1384–93. doi: 10.1164/rccm.201605-1027OC

77. Ali HA, Fouda EM, Salem MA, Abdelwahad MA, Radwan HH. Sputum neutrophil elastase and its relation to pediatric bronchiectasis severity: a cross-sectional study. *Health Sci Rep.* (2022) 5:e581. doi: 10.1002/hsr2.581

78. Ray A, Kolls JK. Neutrophilic inflammation in asthma and association with disease severity. *Trends Immunol.* (2017) 38:942–54. doi: 10.1016/j.it.2017.07.003

79. Fahy JV, Kim KW, Liu J, Boushey HA. Prominent neutrophilic inflammation in sputum from subjects with asthma exacerbation. *J Allergy Clin Immunol.* (1995) 95:843–52. doi: 10.1016/s0091-6749(95)70128-1

80. Vignola AM, Bonanno A, Mirabella A, Riccobono L, Mirabella F, Profita M, et al. Increased levels of elastase and alpha1-antitrypsin in sputum of asthmatic patients. *Am J Respir Crit Care Med.* (1998) 157:505–11. doi: 10.1164/ajrccm.157.2.9703070

81. Wark PA, Johnston SL, Moric I, Simpson JL, Hensley MJ, Gibson PG. Neutrophil degranulation and cell lysis is associated with clinical severity in virus-induced asthma. *Eur Respir J.* (2002) 19:68–75. doi: 10.1183/09031936.02.00226302

82. Jatakanon A, Uasuf C, Maziak W, Lim S, Chung KF, Barnes PJ. Neutrophilic inflammation in severe persistent asthma. *Am J Respir Crit Care Med.* (1999) 160 (5 Pt 1):1532–9. doi: 10.1164/ajrccm.160.5.9806170

83. Koga H, Miyahara N, Fuchimoto Y, Ikeda G, Waseda K, Ono K, et al. Inhibition of neutrophil elastase attenuates airway hyperresponsiveness and inflammation in a mouse model of secondary allergen challenge: neutrophil elastase inhibition attenuates allergic airway responses. *Respir Res.* (2013) 14:8. doi: 10.1186/1465-9921-14-8

84. Weng Q, Zhu C, Zheng K, Wu Y, Dong L, Wu Y, et al. Early recruited neutrophils promote asthmatic inflammation exacerbation by release of neutrophil elastase. *Cell Immunol.* (2020) 352:104101. doi: 10.1016/j.cellimm.2020.104101

85. Fujimoto K, Kubo K, Shinozaki S, Okada K, Matsuzawa Y, Kobayashi T, et al. Neutrophil elastase inhibitor reduces asthmatic responses in allergic sheep. *Respir Physiol.* (1995) 100:91–100. doi: 10.1016/0034-5687(94)00123-h

86. Margaroli C, Moncada-Giraldo D, Gulick DA, Dobosh B, Giacalone VD, Forrest OA, et al. Transcriptional firing represses bactericidal activity in cystic fibrosis airway neutrophils. *Cell Rep Med.* (2021) 2:100239. doi: 10.1016/j.xcrm.2021.100239

87. Shanmugam L, Ravinder SS, Johnson P, Padmavathi R, Rajagopalan B, Kindo AJ. Assessment of phagocytic activity of neutrophils in chronic obstructive pulmonary disease. *Lung India.* (2015) 32:437–40. doi: 10.4103/0970-2113.164159

88. Thomas C, Barnes P, Donnelly L. Reduced phagocytosis of pathogenic bacteria by neutrophils from COPD patients. *Eur Respir J.* (2012) 40 (Suppl 56):387.

89. Lavinskiene S, Vaitkus M, Bieksiene K, Jeroch J, Sakalauskas R. Neutrophil phagocytic activity in AECOPD. *Eur Respir J.* (2011) 38 (Suppl 55):738.

90. Bedi P, Davidson DJ, McHugh BJ, Rossi AG, Hill AT. Blood neutrophils are reprogrammed in bronchiectasis. *Am J Respir Crit Care Med.* (2018) 198:880–90. doi: 10.1164/rccm.201712-2423OC

91. Chalmers JD, Ma A, Turnbull K, Doherty C, Govan JR, Hill A. Impaired neutrophil phagocytosis and receptor expression in non-CF bronchiectasis. *Eur Respir J.* (2013) 42 (Suppl 57):2065. doi: 10.3390/biom11081065

92. da Silva-Martins CL, Couto SC, Muniz-Junqueira MI. Inhaled corticosteroid treatment for 6 months was not sufficient to normalize phagocytosis in asthmatic children. *Clin Transl Allergy.* (2013) 3:28. doi: 10.1186/2045-7022-3-28

93. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. *Science.* (2004) 303:1532–5. doi: 10.1126/science.1092385

94. Fuchs TA, Abed U, Goosmann C, Hurwitz R, Schulze I, Wahn V, et al. Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol.* (2007) 176:231–41. doi: 10.1083/jcb.200606027

95. Yousefi S, Mihalache C, Kozlowski E, Schmid I, Simon HU. Viable neutrophils release mitochondrial DNA to form neutrophil extracellular traps. *Cell Death Differ.* (2009) 16:1438–44. doi: 10.1038/cdd.2009.96

96. Pilsczek FH, Salina D, Poon KK, Fahey C, Yipp BG, Sibley CD, et al. A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to *Staphylococcus aureus*. *J Immunol.* (2010) 185:7413–25. doi: 10.4049/jimmunol.1000675

97. Yipp BG, Petri B, Salina D, Jenne CN, Scott BN, Zbytnuik LD, et al. Infection-induced NETosis is a dynamic process involving neutrophil multitasking in vivo. *Nat Med.* (2012) 18:1386–93. doi: 10.1038/nm.2847

98. Kaplan MJ, Radic M. Neutrophil extracellular traps: double-edged swords of innate immunity. *J Immunol.* (2012) 189:2689–95. doi: 10.4049/jimmunol.1201719

99. Delgado-Rizo V, Martinez-Guzman MA, Iniguez-Gutierrez L, Garcia-Orozco A, Alvarado-Navarro A, Fafutis-Morris M. Neutrophil extracellular traps and its implications in inflammation: an overview. *Front Immunol.* (2017) 8:81. doi: 10.3389/fimmu.2017.00081

100. Manzenreiter R, Kienberger F, Marcos V, Schilcher K, Krautgartner WD, Obermayer A, et al. Ultrastructural characterization of cystic fibrosis sputum using atomic force and scanning electron microscopy. *J Cyst Fibros.* (2012) 11:84–92. doi: 10.1016/j.jcf.2011.09.008

101. Grabcanovic-Musija F, Obermayer A, Stoiber W, Krautgartner WD, Steinbacher P, Winterberg N, et al. Neutrophil extracellular trap (NET) formation characterises stable and exacerbated COPD and correlates with airflow limitation. *Respir Res.* (2015) 16:59. doi: 10.1186/s12931-015-0221-7

102. Keir HR, Shoemark A, Dicker AJ, Perea L, Pollock J, Giam YH, et al. Neutrophil extracellular traps, disease severity, and antibiotic response in bronchiectasis: an international, observational, multicohort study. *Lancet Respir Med.* (2021) 9:873–84. doi: 10.1016/S2213-2600(20)30504-X

103. Linssen RS, Chai G, Ma J, Kummarapurugu AB, van Woensel JBM, Bem RA, et al. Neutrophil extracellular traps increase airway mucus viscoelasticity and slow mucus particle transit. *Am J Respir Cell Mol Biol.* (2021) 64:69–78. doi: 10.1165/rcmb.2020-0168OC

104. Marcos V, Zhou-Suckow Z, Onder Yildirim A, Bohla A, Hector A, Vitkov L, et al. Free DNA in cystic fibrosis airway fluids correlates with airflow obstruction. *Mediators Inflamm.* (2015) 2015:408935. doi: 10.1155/2015/408935

105. Gray RD, Hardisty G, Regan KH, Smith M, Robb CT, Duffin R, et al. Delayed neutrophil apoptosis enhances NET formation in cystic fibrosis. *Thorax.* (2018) 73:134–44. doi: 10.1136/thoraxjnl-2017-210134

106. Dicker AJ, Crichton ML, Pumphrey EG, Cassidy AJ, Suarez-Cuartin G, Sibila O, et al. Neutrophil extracellular traps are associated with disease severity and microbiota diversity in patients with chronic obstructive pulmonary disease. *J Allergy Clin Immunol.* (2018) 141:117–27. doi: 10.1016/j.jaci.2017.04.022

107. Lachowicz-Scroggins ME, Dunican EM, Charbit AR, Raymond W, Looney MR, Peters MC, et al. Extracellular DNA, neutrophil extracellular traps, and inflammasome activation in severe asthma. *Am J Respir Crit Care Med.* (2019) 199:1076–85. doi: 10.1164/rccm.201810-1869OC

108. Konstan MW, Doring G, Heltshe SL, Lands LC, Hilliard KA, Koker P, et al. A randomized double blind, placebo controlled phase 2 trial of BIIL 284 BS (an LTB4 receptor antagonist) for the treatment of lung disease in children and adults with cystic fibrosis. *J Cyst Fibros.* (2014) 13:148–55. doi: 10.1016/j.jcf.2013.12.009

109. Doring G, Bragonzi A, Paroni M, Akturk FF, Cigana C, Schmidt A, et al. BIIL 284 reduces neutrophil numbers but increases *P. aeruginosa* bacteremia and inflammation in mouse lungs. *J Cyst Fibros.* (2014) 13:156–63. doi: 10.1016/j.jcf.2013.10.007

110. Hoyle N, Zhvaniya P, Balarjishvili N, Bolkvadze D, Nadareishvili L, Nizharadze D, et al. Phage therapy against *Achromobacter xylosoxidans* lung infection in a patient with cystic fibrosis: a case report. *Res Microbiol.* (2018) 169:540–2. doi: 10.1016/j.resmic.2018.05.001

111. Lebeaux D, Merabishvili M, Caudron E, Lannoy D, Van Simaey L, Duyvejonck H, et al. A case of phage therapy against pandrug-resistant *Achromobacter xylosoxidans* in a 12-year-old lung-transplanted cystic fibrosis patient. *Viruses.* (2021) 13:60. doi: 10.3390/v13010060

112. Maddocks S, Fabijan AP, Ho J, Lin RCY, Ben Zakour NL, Dugan C, et al. Bacteriophage therapy of ventilator-associated pneumonia and empyema caused by *Pseudomonas aeruginosa*. *Am J Respir Crit Care Med.* (2019) 200:1179–81. doi: 10.1164/rccm.201904-0839LE

113. Dedrick RM, Smith BE, Cristinziano M, Freeman KG, Jacobs-Sera D, Belessis Y, et al. Phage therapy of *Mycobacterium* infections: compassionate-use of phages in twenty patients with drug-resistant Mycobacterial disease. *Clin Infect Dis.* (2022) [Online ahead of print]. doi: 10.1093/cid/ciac453.

114. Nick JA, Dedrick RM, Gray AL, Vladar EK, Smith BE, Freeman KG, et al. Host and pathogen response to bacteriophage engineered against *Mycobacterium abscessus* lung infection. *Cell.* (2022) 185:1860–74.e12. doi: 10.1016/j.cell.2022.04.024

115. Petrovic Fabijan A, Lin RCY, Ho J, Maddocks S, Ben Zakour NL, Iredell JR, et al. Safety of bacteriophage therapy in severe *Staphylococcus aureus* infection. *Nat Microbiol.* (2020) 5:465–72. doi: 10.1038/s41564-019-0634-z

116. Pabary R, Singh C, Morales S, Bush A, Alshafi K, Bilton D, et al. Antipseudomonal bacteriophage reduces infective burden and inflammatory response in murine lung. *Antimicrob Agents Chemother.* (2016) 60:744–51. doi: 10.1128/AAC.01426-15

117. Debarbieux L, Leduc D, Maura D, Morello E, Criscuolo A, Grossi O, et al. Bacteriophages can treat and prevent *Pseudomonas aeruginosa* lung infections. *J Infect Dis.* (2010) 201:1096–104. doi: 10.1086/651135

118. Dufour N, Delattre R, Chevallereau A, Ricard JD, Debarbieux L. Phage therapy of pneumonia is not associated with an overstimulation of the inflammatory response compared to antibiotic treatment in mice. *Antimicrob Agents Chemother.* (2019) 63:e00379-19. doi: 10.1128/AAC.00379-19.

119. Cafora M, Deflorian G, Forti F, Ferrari L, Binelli G, Briani F, et al. Phage therapy against *Pseudomonas aeruginosa* infections in a cystic fibrosis zebrafish model. *Sci Rep.* (2019) 9:1527. doi: 10.1038/s41598-018-37636-x

120. Metzemaekers M, Gouwy M, Proost P. Neutrophil chemoattractant receptors in health and disease: double-edged swords. *Cell Mol Immunol.* (2020) 17:433–50. doi: 10.1038/s41423-020-0412-0

121. Miralda I, Uriarte SM, McLeish KR. Multiple phenotypic changes define neutrophil priming. *Front Cell Infect Microbiol.* (2017) 7:217. doi: 10.3389/fcimb.2017.00217

122. Yamamoto K, Ahyi AN, Pepper-Cunningham ZA, Ferrari JD, Wilson AA, Jones MR, et al. Roles of lung epithelium in neutrophil recruitment during pneumococcal pneumonia. *Am J Respir Cell Mol Biol.* (2014) 50:253–62. doi: 10.1165/rcmb.2013-0114OC

123. Cabrini G, Rimessi A, Borgatti M, Lampronti I, Finotti A, Pinton P, et al. Role of cystic fibrosis bronchial epithelium in neutrophil chemotaxis. *Front Immunol.* (2020) 11:1438. doi: 10.3389/fimmu.2020.01438

124. Trend S, Chang BJ, O'Dea M, Stick SM, Kicic A, Waerp, et al. Use of a primary epithelial cell screening tool to investigate phage therapy in cystic fibrosis. *Front Pharmacol.* (2018) 9:1330. doi: 10.3389/fphar.2018.01330

125. Van Belleghem JD, Clement F, Merabishvili M, Lavigne R, Vaneechoutte M. Pro- and anti-inflammatory responses of peripheral blood mononuclear cells induced by *Staphylococcus aureus* and *Pseudomonas aeruginosa* phages. *Sci Rep.* (2017) 7:8004. doi: 10.1038/s41598-017-08336-9

126. Przerwa A, Zimecki M, Switala-Jelen K, Dabrowska K, Krawczyk E, Luczak M, et al. Effects of bacteriophages on free radical production and phagocytic functions. *Med Microbiol Immunol.* (2006) 195:143–50. doi: 10.1007/s00430-006-0011-4

127. Miernikiewicz P, Klopot A, Soluch R, Szkuta P, Keska W, Hodyra-Stefaniak K, et al. T4 phage tail adhesin Gp12 counteracts LPS-induced inflammation *in vivo*. *Front Microbiol.* (2016) 7:1112. doi: 10.3389/fmicb.2016.01112

128. Nelson AR. The effect of bacteriophage upon the phenomena of leukocytosis and phagocytosis. *J Immunol.* (1928) 15:43–64.

129. Smith GH. Bacteriophage and phagocytosis, i. effect on resistant and dead bacteria. *J Immunol.* (1928) 15:125–40. doi: 10.3390/pharmaceutics14091916

130. d'Herelle F, Smith GH. *The Bacteriophage, its Role in Immunity*. Philadelphia, PA: Williams & Wilkins (1922).

131. Tiwari BR, Kim S, Rahman M, Kim J. Antibacterial efficacy of lytic *Pseudomonas* bacteriophage in normal and neutropenic mice models. *J Microbiol.* (2011) 49:994–9. doi: 10.1007/s12275-011-1512-4

132. Singla S, Harjai K, Katare OP, Chhibber S. Encapsulation of bacteriophage in liposome accentuates its entry in to macrophage and shields it from neutralizing antibodies. *PLoS One.* (2016) 11:e0153777. doi: 10.1371/journal.pone.0153777

133. Broxmeyer L, Sosnowska D, Miltner E, Chacon O, Wagner D, McGarvey J, et al. Killing of *Mycobacterium avium* and *Mycobacterium tuberculosis* by a mycobacteriophage delivered by a nonvirulent mycobacterium: a model for phage therapy of intracellular bacterial pathogens. *J Infect Dis.* (2002) 186:1155–60. doi: 10.1086/343812

134. Kaur S, Harjai K, Chhibber S. Bacteriophage-aided intracellular killing of engulfed methicillin-resistant *Staphylococcus aureus* (MRSA) by murine macrophages. *Appl Microbiol Biotechnol.* (2014) 98:4653–61. doi: 10.1007/s00253-014-5643-5

135. Capparelli R, Parlato M, Borriello G, Salvatore P, Iannelli D. Experimental phage therapy against *Staphylococcus aureus* in mice. *Antimicrob Agents Chemother.* (2007) 51:2765–73. doi: 10.1128/AAC.01513-06

136. Jonczyk-Matysiak E, Lusiak-Szelachowska M, Klak M, Bubak B, Miedzybrodzki R, Weber-Dabrowska B, et al. The effect of bacteriophage preparations on intracellular killing of bacteria by phagocytes. *J Immunol Res.* (2015) 2015:482863. doi: 10.1155/2015/482863

137. Kurzepa-Skaradzinska A, Lusiak-Szelachowska M, Skaradzinski G, Jonczyk-Matysiak E, Weber-Dabrowska B, Zaczek M, et al. Influence of bacteriophage preparations on intracellular killing of bacteria by human phagocytes *in vitro*. *Viral Immunol.* (2013) 26:150–62. doi: 10.1089/vim.2012.0071

138. Hasan U, Chaffois C, Gaillard C, Saulnier V, Merck E, Tancredi S, et al. Human TLR10 is a functional receptor, expressed by B cells and plasmacytoid dendritic cells, which activates gene transcription through MyD88. *J Immunol.* (2005) 174:2942–50. doi: 10.4049/jimmunol.174.5.2942

139. Kawasaki T, Kawai T. Toll-like receptor signaling pathways. *Front Immunol.* (2014) 5:461. doi: 10.3389/fimmu.2014.00461

140. Zhao Y, van Kessel KPM, de Haas CJC, Rogers MRC, van Strijp JAG, Haas PA. Staphylococcal superantigen-like protein 13 activates neutrophils via formyl peptide receptor 2. *Cell Microbiol.* (2018) 20:e12941. doi: 10.1111/cmi.12941

141. Laucirica DR, Garratt LW, Kicic A. Progress in model systems of cystic fibrosis mucosal inflammation to understand aberrant neutrophil activity. *Front Immunol.* (2020) 11:595. doi: 10.3389/fimmu.2020.00595

142. Tanner L, Single AB. Animal models reflecting chronic obstructive pulmonary disease and related respiratory disorders: translating pre-clinical data into clinical relevance. *J Innate Immun.* (2020) 12:203–25. doi: 10.1159/000502489

143. Shao L, Fischer DD, Kandasamy S, Saif LJ, Vlasova AN. Tissue-specific mRNA expression profiles of porcine Toll-like receptors at different ages in germ-free and conventional pigs. *Vet Immunol Immunopathol.* (2016) 171:7–16. doi: 10.1016/j.vetimm.2016.01.008

144. Martinovich KM, Iosifidis T, Buckley AG, Looi K, Ling KM, Sutanto EN, et al. Conditionally reprogrammed primary airway epithelial cells maintain morphology, lineage and disease specific functional characteristics. *Sci Rep.* (2017) 7:17971. doi: 10.1038/s41598-017-17952-4

145. Baldassi D, Gabold B, Merkel O. Air-liquid interface cultures of the healthy and diseased human respiratory tract: promises, challenges and future directions. *Adv Nanobiomed Res.* (2021) 1:2000111. doi: 10.1002/anbr.202000111

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# CBD resistant *Salmonella* strains are susceptible to epsilon 34 phage tailspike protein

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The rise of antimicrobial resistance is a global public health crisis that threatens the effective control and prevention of infections. Due to the emergence of pandrug-resistant bacteria, most antibiotics have lost their efficacy. Bacteriophages or their components are known to target bacterial cell walls, cell membranes, and lipopolysaccharides (LPS) and hydrolyze them. Bacteriophages being the natural predators of pathogenic bacteria, are inevitably categorized as "human friends", thus fulfilling the adage that "the enemy of my enemy is my friend". Leveraging on their lethal capabilities against pathogenic bacteria, researchers are searching for more ways to overcome the current antibiotic resistance challenge. In this study, we expressed and purified epsilon 34 phage tailspike protein (E34 TSP) from the E34 TSP gene, then assessed the ability of this bacteriophage protein in the killing of two CBD-resistant strains of *Salmonella* spp. We also assessed the ability of the tailspike protein to cause bacteria membrane disruption, and dehydrogenase depletion. We observed that the combined treatment of CBD-resistant strains of *Salmonella* with CBD and E34 TSP showed poor killing ability whereas the monotreatment with E34 TSP showed considerably higher killing efficiency. This study demonstrates that the inhibition of the bacteria by E34 TSP was due in part to membrane disruption, and dehydrogenase inactivation by the protein. The results of this work provides an interesting background to highlight the crucial role phage protein such as E34 TSP could play in pathogenic bacterial control.

KEYWORDS

**cannabidiol, resistance, *Salmonella*, antibacterial agent, phage**

## 1. Introduction

Cannabidiol (CBD), a constituent of *the Cannabis sativa* (hemp) plant is a non-psychoactive compound, which is a metabolite [2-(1R,6R)-6-isopropenyl-3-methylcyclohex-2-en-1-yl]-5-pentylbenzene-1,3-diol, ($C_{21}H_{30}O_2$) with a molecular weight of 314.4636. It that plays essential roles in health and physiology. CBD has worldwide applications in medicine and possesses tremendous potential for pharmaceutical relevance, such as anti-microbial, antioxidants, anti-inflammatory, anti-cancer, and anti-convulsant potentials among many others. Several studies have reported the particularly enormous roles CBD plays in antimicrobial (anti-parasitic, antiviral, and antibacterial) infections (1–3). Fernandes et al. described in their studies how

CBD was used to inhibit SARS-COV 2 infection in cells and in mouse models by blocking viral gene expression after gaining access to host cells. Their study relied on CBD and its metabolites 7-OH-CBD but did not include Tetrahydrocannabinol (THC). In other works, the anti-viral properties of CBD has been reported, particularly on SARS-COV2 (4–7). (8) examined the antibacterial properties of CBD extract against two strains of *Salmonella* (Typhimurium and Newington) and demonstrated that CBD was able to disrupt the membrane of the *Salmonella* spp. used in the study. Synergistic studies of polymyxin B and CBD co-therapy demonstrated strong antibacterial activities against *Acinetobacter baumannii* ATCC 19606 and other gram-negative bacteria (*Klebsiella pneumoniae* and *Pseudomonas aeruginosa*) (9). Some non-pathogenic bacteria such as gut microbiota is an integral aspect of human health, and while food and antibiotic consumption does affects the gut microbiota and sometimes provides an easy pathway for infections arising from foodborne pathogens (10), CBD has been demonstrated to boost enzyme activities of these gut bacteria (11, 12).

A whopping 115 million human infections and 370,000 deaths *per annum* are attributed to *Salmonella* infections globally. *Salmonella* is an important foodborne pathogen classified into 2,659 strains based on their surface antigens. *Salmonella* Typhimurium is known to be a pathogen of public health concern (13). *S.* Typhimurium is estimated to have resulted in 16–33 million infection cases and about 500,000–600,000 deaths globally *per annum* (14). Although, non-typhoidal *Salmonella* is a self-limiting disease, which is usually treated without the need for antibiotics, the need for antibiotics are required in immunocompromised individuals. Thus, though antibiotics play an enormous role in mitigating *Salmonella* infections, their efficacy is becoming futile due to the ability of bacteria to develop stringent resistance to antibiotics. This ontogeny has become a canker to health, and treatment of *Salmonella* infections. This foreclosure of *Salmonella* ontogeny means that novel antibiotic therapies must be fabricated to meet the urgent global health demand (15, 16). The economic and sanitary burden caused by antimicrobial resistance (AMR) is immense. An in-depth analysis conducted by a group of researchers revealed that the number of deaths caused by bacterial infections could be as high as 4.95 million annually (17–19). The researchers used a statistical model to analyze 471 million records from 16 different countries. They found that the mortality rate from bacterial infections could be as high as 1.27 million annually (20, 21). One of the most common causes of deaths associated with antimicrobial resistance is intra-abdominal infection. This contributes to the economic impact of the issue, as it costs the United States around USD 20 billion annually.

The natural enemies of bacteria are the bacteriophages. They can attack their target bacteria and are generally host-specific. This specificity attribute of phages allows for the maintenance of the human host's microbiota if used as therapy (22). However, phages are self-limiting, easily cleared from the body, elicits strong immune response, thus making it problematic as a therapeutic agent. Additionally, these phages have narrow host range, thus compounding their use as antibacterial agents against a broad spectrum of bacterial infections. In phage therapy, it is crucial that the preparation of the stocks is free of bacterial toxins such as the bacteria LPS and even the host bacteria as a whole since these by themselves are agents of disease. The elimination of bacterial toxins and bacteria during the preparation steps thus presents a technical challenge and increases the production cost (23–26). To overcome these huddles, a much cheaper and safer approach could be the utilization of the phage's hydrolases which the phage uses as its host attacking machinery.

The E34 phage belongs to the P22-like phages which have a uniquely short tailspike architecture, thus the name podovirus. Their tailspike protein is characterized by the presence of a globular head binding domain, a parallel beta-helix domain, and a beta-prism domain (27). Although the structures that are involved in viral adhesion and infection vary, a broad generalization of their properties can be made due to the presence of the β-helix domain. This unique domain is found in most of the viral and fungal structures that are commonly used in the development of human infectious diseases. Some of these include those of *Chlamydia, Helicobacteria, Borrelia*, and *Rickettsia* (28, 29). The parallel β-helix domains of other bacterial and fungal proteins are also known to be found in various surface and bacterial proteins (30–32).

Usually, parallel β-helix folds are not found in higher eukaryotes. However, these structures are known to exhibit high melting point (Tm), stability, and resistance to detergents at room temperature. Their role in the development of exterior virion structures that can endure harsh environmental conditions is also evidenced by their properties (30).

Most β-sheet proteins have been studied. The first known example of this is the pectate lyases from the *Erwinia* species, which are known to have a unique structure that allows them to infect plant cells (30, 31). These proteins are characterized by a long-arm elongated or solenoid-shaped structure. Most of the P22-like protein families, such as SF6, E15, and P22, have a unique structure that allows them to recognize and attach to host receptors. This structure also allows them to develop an LPS cleaving mechanism.

This study employs the tailspike proteins (TSP) of epsilon 34 (E34) phage in combination with CBD extract to understand its interactions with CBD-resistant *Salmonella* Typhimurium and *Salmonella* Newington. We hypothesized that phage protein-CBD extract combination exhibits antibacterial activity against *S.* Typhimurium and *S.* Newington.

## 2. Materials and methods

### 2.1. Media, chemicals, bacterial strains, and other reagents

All media, enzymes and oligonucleotides for PCR reaction, transformation and induction were purchased from New England Biolabs. Competent cells BL21/DE3 and Novablue cells were purchased from Novagen. *Salmonella* Typhimurium (the P22 phage host cells) *Salmonella* Newington (the E34 phage host cells) were received from other laboratories. The two strains of *Salmonella* were used in this study were lab coded as BV4012 and BV7004 representing *Salmonella* Typhimurium LT2 strain MS1868 (a kind gift from Dr. Anthony R. Poteete, University of Massachusetts) and *Salmonella* Newington as *S.* Newington (also known as *S. enterica* serovar Anatum var. 15+ strain UC1698, a kind gift from Dr. Sherwood R. Casjens, University of Utah) respectively. pET30a-LIC vector was purchased from Novagen, urea, sodium chloride, ammonium sulfate, and all other chemicals used in this research were of HPLC grade.

## 2.2. E34 TSP expression and purification

The transformants were grown on LB agar medium, which was premixed with 50 mg/ml of kanamycin antibiotic which serve to select for only transformers, non-transformers are killed by the 50 mg/ml kanamycin. A final concentration of 1 mM isopropyl-β-D-thiogalactopyranoside (Sigma) was used for induction of E34 TSP expression. The transformants were grown on LB agar medium, which was premixed with 50 mg/ml of kanamycin antibiotic which serve to select for only transformers, non-transformers are killed by the 50 mg/ml kanamycin. A final concentration of 1 mM isopropyl-β-D-thiogalactopyranoside (Sigma) was used for induction of E34 TSP expression. The initial cloning of the E34 TSP gene into Pet30a-LIC has been described elsewhere (33), however, the initial clone contained an extra 43 amino acids. In this work, the extra 43 amino acids have been removed so as to maintain the wild type E34 TSP only. Verification of a cloned insert was obtained by PCR analysis *via* 0.8% agarose gel electrophoresis using gene-specific primers (Forward primer; 5-pET-E34–5'-GACGACGACAAGATGACAGACATTAC AGCC-3′. Reverse primer; 3-pET-E34–5'-GAGGAGAAGCCCGGT TCAAGACCAATACTC-3′).

BL21/DE3 cells containing the pET30a-LIC with the E34 TSP insert were streaked on Luria agar plates containing kanamycin. Streaked plates were incubated at 37°C in Precision Economy incubator overnight. This vector contains a 6HIS region that can be targeted for HIS-tag purification. Single colonies were selected and grown in LB broth primed with Kanamycin in a MaxQ 4,450 incubator (Thermo Scientific) fitted with a shaker running at 121 rpm. At mid-log, IPTG (Sigma) was added to a final concentration of 1 mM IPTG to induce cells. An uninduced control sample served as control for subsequent protein analysis. After 6 h, the bacteria were pelleted at 10,000 rpm in an Avanti J XP centrifuge fitted with JA14 rotor chilled at 4°C. Pelleted cells were then re-suspended in lysis buffer consisting of 50 mM Tris at pH7.4, 5 mM $MgCl_2$, 0.1 mg/ml lysozyme, 0.1 mg/ml DNase, 0.05 mg/ml RNASE, 0.2 mg/ml DTT and subjected to three cycles of freeze–thaw–freeze. Samples were then centrifuged at 17,000 rpm for 30 min and the supernatant decanted into 50 ml tubes and stored at−20°C as the E34 TSP lysate. The fractionation of E34 TSP was then carried out using FPLC (GE/Amersham Biosciences-AKTA) connected to a desktop computer Pentium 4 running UNICORN software. In brief, 5 ml Cobalt-NTA FPLC columns (Co-NTA) was used in the FPLC fractionation of the E34 phage TSP at pressure of 0.27 MPa, flow rate of 1 ml/min. The samples that fell under the curve (7–8) were pooled together and concentrated using Amicon Ultra concentrators (Millipore). The gradient of 4.0 ml (100%) of buffer B served as the mobile phase. The buffer B consisted of 20 mM phosphate, 400 mM NaCl, 250 mM imidazole solution, pH 7.4. Then 3 fractions consisting of 1.0 ml each were collected and pooled together and fractions enriched to the desired concentrations using Amicon concentrators (MilliporeSigma). Purified samples were then run on 10% SDS PAGE to determine the purity of the protein.

## 2.3. Cannabidiol extract stock preparation and serial dilutions

The CBD extract hemp variety 'Suver Haze' CBD extract stock was obtained from Sustainable CBD LLC., and the extraction process has been published elsewhere (8). Valizadehderakhshan et al. have demonstrated other excellent and current method for obtaining CBD such as refining Cannabidiol using Wiped-Film Molecular Distillation (34). The stock was diluted with EtOH and vortexed to a final concentration of 50 mg/ml CBD extract and 4% EtOH. This was further diluted serially with 4% EtOH to produce our working concentrations of 250 μg/ml, 125 μg/ml, 62.5 μg/ml, 31.25 μg/ml, 15.62, and 7.81 μg/ml of CBD extract.

## 2.4. Creating CBD resistant strains

To investigate the antimicrobial activity mechanism of CBD, we generated CBD resistance following a procedure published by (35). *S.* Typhimurium strain BV4012 and *S.* Newington strain BV7004 were subjected to CBD resistance development by growing them for an extended time (7 days) in media supplemented with CBD extract. Initially, low doses of CBD extract (1–5 μg/ml), followed by selecting resistant colonies and growing them in CBD extract concentrations of 10–50 μg/ml. Finally, resistant colonies from these were plated on LB agar supplement with 50 μg/ml of CBD extract. Colonies formed were then grown again in LB broth to reach log phase. Resistant strains were then pelleted and resuspended in 1X PBS containing 50% (wt/vol) polyethylene glycol 8,000 (PEG 8000) (Promega Corporation, United States), 50 mM Mg2+ at pH 7.4. Finally, resistant bacterial samples were aliquoted into microcentrifuge tubes and stored at−80°C until use. Subsequently, CBD resistant strains of *S.* Typhimurium and *S.* Newington were restreaked on LB agar.

## 2.5. Assessments of CBD extract, E34 TSP, and their combination treatment of *Salmonella* spp.

Serial dilutions of CBD extract stock solutions were carried out to obtain the final concentrations of 250, 125, 62.5, 31.25, 15.62, and 7.81 μg/ml. Two *Salmonella* strains BV4012 (*S.* Typhimurium) and BV7004 (*S.* Newington) were grown in LB broth to logarithmic growth phase and diluted to $OD_{600}$s of approximately 0.08 and 0.25. The CFUs were determined *via* plating on LB agar to consist of concentration of approximately of $1 \times 10^4$ and $1 \times 10^8$ CFU/ml. Then, aliquots of 100 μl of the bacterial cells were seeded in a 96-well microtiter plate (Fisherbrand™, Fisher Scientific, Fair Lawn, NJ, United States) at a density of $1 \times 10^4$ per mL to mimic early log growth phase of the bacteria, or at a density of $1 \times 10^8$ per mL to mimic log phase. Then, 50 μl of each solution of the protein, or the CBD extract or the combinations was added to the bacterial cells. The experimental groups were treated with 250, 125, 62.5, 31.25, 15.62, and 7.81 μg/ml concentrations of CBD extract, or 44.5, 22.25, 11.12, 5.56, 2.78, 1.39 μg/ml of E34 TSP, or the combinations of CBD extract and E34 TSP in varying concentrations. The controls consisted of $dH_2O$ group (negative control) and 2% SDS group (positive control) (see Table 1). The samples were incubated at 37°C for set time points. The growth kinetics of bacteria were determined by reading their optical densities at wavelength of 600 nm using SpectraMax plate reader [(Molecular Devices SpectraMax® ABS Plus) (Molecular Devices LLC., San Jose, CA, United States)]. The experiments were carried out in triplicates.

TABLE 1 Treatments.

| Treatments | Concentrations/μg/mL | Remark |
|---|---|---|
| E34 TSP | 44.5 | 44.5 to 11.25 μg/ml used in monotreatment analysis for early and late log phases of the bacteria. |
| | 22.25 | |
| | 11.12 | |
| | 5.56 | 44.5 t0 1.39 μg/ml used in dose dependent treatment of the bacteria. |
| | 2.78 | |
| | 1.39 | |
| CBD | 250 | 250 to 7.81 μg/ml CBD was used in the dose dependent treatment analysis CBD against the bacteria |
| | 125 | |
| | 62.5 | |
| | 31.25 | |
| | 15.62 | |
| | 7.81 | |
| E34 TSP + CBD | 44.5 E34 TSP + 250 CBD | 44.5 to 11.25 μg/ml used in combination treatment with 250 μg/ml of CBD. |
| | 22.25 E34 TSP + 250 CBD | |
| | 11.12 E34 TSP + 250 CBD | |
| Controls | LB/$dH_2O$/SDS | |

## 2.6. Membrane integrity assessment *via* propidium iodide and SYTO-9 staining

To assess the capacity of CBD extract, E34 TSP and CBD extract-E34 TSP combination to disrupt the cytoplasmic membrane integrity, the membrane impermeable fluorescent DNA intercalating dye Propidium iodide (PI) and SYTO-9 were employed which is a quick and accurate method to determine the proportion of dead and live cells in cell cultures. The fluorescence produced by the propidium iodide (PI) when it binds to DNA is used to identify dead cells (36). However, live cells with intact membranes present a barrier to PI, thus only cells with disrupted membranes can be stained with PI. Hence, this is used in assessing membrane integrity and cell death since dead cells will allow the penetration of PI into their cytoplasm. Fluorescence microscopy was utilized to observe the effects of CBD extract, or E34 TSP, or the combination of CBD extract and E34 TSP treatment on *Salmonella* cells. CBD resistant strains of *Salmonella*–strains *S.* Typhimurium and *S.* Newington–were grown to mid-log phase, diluted to cell concentration of $1 \times 10^8$ and then treated with CBD extract, E34 TSP, or the combination of CBD extract and E34 TSP. Two controls, $dH_2O$ and 2% SDS served as the negative and positive controls, respectively. Samples were then stained with 1 X SYTO-9 and 40 μg/ml of propidium iodine and left at room temperature for 25 min, covered with aluminum foil. Samples were then observed using an EVOS FLC microscope (Life Technologies Corporation, Carlsbad, CA, United States).

## 2.7. Assessment of membrane lysis *via* genomic DNA migration

Bacteria cell membrane plays several crucial functions; however, the most fundamental function is to act as a storage sac for all the cytoplasmic content. Thus, if lysed, the cytoplasmic content which consists of the bacterial proteins and nucleic acid will leak. Taking advantage of this important role of cytoplasmic membrane, we hypothesized that the genomic materials of lysed cells will migrate in agarose gel matrix, whereas bacteria with intact cell membranes will retain their genomic material and thus block the nucleic acid from migrating through the agarose gel's matrix when subjected to electrophoresis. To investigate the ability of E34 TSP, CBD extract or CBD extract-E34 TSP treatment to cause lysis of bacteria membrane, we pelleted 500 μl of *S.* Typhimurium that was previously growing at mid-log phase and resuspended the bacteria in 500 μl of 1X PBS. 100 μl of the bacteria sample was aliquoted into centrifuge tubes and 100 μl of 44.5 μg/ml E34 TSP, or 22.25 μg/ml of E34 TSP, or 250 μg/ml of CBD extract, or 125 μg/ml of CBD extract were added to the bacteria samples and incubated for 1 h. Sample of the resuspended bacteria which did not receive the CBD or E34 TSP treatments but rather media only served as control. Subsequently, 30 μl of each treatment sample was loaded into a 0.8% agarose gel and run for 75 min at 90 volts. Gel was then stained with Ethidium bromide and DNA bands visualized using ChemiDoc XRS imager.

## 2.8. Bacterial dehydrogenase activity assay

The combined dehydrogenase enzymatic activity of *Salmonella* spp. was assayed to assess the effect of CBD extract, E34 TSP or CBD extract-E34 TSP combination treatment on the bacterial cells in culture. In brief, *Salmonella* cells growing at mid-log phase were diluted to an $OD_{600}$ of 0.2. Then, 100 μl of the bacterial sample was placed into each well of a 96-microplate and treated to varying concentrations of CBD extract, E34 TSP or CBD extract-E34 TSP. Then 50 μl of the non-toxic resazurin was added to each well. The conversion of resazurin to resorufin produced a sharp color change.

The color change was monitored using SpectraMax plate reader (Molecular Devices SpectraMax® ABS Plus) (Molecular Devices LLC., San Jose, CA, United States) and absorbance readings recorded at 590 nm (37).

## 2.9. Statistical analysis

Two different *Salmonella* species were used for these experiments, and the experiments were carried out in triplicates, and results are presented as means ± SEM, *p*-values lower or equal to 0.05 were considered significant using student paired *t*-test. All statistical analyzes were performed, and graphs were plotted on Microsoft Excel (Microsoft 2010), microscopic images were processed using ImageJ (an opensource NIH software).

# 3. Results

## 3.1. Verification of E34 TSP gene insert in PET30 a-LIC vector and SDS PAGE analysis of expressed E34 TSP

As shown in Figure 1A, lane 8, our PCR product produced the 1.818 kbp size insert which is the size of E34 TSP gene. In Figure 1B, the expressed E34 TSP migrated to a size consistent with its trimeric molecular weight size (see lane induced lysate and fractionated E34 TSP 1) this unusual trimeric migration property of E34 TSP has been well documented, and has been implicated on the non-covalent interactions between the three subunits of the protein (38–40), similar observation is noted of most other P22-like phages' tailspike proteins (41–43). The purification process carried out *via* FPLC as shown in Figure 2 ensured the removal of over 90% contaminating proteins as shown in the fractionated E34 TSP1. Induction of E34 TSP was achieved as a thicker band could be visibly seen in the induced lane as compared to the uninduced lane where the E34 TSP band could barely be observed.

## 3.2. Effect of E34 TSP and CBD extract treatments on *Salmonella* Typhimurium and *Salmonella* Newington growth at early and late log phases

At log phase, most bacterial disease symptoms begin to surface, and it is the growth phase which shows most dramatic changes in both bacteria number and disease severity. To assess the effect of E34 TSP, CBD extract and CBD extract-E34 TSP combination treatments on *S.* Typhimurium and *S.* Newington growth at early and late log phases, cells growing at early or late log $OD_{600}$'s were subjected to E34 TSP, CBD extract or CBD extract-E34 TSP combination treatments as depicted in Figure 3.

As shown in Figures 3A,B, treatment of *S.* Newington with CBD extract, E34 TSP and the combination of the two produced

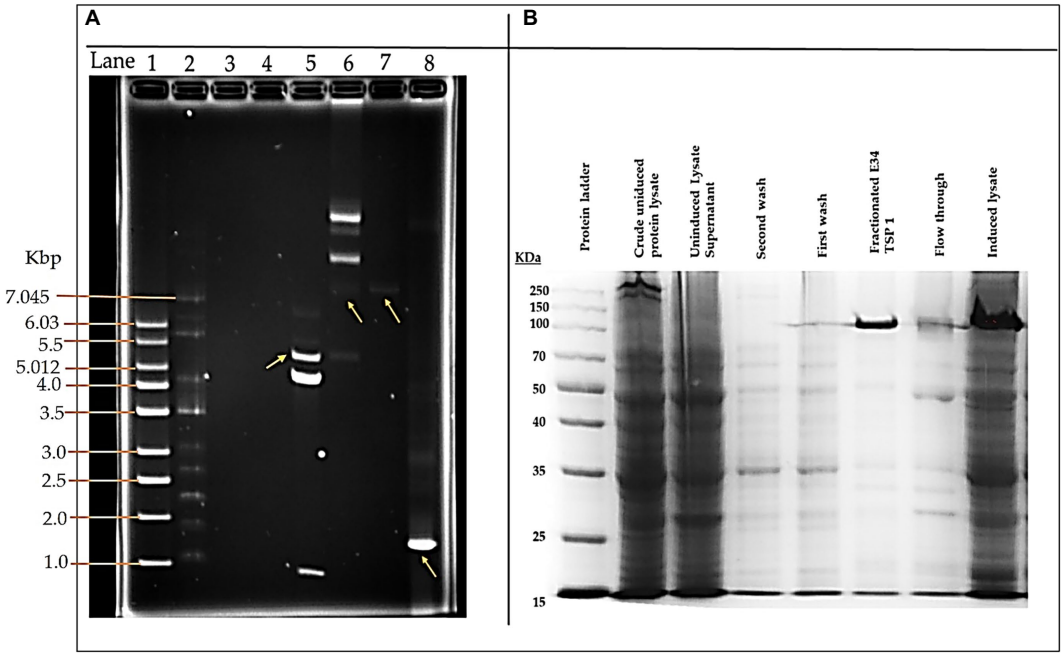


FIGURE 1
pET30a-LIC-E34 TSP clone verification and E34 TSP protein purification analysis. **(A)** Analysis of pET30a-E34 TSP clone in a 0.8% agarose gel. **(B)** Analysis of E34 TSP lysate and fraction using 10% SDS PAGE. Lane 1=1kb DNA ladder, from Millipore Sigma. Lane 2=NEB supercoil ladder; New England BioLabs. Lane 3=miniprep for pET30a-E34 DNA in non-transformed cells. Lane 4=miniprep for pET30a-E34 DNA in unsuccessfully transformed cells. Lane 5=miniprep for pET30a-E34 DNA in successfully transformed cells; Nde1 digested pET30a-E34 DNA. Lane 6=miniprep for pET30a-E34 DNA in successfully transformed cells; undigested pET30a-E34 DNA. Lane 7=E34 WT DNA. Lane 8=PCR product from pET30a-E34. **(B)** SDS-PAGE analysis of E34 TSP protein induced by IPTG, samples separated by 10% polyacrylamide gel, and visualized *via* Coomassie brilliant blue R-250. Induction of the E34 TSP was confirmed *via* the SDS PAGE. Comparing the induced lane and uninduced lane, it is observed that there was an overexpression of E34 TSP as indicated by the thicker band in the induced lane than the uninduced lanes.

interesting findings. We observed that the relative $OD_{600}$ of E34 TSP treated samples were generally lower than the combined treatment for the early log-phase at the 10 h time point of culture. The data revealed a decrease in bacterial relative $OD_{600}$s in both E34 TSP treated samples than the combination treatment. The exception however was observed in the 22.25 μg/ml E34 TSP and the 250 μg/ml CBD extract combination treatment which depicted a drastic increase in relative $OD_{600}$. The 250 μg/ml CBD extract treatment only produced relative $OD_{600}$ comparable to the media control. In the 44.50 μg/ml E34 TSP and the 250 μg/ml CBD extract combination treatment, we observed approximately triple jump in relative $OD_{600}$ as compared to the 44.5 μg/ml E34 TSP monotreatment of *S.* Newington Figure 3. Similar trends were observed in the late log phase treatment of *S.* Newington to E34 TSP and CBD extract as depicted in Figure 3B.

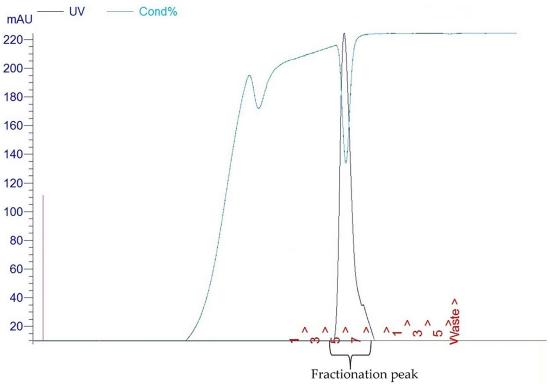


FIGURE 2
Fast protein liquid chromatography chromatograms (FPLC) of E34 TSP.

In Figures 3C,D, treating *S.* Typhimurium (ST) with E34 TSP showed a generally lower relative inhibition characteristic after 10 h (especially at the higher concentrations) than the E34 TSP and CBD extract combination treatments. For instance, it was observed that at higher concentrations of E34 TSP (44.5 μg/ml) alone, there was a drastic reduction in relative $OD_{600}$, however its combination with CBD extract (i.e., 44.5 μg/ml E34 TSP and 250 μg/ml CBD extract) performed poorly against the bacteria at both early and late log phases. Thus, when E34 TSP was combined with CBD extract at 44.5 μg/ml and 250 μg/ml respectively, the treatment performed poorly against the bacteria. Treatment of the bacteria with CBD extract alone did not reduce the relative $OD_{600}$ in the early and late growth phases of the bacteria.

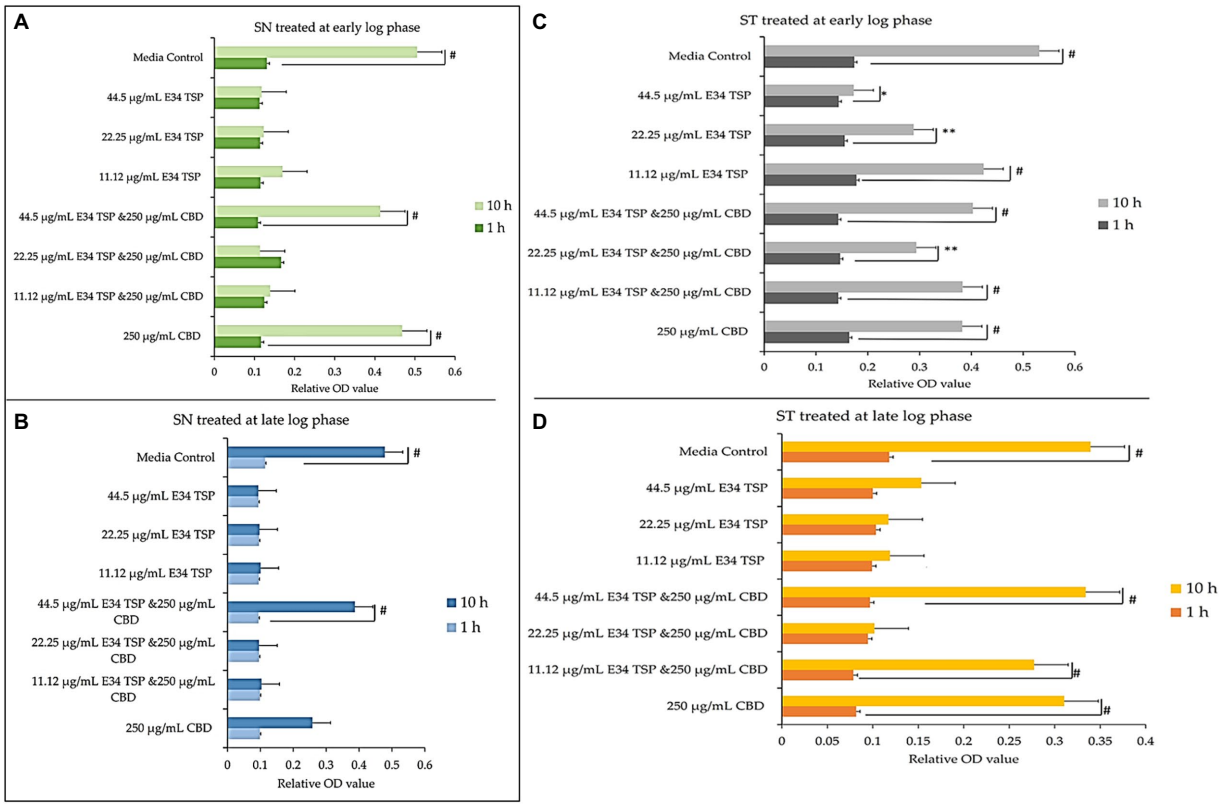


FIGURE 3
**(A,B)** Effect of CBD extract /E34 TSP treatment of *S.* Newington in both early and late log phase of the bacteria species. Studies were initiated an initial $OD_{600}$ of approximately 0.1. **(A)** CBD extract and E34 TSP treatment to *S.* Newington (SN) at early log phase. # value of $p \leq 0.00354$. **(B)** CBD extract and E34 TSP treatment to *S.* Newington (SN) at late log phase. Data shown are from three independent experiments expressed as means ± SEM. # value of $p \leq 0.039$. **(C,D)** Effect of CBD extract and E34 TSP treatment of *S.* Typhimurium in both early and late log phase of the bacteria species. **(C)** CBD extract and E34 TSP treatment to *S.* Typhimurium (ST) at early log phase. # value of $p \leq 0.0001$, * and ** value of $p \leq 0.025$. **(D)** CBD extract and E34 TSP treatment to *S.* Typhimurium (ST) at late log phase. Studies were initiated an initial $OD_{600}$ of approximately 0.1. Data shown are from three independent experiments expressed as means ± SEM. # value of $p \leq 0.0103$.

## 3.3. Immunofluorescent analysis of E34 TSP, CBD extract, and CBD extract-E34 TSP combination treatment on *Salmonella* Typhimurium and *Salmonella* Newington

To investigate the effect of E34 TSP, CBD extract, and CBD extract-E34 combination on *S.* Typhimurium and *S.* Newington viability through the disruption of the bacterial cytoplasmic membrane integrity, the membrane impermeable fluorescent DNA intercalating dye Propidium iodide (PI) and the membrane permeable green, fluorescent dye, SYTO-9 was employed. In brief, CBD resistant strains *S.* Typhimurium and *S.* Newington were grown to mid-log phase, diluted to cell concentration of $1 \times 10^8$ and then treated with CBD extract, E34 TSP, or the combination of CBD extract and E34 TSP. Two controls, $dH_2O$ and 2% SDS served as the negative and positive controls, respectively. Figures 4–8 illustrates the effects of treatment on the viabilities of *S.* Typhimurium and *S.* Newington.

## 3.4. Time dependent analysis of E34 TSP and CBD extract treatments on *Salmonella* Typhimurium and *Salmonella* Newington growth at lag phase

To understand the effect of time kinetics of E34 TSP, CBD extract, or their combination treatment to *S.* Typhimurium and *S.* Newington, we subjected bacterial cells growing at lag phase (approximately 0.081 $OD_{600}$) to E34 TSP and CBD extract treatment.

At the lag phase treatment, while all performed better than the control, treatment of *S.* Typhimurium to 44.5 µg/ml and 22.25 µg/ml of E34 TSP performed significantly higher in inhibiting the bacterial growth. The combination treatment of 250 µg/ml CBD extract and 44.5 µg/ml E34 TSP also performed significantly better in inhibiting the *S.* Typhimurium growth than the 250 µg/ml and 22.25 µg/ml combination treatment or the 250 µg/ml CBD extract monotreatment (Figure 9A). In Figure 9B however, only E34 TSP treatment at concentrations 22.25 µg/ml and 44.5 µg/ml showed high inhibition of *S.* Newington, all other treatments showed poor inhibition kinetics. Comparatively, as shown in Figure 9C, the control group as expected showed the highest increase in $OD_{600}$ of 38% compared to the other treatments, followed by the 250 µg/ml CBD extract and 22.25 µg/ml E34 TSP combination treatment which recorded 22%. Thus, indicating that these two treatments performed the poorest in inhibiting *S.* Typhimurium growth at lag phase at the 12 h time point. The 22.25 µg/ml E34 TSP treatment showed the best inhibitory characteristic at the 12 h time point, recording 10%.

In treating *S.* Newington, as shown in Figure 9D, the best inhibitory effect again was observed in the 22.25 µg/ml E34 TSP treatment, which recorded a comparative rate of 16% at the 1 h time point and reduced this to 9% at the 12 h time point.

## 3.5. Dose dependent analysis of E34 TSP and CBD extract treatment on *Salmonella* Typhimurium and *Salmonella* Newington

To investigate how the concentration of E34 TSP, CBD extract or CBD extract-E34 TSP combinations could inhibit bacterial cell growth, we treated *S.* Typhimurium and *S.* Newington to varying concentrations of E34 TSP, CBD extract, and combinations of CBD extract -E34 TSP. Figures 10A–D show the inhibition curves at the various treatment doses.

## 3.6. E34 TSP treatment potentiate bacterial membrane disruption

The nucleic acid migration analysis of *S.* Typhimurium which whole bacteria whole cells were treated to CBD extract, or E34 TSP as shown in Figure 11 indicates that E34 TSP causes lysis of *S.* Typhimurium cells.

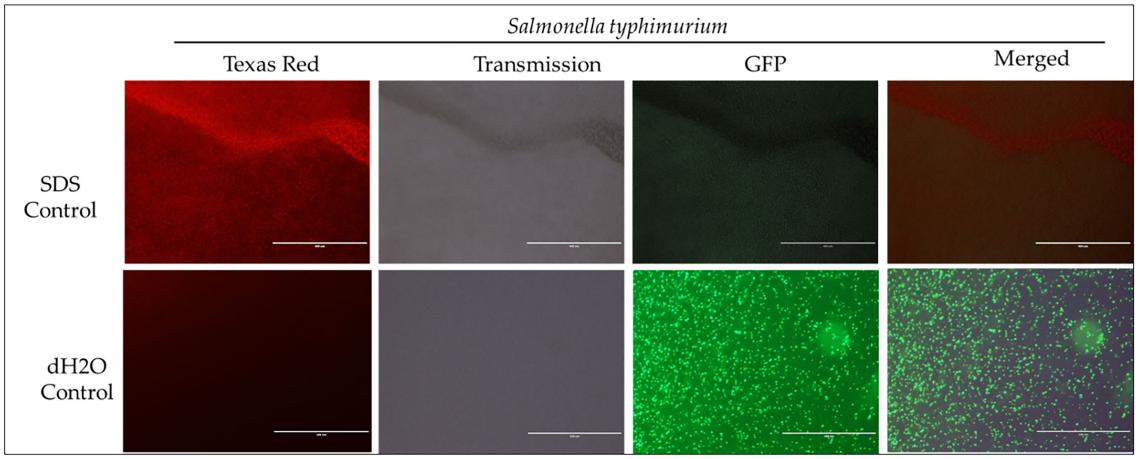


FIGURE 4
Immunofluorescence analysis of *S.* Typhimurium (ST) treated to 2% SDS as a positive control, while $dH_2O$ as a negative control. Scale bar=100µm. The immunofluorescence studies reveal that 2% SDS had killed *S.* Typhimurium (as shown in complete red fluorescence), whereas the $dH_2O$ revealed live bacteria growing in the sample as indicated by the green fluorescence.

### 3.7. Analysis of dehydrogenase activity of treated *Salmonella* Typhimurium and *Salmonella* Newington

The dehydrogenase of *Salmonella* is one critical reductase that is modulated by various metabolites, which include among others the glycolytic TCA cycle and related intermediates, such as PEP, pyruvate, acetyl CoA, citrate, etc. Another set of metabolites that actively regulate *Salmonella* dehydrogenase activity are the purine nucleotides (AMP, ADP, ATP, CAMP, GMP, GDP, and GTP). For instance, ATP is a potent activator of glutamate dehydrogenase (44). Thus, to assess the effect of CBD extract treatment to dehydrogenase of *Salmonella* spp. used in this study, we employed resazurin assay.

For *S.* Typhimurium, the effect of CBD extract on the bacteria dehydrogenase activity seems to negatively correlate with the concentration of CBD extract at the 1 h time point. E34 TSP showed no significant differences in all doses. The *S.* Newington strain showed lower relative values when treated to E34 TSP at all concentrations except at 2.78 μg/ml and 1.39 μg/ml at the 1 h time point. CBD extract at all concentrations showed no significant effect on *S.* Newington at 1 h time point. In general, 1 h post treatment of *S.* Typhimurium with CBD extract showed far lower dehydrogenase activity than E34 TSP treatments of the same strain. This is unexpected since our previous results demonstrated that E34 TSP showed higher killing of *S.* Typhimurium than CBD extract (Figure 3). This might be due in part to the inability of E34 TSP to inhibit the dehydrogenase enzyme in *S.* Typhimurium whereas CBD does inhibit it. A question remaining however, is why then we have less killing effect of CBD extract than E34 TSP in *S.* Typhimurium. To answer this, we infer that, there might be a possible alternative dehydrogenase pathway utilized by *S.* Typhimurium in its energy metabolism. In treating *S.* Newington however, E34 TSP treatments showed relatively lower dehydrogenase activity compared to the CBD extract treatment.

For *S.* Typhimurium, the effect of CBD extract and E34 TSP was not hugely different from each other except the 31.25 μg/ml CBD extract treatment which considerably limited the dehydrogenase activity. The treatment of *S.* Newington to E34 TSP however, showed general shutdown of the dehydrogenase enzyme at the 5 h time point. Interestingly, CBD extract treatment to the same strain produced slightly higher dehydrogenase activity as compared to the E34 TSP treatment. This trend is in consonance with the killing ability of E34 TSP on *S.* Newington than CBD does. All SDS controls showed the lowest activity indicative of dead bacteria. Comparing the 5 h treatment to 1 h treatment, all the samples showed significantly lower dehydrogenase activity at 5 h than 1 h time point. This is indicative of the killing ability of all the treatments.

## 4. Discussion

Due to the emergence of multidrug-resistant bacteria, the use of bacteriophage as an alternative to antibiotics has been reconsidered. However, this approach can be very effective when combined with other agents. The combination of these two agents can provide better bacterial suppression and lower the chances of the development of resistance. It also allows for more efficient penetration into the bacterial population. Although neutral effects have been observed in some studies, combined approaches are still considered important in preventing the development of multidrug-resistant bacteria (45). The main culprit for multi-drug resistance has been excessive use of antibiotics, which jeopardizes their effectiveness for controlling these pathogenic agents (46). Considering this, bacteriophages and their derivatives are becoming more widely accepted as viable complementary techniques for use in food safety, health, and medicine.

In our previous studies, we demonstrated the antimicrobial effect of CBD extract against the Gram-negative bacterium *S.* Typhimurium (8), and in a separate publication, we also revealed that CBD synergizes with ampicillin and polymyxin B in killing *S.* Typhimurium ((47)). E34 phage protected Vero cells from *Salmonella* infection (38). In this work, E34 phage's LPS hydrolase, which is the E34 TSP, was expressed from a previously published clone (38), and used in combination with CBD extract to investigate the killing abilities of the two antimicrobial agents against two bacterial strains of *Salmonella*. The proceeding sections provides data obtained from multiple biological assays carried out to determine the antimicrobial activities of CBD extract and E34 TSP against CBD-resistant strains of *S.* Typhimurium and *S.* Newington.

E34 phage is a bacteriophage that infects *S.* Newington, and it uses its hydrolase machinery which is the tailspike protein (TSP) for the initial interaction and anchoring of the phage particle to the LPS of the cell and subsequent hydrolysis and anchoring of the LPS to the membrane of the bacteria. In this study, the E34 TSP was expressed under the control of the T7 promoter in pET30a-LIC vector, purified and combined with CBD as an antibacterial agent against CBD-resistant strains of *Salmonella*. Validation of the cloned insert was achieved *via* PCR reaction using two primers that amplified exactly the tailspike gene, gp19 in the clone. An agarose gel electrophoresis of PCR product demonstrated that it carried the exact size of 1.818 kbp insert (Figure 1A). Non-recombinants control did not yield any band, indicative of the absence of the insert. Cobalt-NTA column was used for affinity purification *via* FPLC (chromatograph shown in Figure 2), and samples were run on SDS PAGE to validate the purity of the protein.

The ability of CBD extract to potently inhibit *S.* Typhimurium was previously demonstrated in our laboratory (8), other works by (48) also revealed that CBD is a novel antibiotic adjuvant that potentiates the effect of the bacitracin against Gram-positive bacteria (e.g., *Staphylococcus aureus*, *Listeria monocytogenes*, and *Enterococcus faecalis*). The same group in another study explored the mechanism of resistance to CBD by *Staphylococcus aureus*. They discovered through resistant strains genome sequencing that the farE/farR system encoding a fatty acid efflux pump (FarE) and its regulator (FarR) were mutated, which showed diminished susceptibility to both CBD and bacitracin (35).

Proceeding from lag phase, bacteria enter log phase, a phase characterized by exponential growth. One major biological characteristic of this phase is the high metabolic activities occurring, which is due in part to high DNA replication, RNA translation, cell wall biosynthesis, and in part due to high cell division. While bacteria are metabolically hyperactive at this phase, they are also most vulnerable too, it is in this phase that antibiotics and other antibacterial agents can produce their highest potency. Usually, most of these agents target bacteria cell wall synthesis (e.g., Beta-lactams), or protein synthesis (e.g., thermorubin, (49)), DNA transcription

(e.g., Anthracyclines (50)), and RNA translation (e.g., spectinomycin (51)). In this work, we investigated the effect of treating *S.* Newington and *S.* Typhimurium to CBD extract, E34 TSP, and the combination of the two agents at both early and late log phase of the bacteria. As shown in Figure 3 while generally all treatments performed better in *S.* Newington than in *S.* Typhimurium, the E34 TSP treatments gave the best inhibition than both CBD extract and CBD extract-E34 TSP combination. While it is predictable that E34 TSP monotreatment will perform better than CBD extract in reducing the bacterial growth, since these strains were CBD resistant, it is surprising that the combination treatment performed poorly. While these results necessitate a multiple dose analysis of CBD extract, and E34 TSP combinations, it seems safe to infer that there is probably an unknown interaction between the CBD extract and E34 TSP. This might be due in part to CBD in the CBD extract probably binding to the catalytic site of the E34 TSP, thus blocking it from its endorhamnosidase activity, however this observation does not answer for lower concentration of E34 TSP in combination with CBD extract which showed higher bacterial $OD_{600}$. Since these strains are CBD-resistant, inactivated E34 TSP and high amount CBD extract will possibly still show minimal effect on the bacteria, and such should be highly pronounced at the lower E34 TSP concentrations. Figures 4–8 depict the immunofluorescent images showing the ability of E34 TSP to kill *S.* Typhimurium and *S.* Newington. However, as shown in Figure 5, the bacteria seemed to pool into micro clusters in high CBD concentrations that possibly provided unique shields against the CBD. This clustering might enable the development of biofilm by the bacteria, thus enhancing their CBD resistance (52). Micro clustering enables quicker cell–cell communication in the form of quorum sensing or membrane vesicle trafficking. The membrane vesicles in bacteria play a role in cell–cell communication between bacteria themselves and between bacteria their hosts. Membrane vesicles are an important component of anti-bacterial resistance and thus have gained relevance in antibacterial resistance research. Given this, (53) revealed that CBD affected the membrane vesicle profile and membrane vesicle release of bacteria. They reported that CBD had a strong inhibitory influence on membrane vesicle release of Gram-negative bacteria such as *E. coli* VCS257, and negligible inhibitory influence of CBD on Gram-positive bacteria such as *S. aureus* subsp. *aureus* Rosenbach 1884 membrane vesicle release. Thus, the micro clustering might be a defensive maneuver to overcome CBD anti-vesicle release property.

The commencement of disease symptoms mostly starts after the lag phase of bacterial infection. This initial phase of the bacterial growth is characterized by cellular activity that typically involves the synthesis of proteins without much growth. To understand the effect of CBD on our CBD-resistant strains growing at lag phase, we treated *S.* Typhimurium and *S.* Newington to CBD, E34 TSP and CBD-E34 TSP combination. As it is well known, CBD is recognized as a potent antimicrobial. (54) Studied the antimicrobial characteristics of CBD-resistance in *Staphylococcus aureus*, *Streptococcus pneumonia*, and *Clostridioides difficile*. Their findings suggested that CBD has an impeccable impact on biofilm and topical *in vivo* efficacy. In this study, at the lag phase, while all treatments performed better than the control, monotreatment of *S.* Typhimurium and *S.* Newington to 44.5 µg/ml and 22.25 µg/ml of E34 TSP performed significantly higher in inhibiting the bacterial growth. The results corroborated with the data obtained in the log phase treatment indicating that bacteria growth phase did not significantly affect the performances of the treatments.

As shown in Figure 10A, CBD showed slightly higher inhibition at higher doses, but did not exhibit time dependent inhibition of

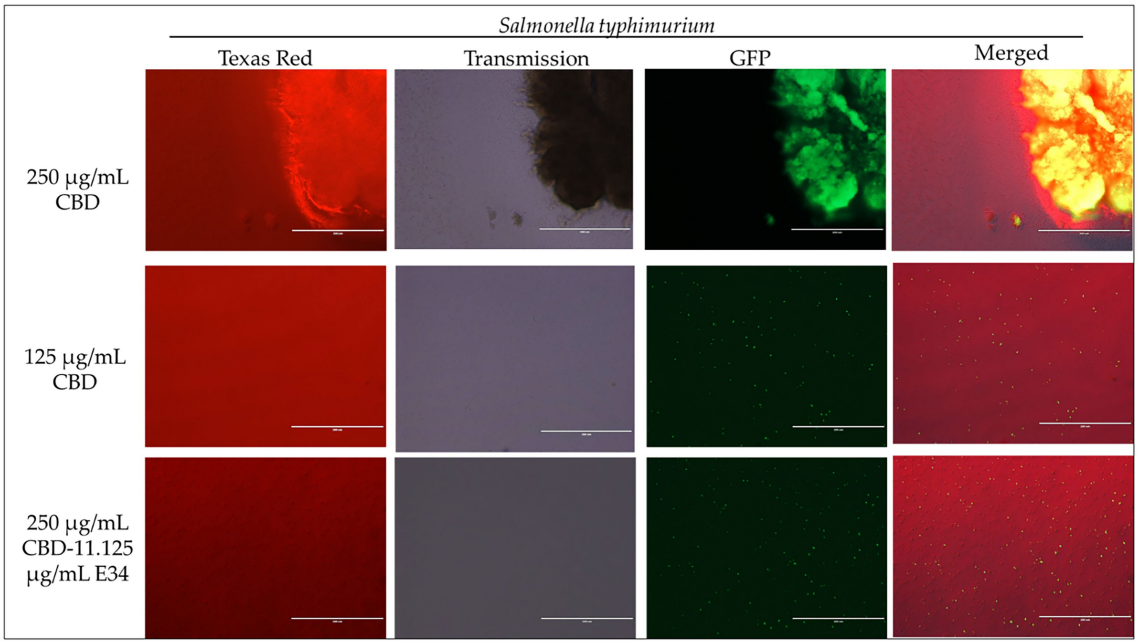


FIGURE 5
Immunofluorescence analysis of *S.* Typhimurium (ST) at high concentrations of CBD extract (250µg/ml, 125µg/ml, and 250µg/ml+11.125µg/ml E34 TSP) treatment. As shown in all panels, treatment at higher concentrations killed the *S.* Typhimurium. However, bacteria seemed to cluster together into biofilms enhancing their ability to survive (See top panel).

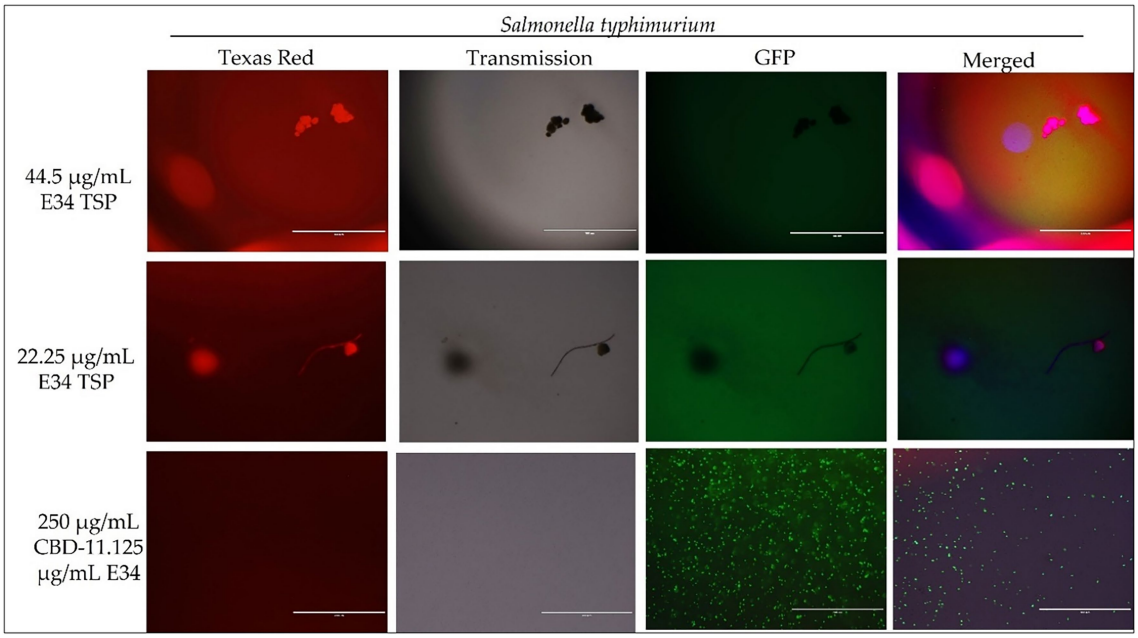


FIGURE 6
Immunofluorescence analysis of *S.* Typhimurium (ST) at higher concentrations of E34 TSP; 44.5µg/ml E34 TSP, 22.25µg/ml E34 TSP, and the combination treatment of 250µg/ml CBD extract +11.125µg/ml E34 TSP. As shown in all panels, treatment at higher concentrations killed the *S.* Typhimurium. However, bacteria seemed to survive the combination treatment of CBD extract and E34 TSP especially at the higher concentration of 250µg/ml CBD extract.

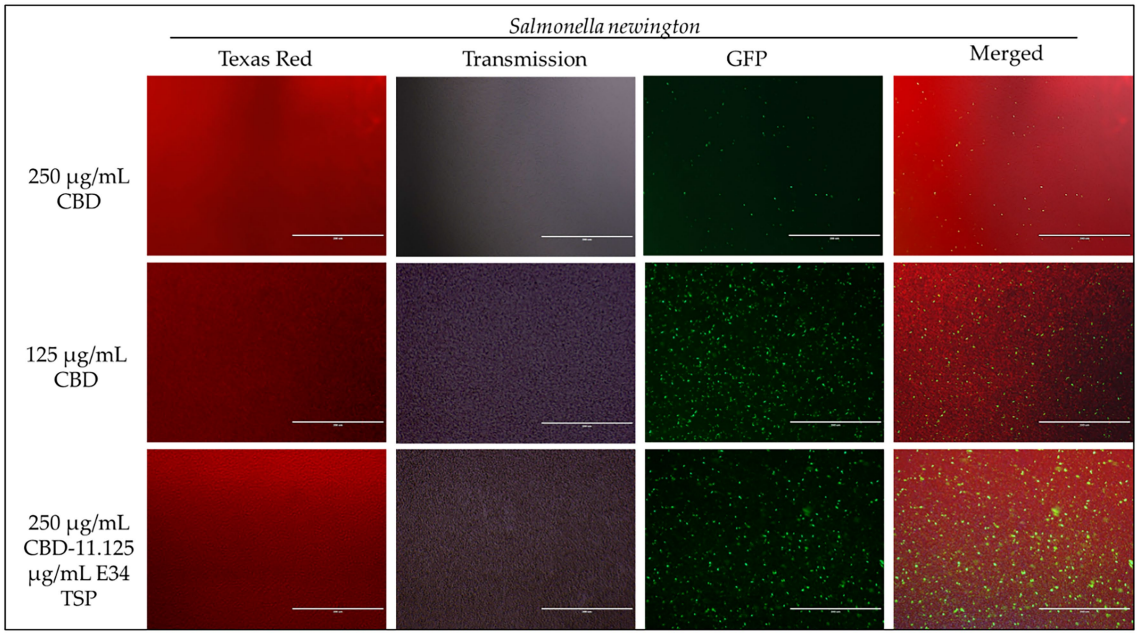


FIGURE 7
Immunofluorescence analysis of *S.* Newington (SN) at higher concentrations of CBD extract (250µg/ml, 125µg/ml, and 250µg/ml+11.125µg/ml E34 TSP) treatments. Generally, there is observed higher killing of *S.* Newington in all treatments. Comparatively, there seems to be an observed lower killing of *S.* Newington at the combination treatment.

*S.* Newington. E34 TSP demonstrated both time and dose dependent inhibition of *S.* Typhimurium (Figure 10B). As expected, CBD extract failed to show any significant inhibition of *S.* Newington in all treatments (Figure 10C). Treatment of *S.* Newington to E34 TSP showed time and dose dependent inhibition (Figure 10D).

Given that there was observed substantial inhibition of *S.* Typhimurium and *S.* Newington caused by E34 TSP on the

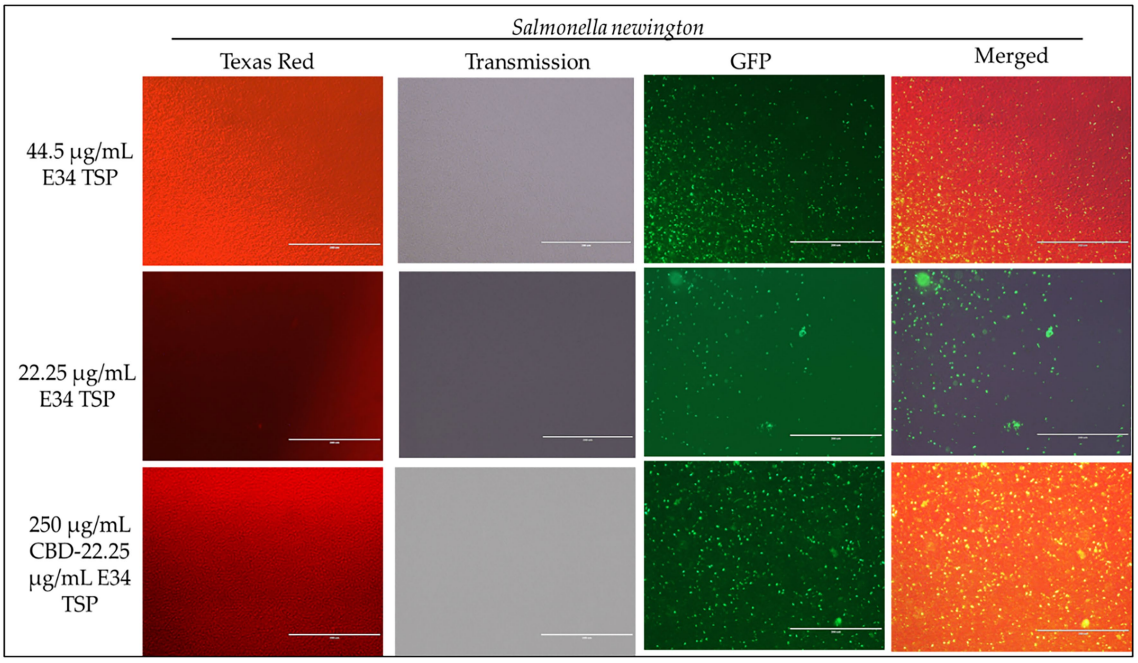


FIGURE 8
Immunofluorescence analysis of *S*. Newington (SN) treated to low concentrations of E34 TSP; 44.5µg/ml, 22.25µg/ml, and a combination treatment of 250µg/ml CBD extract +22.25µg/ml E34 TSP. The immunofluorescence images showed that the combination treatment (CBD extract and E34 TSP) had poorer membrane disruptive activities against *S*. Newington as compared to the monotreatment with E34 TSP.

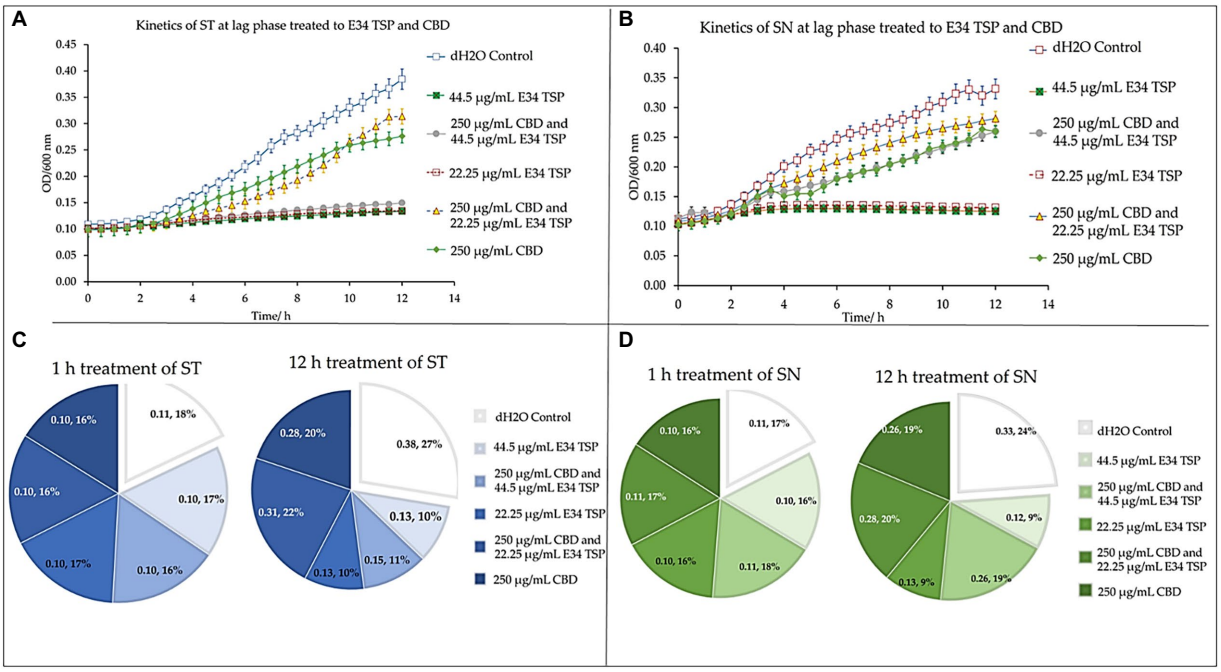


FIGURE 9
Kinetics of combined treatment of E34 TSP with CBD extract on **(A)** *S*. Typhimurium (ST) in the lag growth phase, **(B)** *S*. Newington (SN) in the lag growth phase. **(C)** Pie chart illustrating the relative percent changes in $OD_{600}$ of *S*. Typhimurium among treatments. **(D)** Pie chart illustrating the relative percent changes in $OD_{600}$ of *S*. Newington among treatments. Studies were initiated an initial $OD_{600}$ of approximately 0.1 Data shown are from three independent experiments expressed as means ± SEM.

CBD-resistant strains, we found it important to investigate the possible mechanism of the bacterial killing. We reasoned that it might be due to membrane integrity disruption. For this reason, we analyzed E34 TSP and CBD extract treated samples of CBD-resistant

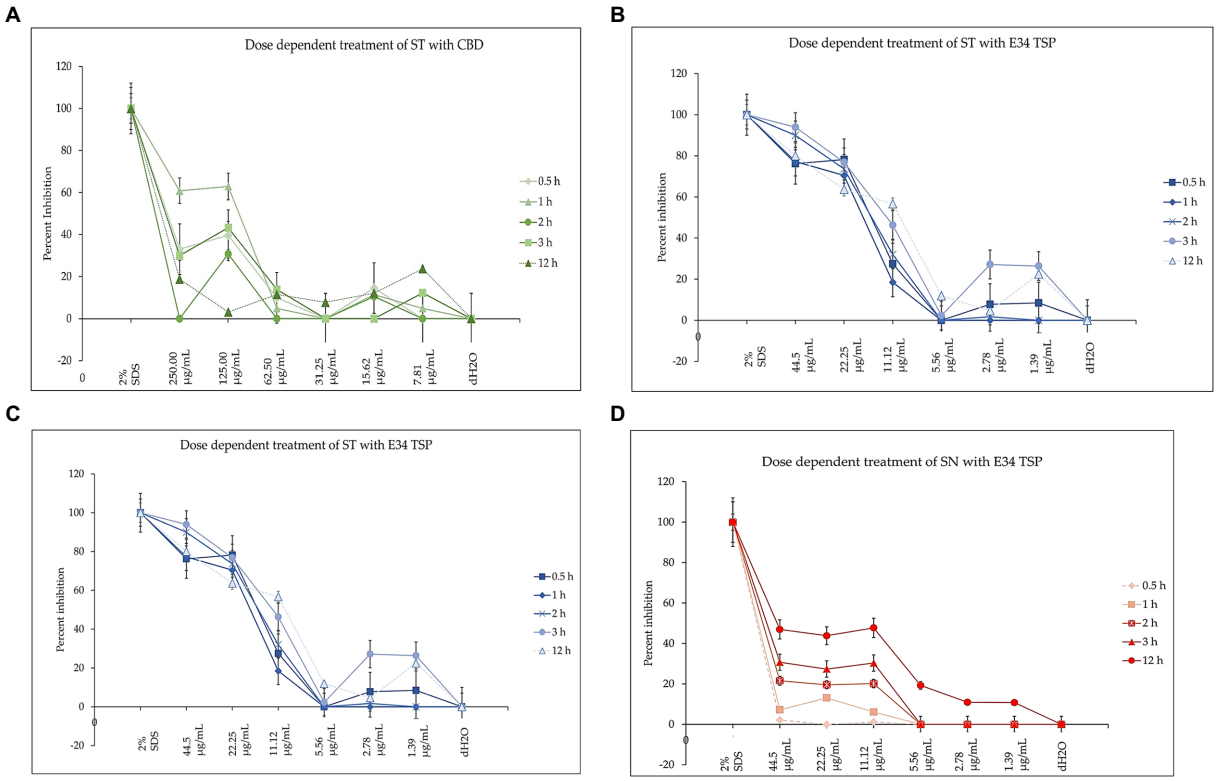


FIGURE 10
**(A)** The effect of CBD extract on *S.* Typhimurium presented as means ± standard deviation. Data shown are from three independent experiments expressed as means ± SEM. As can be seen, CBD extract although showed slightly higher inhibition at higher doses, it seemed not to exhibit time dependent inhibition of *S.* Newington. **(B)** The effect of E34 TSP on *S.* Typhimurium presented as means ± standard deviation. Data shown are from three independent experiments expressed as means ± SEM. As can be seen, E34 TSP seems to inhibit *S.* Typhimurium in both time and dose dependent nature. **(C)** The effect of CBD extract on *S.* Newington presented as means ± standard deviation. Data shown are from three independent experiments expressed as means ± SEM. As shown, CBD extract did not show any significant inhibition of *S.* Newington in all treatments. **(D)** The effect of E34 TSP on *S.* Newington presented as means ± standard deviation. Data shown are from three independent experiments expressed as means ± SEM. As can be observed, E34 TSP seems to inhibit *S.* Newington in both time and dose dependent nature.

*S.* Typhimurium using 0.8% agarose gel. Previous works by (48) revealed that CBD is an effective helper compound in combination with bacitracin to kill Gram-positive bacteria *via* possible membrane disruption. Furthermore, (8) also demonstrated the CBD extract in combination with polymyxin B was potent bactericidal agent whose mechanism of action was membrane lysis. In this study, treatment of CBD resistant strains of *S.* Typhimurium to CBD extract produced relatively denser bands than the control group as indicated in Figure 11. The densities of the bands designated as non-migrated bacterial DNA showed highest in the Control group, followed by the CBD extract treatment group, E34 TSP treatment group showed the lowest density of bands in the non-migrated bacterial DNA. This points to the possibility that E34 TSP caused the highest bacterial membrane lysis, thus genomic content was free to migrate out of the cells. As depicted in the Migrated Bacterial DNA, the E34 TSP treated samples also gave the highest densities compared to both the control group and the CBD extract treated samples. Another interesting observation was the presence of short DNA fragments. These fragments were due to possible endonuclease activity during the treatment administration. As can be observed in Figure 11, most of these fragments are centered on the E34 TSP treated lanes, indicating further the possibility of E34 TSP exerting a much higher lytic activity on *S.* Typhimurium than CBD. The inability of CBD extract to exert high lytic action on *S.* Typhimurium could be due in part to the resistance of these strains to CBD. We infer that, the resistance mechanism is possibly *via* membrane content modifications that enforced lesser susceptibility to CBD interactions. Furthermore, CBD has been demonstrated to show potent membrane disruption as its primary mechanism of attack (54). Intuitively, the same study also hinted for the first time how CBD deactivates the "urgent threat" pathogen *Neisseria gonorrhoeae*. Similarly, (55), reported how CBD extract was effective against *Neisseria gonorrhoeae*, *Neisseria meningitides*, *Moraxella catarrhalis*, and *Mycobacterium tuberculosis*. Their CBD formulation, however, was in combination with polymyxin B which is a known antibiotic that shows very potent membrane disruption activity (56). They stated that the activities were achieved at a polymyxin B concentration of ≤2 µg/ml and CBD concentration of ≤ 4 µg/ml, especially against *Klebsiella pneumonia*, *Escherichia coli*, and *Acinetobacter baumannii*. In our research work, we showed that CBD caused very slight membrane disruption compared to E34 TSP which caused a much higher membrane disruption (Figure 11). The lower membrane disruption of CBD extract might be attributable to the resistant strains used for the study.

In bacteria, respiratory chains are composed of various types of transport constituents, such as cytochromes, quinones, iron–sulfur proteins, and flavoproteins. The differential transport of protons and

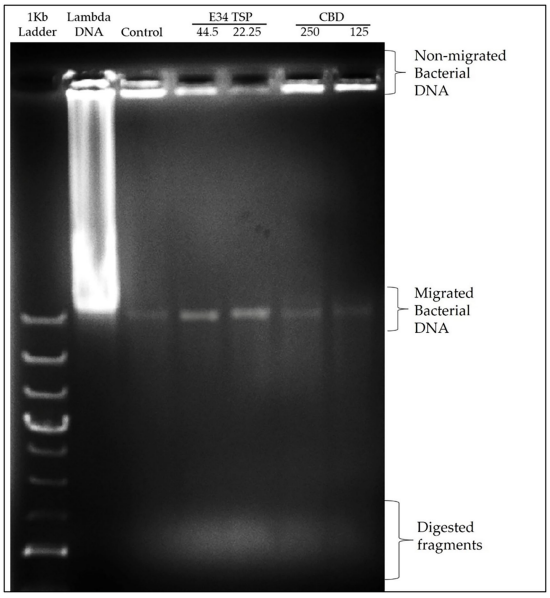


FIGURE 11
Agarose gel analysis of *S.* Typhimurium treated to CBD extract, or E34 TSP.

electrons through the cytoplasm leads to the formation of a proton gradient membrane. This membrane can be used to drive the formation of ATP through the F1/F0 ATPase (57–59). Electrons are transported through a variety of redox carriers to reach the oxygen-rich environment during respiration. They can also be obtained from alternative terminal electron acceptors when oxygen is unavailable (60). The glutamate dehydrogenase of *Salmonella* is one critical reductase that is modulated by various metabolites, which include among others the glycolytic, TCA cycle and related intermediates, such as PEP, pyruvate, acetyl CoA, citrate, etc. Another set of metabolites that actively regulate *Salmonella* glutamate dehydrogenase activity are the purine nucleotides (AMP, ADP, ATP, CAMP, GMP, GDP, and GTP). For instance, ATP is a potent activator of glutamate dehydrogenase (44). Thus, to assess the effect of CBD treatment to dehydrogenase of *Salmonella* spp. used in this study, we employed resazurin assay. As shown in Figure 12A, the effect of CBD on *S.* Typhimurium dehydrogenase activity seems to negatively correlate with the concentration of CBD at the 1 h time point whereas E34 TSP showed no significant difference in all doses. In general, 1 h posttreatment of *S.* Typhimurium with CBD showed far lower dehydrogenase activity than E34 TSP treatments of the same strain. This is unexpected since our previous results demonstrated that E34 TSP showed higher killing of *S.* Typhimurium than CBD (Figure 3). This might be explained in part by proposing that E34 TSP does not interact with dehydrogenase enzyme in *S.* Typhimurium whereas CBD does possibly interact with the enzyme. The concern, however, is why the less killing effect of CBD observed in *S.* Typhimurium than E34 TSP. To answer this, we infer that there might be a possible alternative dehydrogenase pathway utilized by *S.* Typhimurium in energy metabolism especially at the 1 h time point. In treating *S.* Newington however, E34 TSP treatments showed relatively lower dehydrogenase activity compared to the CBD treatment.

As shown in Figure 12B, treatment of *S.* Typhimurium and *S.* Newington showed a general decrease in dehydrogenase activity in 5 h. The most pronounced decrease however was observed in *S.* Newington treated to E34 TSP which demonstrated a complete inactivation of the enzyme at the 5 h time point. Interestingly, CBD treatment to the same strain produced slightly higher dehydrogenase activity as compared to the E34 TSP treatment. This trend is in consonance with the killing ability of E34 TSP on *S.* Newington than CBD does. All SDS controls showed the lowest activity indicative of dead bacteria. Comparing the 5 h treatment to 1 h treatment, all the samples showed significantly lower dehydrogenase activity at 5 h than 1 h time point. This is indicative of the killing ability of all the treatments.

To halt nosocomial infections, intensive research on antimicrobial surfaces is necessary. Currently, bacteriophages are emerging as alternative candidates for antimicrobial surfaces (61). Bacteriophages or their products can be selective in the destruction of bacteria since they are their natural enemies. More interesting is their host specificity which allows the bacteriophages avoid killing the human host's microbiota if used as therapy (22). In this study, E34 TSP has been demonstrated to be effective in killing two CBD-resistant strains of *Salmonella* (*S.* Typhimurium and *S.* Newington). Although much in-depth studies are required to ascertain both the pharmacodynamics and pharmacokinetics of E34 TSP, most phages or their products are self-limiting, and show quick clearance from the body (62), thus, if E34 TSP is formulated into a drug, it could be beneficial in treating most *Salmonella* infections. The added benefits of using the E34 phage tailspike protein instead of the whole bacteriophage is because most whole phages can elicit strong immune response and have narrow host range, therefore making it difficult to be used as antibacterial agents. In this work, these challenges are eliminated, since only the spike protein is used. The mechanisms of action of the E34 TSP include the binding of tailspike protein to bacterial surface proteins, e.g., the outermembrane protein A (ompA) as well as the LPS of the bacteria (38). This protein is responsible for processing through and hydrolyzing the LPS of the bacteria (63). We have demonstrated that the E34 TSP showed broader spectrum of activity since it showed antibacterial activity against *S.* Typhimurium which is not E34 phage's host. Future studies will include investigating the effectiveness of this protein in killing other bacterial strains unrelated to *Salmonella* to ascertain the spectrum of activity of the protein.

## 5. Conclusion

Given the rapid antimicrobial resistance development, it is crucial to look for other avenues to treat antimicrobial resistant bacteria. In our previous communication, we demonstrated the effectiveness of CBD as an antibacterial agent against *Salmonella* even at very low concentrations. We however observed that *Salmonella* developed resistance. In this work, we demonstrated that even strains that are resistant to the potent CBD could still be inhibited by a phage protein such as E34 TSP. Another important observation was the ability of the tailspike protein of E34 phage that is specific for only *Salmonella* Newington, showing antibacterial efficacy against *Salmonella* Typhimurium which is not

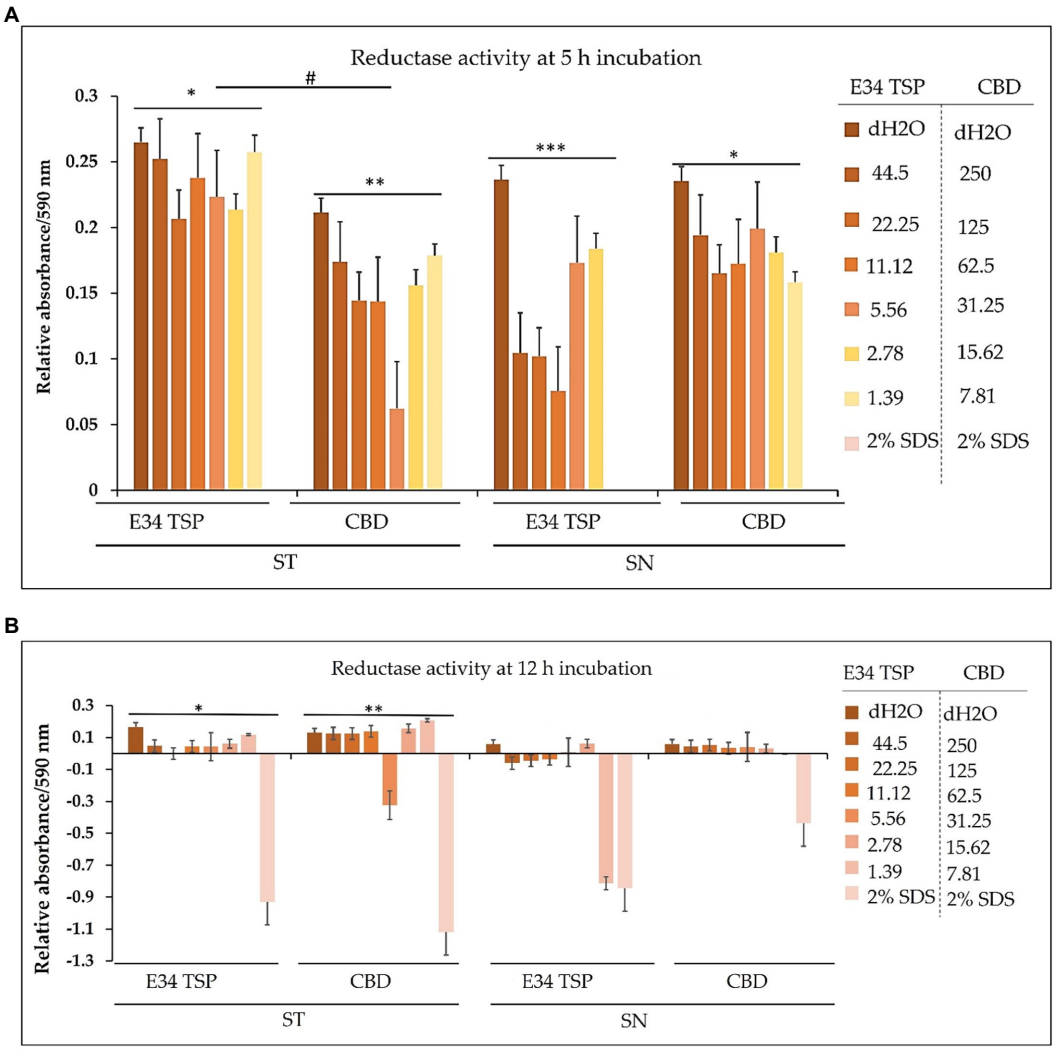


FIGURE 12
**(A)** Chart illustrating the *S.* Typhimurium and *S.* Newington dehydrogenase activity in 1h after treatment to varying concentrations of CBD extract or E34 TSP. Data shown are from three independent experiments expressed as means ± SEM. * and ** value of $ps \leq 0.0000024$, *** and ****value of $ps \leq 0.0032$, # $p$-value=0.0038. **(B)** Chart illustrating the *S.* Typhimurium and *S.* Newington dehydrogenase activities in 5h after treatment to varying concentrations of CBD extract, or E34 TSP. Data shown are from three independent experiments expressed as means ± SEM. * and ** $p$-values$\leq 0.000367$.

its host. This is an interesting finding especially for health application since, this protein has indicated it ability to be used against multiple *Salmonella* strains, possibly indicating its broad-spectrum property. We also showed that the inhibition of the bacteria by E34 TSP was due in part to membrane disruption, and dehydrogenase inactivation by the protein. A CBD-E34 TSP combination treatment resulted in lower killing ability of the treatment. This finding indicates that CBD might have had an unknown interaction with the protein which may cancel their individual or combine efficacy in killing the bacteria. Nonetheless, further research is needed to fully elucidate the mechanism of action of E34 TSP only, E34 TSP and CBD extract combination in the killing of *Salmonella*. Furthermore, it will be very interesting to explore the genetic basis of the resistance development of these two strains of bacteria to CBD. In conclusion, this work highlights the crucial role phage protein such as E34 TSP could play in pathogenic bacterial control.

## Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

## Author contributions

JA: conceptualization. II and JA: methodology, software, visualization, and writing–original draft preparation. JA, OA, and MS-F: validation and resources. JA, JX, MS-F, and OA: formal analysis. II, JA, AA, JX, MS-F, and OA: investigation. JA, JX, and OA: data curation. II, JA, JX, and OA: writing–review and editing. JA and OA: supervision. OA: project administration and funding acquisition. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## References

1. Liu, Y, Jing, SX, Luo, SH, and Li, SH. Non-volatile natural products in plant glandular trichomes: chemistry, biological activities and biosynthesis. *Nat Prod Rep*. (2019) 36:626–65. doi: 10.1039/C8NP00077H

2. Nuutinen, T. Medicinal properties of terpenes found in *Cannabis sativa* and *Humulus lupulus*. *Eur J Med Chem*. (2018) 157:198–228. doi: 10.1016/j.ejmech.2018.07.076

3. Seltzer, ES, Watters, AK, MacKenzie, D Jr, Granat, LM, and Zhang, D. Cannabidiol (CBD) as a promising anti-cancer drug. *Cancers*. (2020) 12:3203. doi: 10.3390/cancers12113203

4. Fernandes, MF, Chan, JZ, Hung, CCJ, Tomczewski, MV, and Duncan, RE. Effect of cannabidiol on apoptosis and cellular interferon and interferon-stimulated gene responses to the SARS-CoV-2 genes ORF8, ORF10 and M protein. *Life Sci*. (2022) 301:120624. doi: 10.1016/j.lfs.2022.120624

5. Janecki, M, Graczyk, M, Lewandowska, AA, and Pawlak, Ł. Anti-inflammatory and antiviral effects of cannabinoids in inhibiting and preventing SARS-CoV-2 infection. *Int J Mol Sci*. (2022) 23:4170. doi: 10.3390/ijms23084170

6. Liu, C, Puopolo, T, Li, H, Cai, A, Seeram, NP, and Ma, H. Identification of SARS-CoV-2 Main protease inhibitors from a library of minor cannabinoids by biochemical inhibition assay and surface Plasmon resonance characterized binding affinity. *Molecules*. (2022) 27:6127. doi: 10.3390/molecules27186127

7. McGrail, J, Martín-Banderas, L, and Durán-Lobato, M. Cannabinoids as emergent therapy against COVID-19. *Cannabis Cannabinoid Res*. (2022) 7:582–90. doi: 10.1089/can.2022.0018

8. Gildea, L, Ayariga, JA, Ajayi, OS, Xu, J, Villafane, R, and Samuel-Foo, M. *Cannabis sativa* CBD extract shows promising antibacterial activity against *Salmonella* Typhimurium and *S.* Newington. *Molecules*. (2022) 27:2669. doi: 10.3390/molecules27092669

9. Hussein, M, Allobawi, R, Levou, I, Blaskovich, MA, Rao, GG, Li, J, et al. Mechanisms underlying synergistic killing of Polymyxin B in combination with Cannabidiol against *Acinetobacter baumannii*: a metabolomic study. *Pharmaceutics*. (2022) 14:786. doi: 10.3390/pharmaceutics14040786

10. Ayariga, JA, Ibrahim, I, Gildea, L, Abugri, J, and Villafane, R. Microbiota in a long survival discourse with the human host. *Arch Microbiol*. (2023) 205:1–17. doi: 10.1007/s00203-022-03342-6

11. Ibrahim, I, Syamala, S, Ayariga, JA, Xu, J, Robertson, BK, Meenakshisundaram, S, et al. Modulatory effect of Gut microbiota on the Gut-brain, Gut-bone axes, and the impact of cannabinoids. *Meta*. (2022) 12:1247. doi: 10.3390/metabo12121247

12. Konieczka, P, Szkopek, D, Kinsner, M, Fotschki, B, Juśkiewicz, J, and Banach, J. *Cannabis*-derived cannabidiol and nanoselenium improve gut barrier function and affect bacterial enzyme activity in chickens subjected to *C. perfringens* challenge. *Vet Res*. (2020) 51:141–14. doi: 10.1186/s13567-020-00863-0

13. Qin, X, Yang, M, Cai, H, Liu, Y, Gorris, L, Aslam, MZ, et al. Antibiotic resistance of *Salmonella* Typhimurium monophasic variant 1, 4, [5], 12: i: -in China: a systematic review and meta-analysis. *Antibiotics*. (2022) 11:532. doi: 10.3390/antibiotics11040532

14. Gut, AM, Vasiljevic, T, Yeager, T, and Donkor, ON. *Salmonella* infection–prevention and treatment by antibiotics and probiotic yeasts: a review. *Microbiology*. (2018) 164:1327–44. doi: 10.1099/mic.0.000709

15. Balasubramanian, R, Im, J, Lee, JS, Jeon, HJ, Mogeni, OD, Kim, JH, et al. The global burden and epidemiology of invasive non-typhoidal *Salmonella* infections. *Hum Vaccin Immunother*. (2019) 15:1421–6. doi: 10.1080/21645515.2018.1504717

16. Rogers, AW, Tsolis, RM, and Bäumler, AJ. *Salmonella* versus the microbiome. *Microbiol Mol Biol Rev*. (2021) 85:e00027. doi: 10.1128/MMBR.00027-19

17. Huttner, A, Harbarth, S, Carlet, J, Cosgrove, S, Goossens, H, Holmes, A, et al. Antimicrobial resistance: a global view from the 2013 world healthcare-associated infections forum. *Antimicrob Resist Infect Control*. (2013) 2:31. doi: 10.1186/2047-2994-2-31

18. Laxminarayan, R, Duse, A, Wattal, C, Zaidi, AK, Wertheim, HF, Sumpradit, N, et al. Antibiotic resistance-the need for global solutions. *Lancet Infect Dis*. (2013) 13:1057–98. doi: 10.1016/S1473-3099(13)70318-9

19. O'Neill, J. *Tackling Drug-Resistant Infections Globally: Final Report and Recommendations*. London: Government of the United Kingdom (2016).

20. Murray, CJ, Ikuta, KS, Sharara, F, Swetschinski, L, Aguilar, GR, Gray, A, et al. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *Lancet*. (2022) 399:629–55. doi: 10.1016/S0140-6736(21)02724-0

21. Smith, R, and Coast, J. The true cost of antimicrobial resistance. *BMJ*. (2013) 346:f1493. doi: 10.1136/bmj.f1493

22. Scarpellini, E, Ianiro, G, Attili, F, Bassanelli, C, De Santis, A, and Gasbarrini, A. The human gut microbiota and virome: potential therapeutic implications. *Dig Liver Dis*. (2015) 47:1007–12. doi: 10.1016/j.dld.2015.07.008

23. Borysowski, J, Weber-Dąbrowska, B, and Górski, A. Bacteriophage endolysins as a novel class of antibacterial agents. *Exp Biol Med*. (2006) 231:366–77. doi: 10.1177/153537020623100402

24. Fischetti, VA. Bacteriophage lytic enzymes: novel anti-infectives. *Trends Microbiol*. (2005) 13:491–6. doi: 10.1016/j.tim.2005.08.007

25. López, R, García, E, and García, P. Enzymes for anti-infective therapy: phage lysins. *Drug Discov Today Ther Strateg*. (2004) 1:469–74. doi: 10.1016/j.ddstr.2004.09.002

26. Rahman, MU, Wang, W, Sun, Q, Shah, JA, Li, C, Sun, Y, et al. Endolysin, a promising solution against antimicrobial resistance. *Antibiotics*. (2021) 10:1277. doi: 10.3390/antibiotics10111277

27. Kim, J.. *Roles of Intermediate Conformation and Transient Disulfide bonding on Native Folding of P22 Tailspike Protein*. University of Delaware (2006), pp. 10–30. Available at: https://www.proquest.com/pagepdf/305324578?accountid=8243

28. Anantharaman, V, Koonin, EV, and Aravind, L. Regulatory potential, phyletic distribution and evolution of ancient, intracellular small-molecule-binding domains. *J Mol Biol*. (2001) 307:1271–92. doi: 10.1006/jmbi.2001.4508

29. Smith, KS, and Ferry, JG. Prokaryotic carbonic anhydrases. *FEMS Microbiol Rev*. (2000) 24:335–66. doi: 10.1111/j.1574-6976.2000.tb00546.x

30. Bradley, P, Cowen, L, Menke, M, King, J, and Berger, B. BETAWRAP: successful prediction of parallel β-helices from primary sequence reveals an association with many microbial pathogens. *Proc Natl Acad Sci*. (2001) 98:14819–24. doi: 10.1073/pnas.251267298

31. Junker, M, Schuster, CC, McDonnell, AV, Sorg, KA, Finn, MC, Berger, B, et al. Pertactin β-helix folding mechanism suggests common themes for the secretion and folding of autotransporter proteins. *Proc Natl Acad Sci*. (2006) 103:4918–23. doi: 10.1073/pnas.0507923103

32. Reithofer, V, Fernández-Pereira, J, Alvarado, M, de Groot, P, and Essen, LO. A novel class of Candida glabrata cell wall proteins with β-helix fold mediates adhesion in clinical isolates. *PLoS Pathogens*, (2021) 17:e1009980. doi: 10.1371/journal.ppat.1009980

33. Ayariga, JA, Abugri, DA, Amrutha, B, and Villafane, R. Capsaicin potently blocks *Salmonella* Typhimurium invasion of Vero cells. *Antibiotics*. (2022) 11:666. doi: 10.3390/antibiotics11050666

34. Valizadehderakhshan, M, Kazem-Rostami, M, Shahbazi, A, Azami, M, Bhowmik, A, and Wang, L. Refining Cannabidiol using wiped-film molecular distillation: experimentation, process modeling, and prediction. *Ind Eng Chem Res*. (2022) 61:6628–39. doi: 10.1021/acs.iecr.2c00290

35. Wassmann, CS, Rolsted, AP, Lyngsie, MC, Torres-Puig, S, Kronborg, T, Vestergaard, M, et al. The menaquinone pathway is important for susceptibility of Staphylococcus aureus to the antibiotic adjuvant, cannabidiol. *Microbiol Res*. (2022) 257:126974. doi: 10.1016/j.micres.2022.126974

36. Zhou, S., Cui, Z., and Urban, J.. *Dead Cell Counts during Serum Cultivation are Underestimated by the Fluorescent Live/Dead Assay*. *6*, Weinheim: WILEY-VCH Verlag, (2011) pp. 513–518.

37. Alonso, B, Cruces, R, Pérez, A, Sánchez-Carrillo, C, and Guembe, M. Comparison of the XTT and resazurin assays for quantification of the metabolic activity of Staphylococcus aureus biofilm. *J Microbiol Methods*. (2017) 139:135–7. doi: 10.1016/j.mimet.2017.06.004

38. Ayariga, JA, Gildea, L, Wu, H, and Villafane, R. The E34 phage Tailspike protein: an in vitro characterization, structure prediction, potential interaction with *S*. Newington LPS and cytotoxicity assessment to animal cell line. *J Clin Trials*. (2021) 14:2. doi: 10.1101/2021.09.20.461090

39. Guan, L, Ehrmann, M, Yoneyama, H, and Nakae, T. Membrane topology of the xenobiotic-exporting subunit, MexB, of the MexA, B-OprM extrusion pump in Pseudomonas aeruginosa. *J Biological Chemistry*, (1999) 274:10517–22. doi: 10.1074/jbc.274.15.10517

40. Villafane, R, Zayas, M, Gilcrease, EB, Kropinski, AM, and Casjens, SR. Genomic analysis of bacteriophage ε34 of Salmonella entericaserovar Anatum (15+). *BMC microbiology*, (2008) 8:1–13. doi: 10.1186/1471-2180-8-227

41. Goldenberg, D, and King, J. Trimeric intermediate in the in vivo folding and subunit assembly of the tail spike endorhamnosidase of bacteriophage P22. *Proceedings of the National Academy of Sciences*, (1982) 79:3403–7. doi: 10.1073/pnas.79.11.340

42. Freiberg, C, and Brötz-Oesterhelt, H. Functional genomics in antibacterial drug discovery. *Drug Disc. Today*, (2005) 10:927–35. doi: 10.1016/S1359-6446(05)03474-4340

43. Williams, J, Venkatesan, K, Ayariga, A, Jackson, D, Wu, H, and Villafane, R. A genetic analysis of an important hydrophobic interaction at the P22 tailspike protein N-terminal domain. *Archiv. Virology*. (2018) 163:1623–33. doi: 10.1007/s00705-018-3777-y

44. Coulton, JW, and Kapoor, M. Studies on the kinetics and regulation of glutamate dehydrogenase of *Salmonella* Typhimurium. *Can J Microbiol*. (1973) 19:439–50. doi: 10.1139/m73-072

45. Barbu, EM, Cady, KC, and Hubby, B. Phage therapy in the era of synthetic biology. *Cold Spring Harb Perspect Biol*. (2016) 8:a023879. doi: 10.1101/cshperspect.a023879

46. Tagliaferri, TL, Jansen, M, and Horz, HP. Fighting pathogenic bacteria on two fronts: phages and antibiotics as combined strategy. *Front Cell Infect Microbiol*. (2019) 9:22. doi: 10.3389/fcimb.2019.00022

47. Gildea, L, Ayariga, J, Xu, J, Villafane, R, Boakai, R, Samuel-Foo, M, et al. *Cannabis sativa* CBD extract exhibits synergy with broad-Spectrum antibiotics against *Salmonella* Typhimurium. *Preprints*. (2022):2022090143. doi: 10.20944/preprints202209.0143.v1

48. Wassmann, CS, Højrup, P, and Klitgaard, JK. Cannabidiol is an effective helper compound in combination with bacitracin to kill gram-positive bacteria. *Sci Rep*. (2020) 10:1–12. doi: 10.1038/s41598-020-60952-0

49. Bulkley, D, Johnson, F, and Steitz, TA. The antibiotic thermorubin inhibits protein synthesis by binding to inter-subunit bridge B2a of the ribosome. *J Mol Biol*. (2012) 416:571–8. doi: 10.1016/j.jmb.2011.12.055

50. Bilardi, RA, Kimura, KI, Phillips, DR, and Cutts, SM. Processing of anthracycline-DNA adducts via DNA replication and interstrand crosslink repair pathways. *Biochem Pharmacol*. (2012) 83:1241–50. doi: 10.1016/j.bcp.2012.01.029

51. Borovinskaya, MA, Shoji, S, Holton, JM, Fredrick, K, and Cate, JH. A steric block in translation caused by the antibiotic spectinomycin. *ACS Chem Biol*. (2007) 2:545–52. doi: 10.1021/cb700100n

52. Horstmann, JC, Laric, A, Boese, A, Yildiz, D, Röhrig, T, Empting, M, et al. Transferring microclusters of *P. aeruginosa* biofilms to the air–liquid Interface of bronchial epithelial cells for repeated deposition of aerosolized tobramycin. *ACS Infect Dis*. (2021) 8:137–49. doi: 10.1021/acsinfecdis.1c00444

53. Kosgodage, US, Matewele, P, Awamaria, B, Kraev, I, Warde, P, et al. Cannabidiol is a novel modulator of bacterial membrane vesicles. *Front Cell Infect Microbiol*. (2019) 9:324. doi: 10.3389/fcimb.2019.00324

54. Blaskovich, MA, Kavanagh, AM, Elliott, AG, Zhang, B, Ramu, S, Amado, M, et al. The antimicrobial potential of cannabidiol. *Commun Biol*. (2021) 4:1–18. doi: 10.1038/s42003-020-01530-y

55. Abichabki, N, Zacharias, LV, Moreira, NC, Bellissimo-Rodrigues, F, Moreira, FL, Benzi, JR, et al. Potential cannabidiol (CBD) repurposing as antibacterial and promising therapy of CBD plus polymyxin B (PB) against PB-resistant gram-negative bacilli. *Sci Rep*. (2022) 12:1–15. doi: 10.1038/s41598-022-10393-8

56. Grau-Campistany, A, Manresa, Á, Pujol, M, Rabanal, F, and Cajal, Y. Tryptophan-containing lipopeptide antibiotics derived from polymyxin B with activity against gram positive and gram-negative bacteria. *Biochim Biophys Acta*. (2016) 1858:333–43. doi: 10.1016/j.bbamem.2015.11.011

57. Ingledew, WJ, and Poole, RK. The respiratory chains of Escherichia coli. *Microbiol Rev*. (1984) 48:222–71. doi: 10.1128/mr.48.3.222-271.1984

58. Russell, JB. The energy spilling reactions of bacteria and other organisms. *Microb Physiol*. (2007) 13:1–11. doi: 10.1159/000103591

59. Syroeshkin, AV, Bakeeva, LE, and Cherepanov, DA. Contraction transitions of F1-F0 ATPase during catalytic turnover. *Biochim Biophys Acta*. (1998) 1409:59–71. doi: 10.1016/S0005-2728(98)00150-9

60. Tielens, AG, and Van Hellemond, JJ. The electron transport chain in anaerobically functioning eukaryotes. *Biochim Biophys Acta*. (1998) 1365:71–8. doi: 10.1016/S0005-2728(98)00045-0

61. Page, K, Wilson, M, and Parkin, IP. Antimicrobial surfaces and their potential in reducing the role of the inanimate environment in the incidence of hospital-acquired infections. *J Materials Chemistry*. (2009) 19:3819–31. doi: 10.1039/B818698G

62. Hosseinidoust, Z, Van de Ven, TG, and Tufenkji, N. Bacterial capture efficiency and antimicrobial activity of phage-functionalized model surfaces. *Langmuir*. (2011) 27:5472–80. doi: 10.1021/la200102z

60. Zayas, MV, and Villafane, R. The tailspike protein of the Salmonella phage [epsilon] 34. *FASEB J*. (2007) 21:A979. doi: 10.1096/fasebj.21.6.A979-c

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# Characterization and genomic study of EJP2, a novel jumbo phage targeting antimicrobial resistant *Escherichia coli*

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The emergence of antimicrobial resistance (AMR) *Escherichia coli* has noticeably increased in recent years worldwide and causes serious public health concerns. As alternatives to antibiotics, bacteriophages are regarded as promising antimicrobial agents. In this study, we isolated and characterized a novel jumbo phage EJP2 that specifically targets AMR *E. coli* strains. EJP2 belonged to the *Myoviridae* family with an icosahedral head (120.9±2.9 nm) and a non-contractile tail (111.1±0.6 nm), and contained 349,185bp double-stranded DNA genome with 540 putative ORFs, suggesting that EJP2 could be classified as jumbo phage. The functions of genes identified in EJP2 genome were mainly related to nucleotide metabolism, DNA replication, and recombination. Comparative genomic analysis revealed that EJP2 was categorized in the group of Rak2-related virus and presented low sequence similarity at the nucleotide and amino acid level compared to other *E. coli* jumbo phages. EJP2 had a broad host spectrum against AMR *E. coli* as well as pathogenic *E. coli* and recognized LPS as a receptor for infection. Moreover, EJP2 treatment could remove over 80% of AMR *E. coli* biofilms on 96-well polystyrene, and exhibit synergistic antimicrobial activity with cefotaxime against AMR *E. coli*. These results suggest that jumbo phage EJP2 could be used as a potential biocontrol agent to combat the AMR issue in food processing and clinical environments.

KEYWORDS

**bacteriophage, jumbo phage, antimicrobial resistance, *Escherichia coli*, antimicrobial agent, genome and phylogenetic analysis**

## Introduction

*Escherichia coli* is a main opportunistic pathogen that commonly colonizes in the gastrointestinal tract of both animals and humans (Kim et al., 2021), causing a range of intestinal and extra-intestinal disease. Various antibiotics have been applied orally or via injection to control this bacteria, but *E. coli* species have represented a capability to easily acquire the resistance genes by horizontal gene transfer (Poirel et al., 2018). Antimicrobial resistance (AMR) *E. coli*, such as Extended-Spectrum Beta-Lactamase (ESBL)-producing *E. coli*, exhibit a wide spectrum of resistance against all $\beta$-lactam antibiotics and other class of antibiotics such as fluoroquinolones, aminoglycosides (Coque et al., 2008; Dd Pitout, 2013). The emergence and prevalence of AMR in *E. coli* has become serious public health threats (Roca et al., 2015) and a number of studies have reported the isolation of AMR *E. coli* from patients, animals, and food

chain (Rupp and Fey, 2003; Poirel et al., 2018; Park et al., 2019; Martak et al., 2022). Hence, World Health Organization (WHO) declared that AMR *E. coli* is one of the most urgent strains for which novel antimicrobials are needed (Shrivastava et al., 2018). Currently, the use of antibiotics is limited due to rapid acquisition of AMR in *E. coli* (Zurfuh et al., 2016; Parvez and Khan, 2018; Wu et al., 2018), so the development of alternative antimicrobial agents is necessary to address these concerns.

Bacteriophages (phages) are viruses that infect specific bacterial species and are the most abundant biological entities on earth (Bragg et al., 2014). In comparison to conventional antibiotics, phages have several advantages for use as alternative antimicrobial agents, such as harmlessness to human and commensal bacteria, host specificity, and low cost for production compared to development of novel antibiotics (Loc-Carrillo and Abedon, 2011). Phages are classified into two categories based on their life cycles; virulent and temperate phage (Kutter and Sulakvelidze, 2004) and the use of virulent phage is considered as a suitable strategy to control pathogens (Hassan et al., 2021). After the phage products, Listshield (Intralytix, Inc., Baltimore, MD, United States), was firstly approved by U.S. Food and Drug Administration (FDA) in 2006, a variety of phage products have commodified to prevent *E. coli* infection (Huang et al., 2022). *E. coli* phages (coliphages) are commonly isolated from environment. Although many studies of coliphages in terms of genetics and molecular biology have provided insights into the phage biology (Kutter and Sulakvelidze, 2004), but what we know about phage is just the tip of the iceberg.

Phages with a genome size from 200 to 500 kb are classified as jumbo phages (Yuan and Gao, 2017). There are over 22,000 registered phages in the NCBI database, and out of these, 581 phages can be classified as jumbo phages. Jumbo phages have distinct characteristics compared to phage with genome size under 200 kb. Nazir et al. (2021) reported that most jumbo phages morphologically represent head and tail sizes of over 100 nm (Nazir et al., 2021). Jumbo phages possess genomes that contain numerous genes associated with genome replication, modification and nucleotide metabolism, enabling them to replicate independently from the host. Genes with similar function in jumbo phage genomes are typically dispersed or organized into sub-clusters throughout the genome (Ceyssens et al., 2014; Guan and Bondy-Denomy, 2020). Transcription of early phage genes is commonly regulated by phage-encoded RNA polymerases (RNAPs) (Mesyanzhinov et al., 2002; Leskinen et al., 2016; Imam et al., 2019), and non-virion RANP is responsible for transcription of middle or late phage genes (Orekhova et al., 2019). In addition, jumbo phages possess a range of tRNAs and aminoacyl-tRNA synthetases, which can replace cleaved host tRNA to maintain translation of viral proteins. Iyer et al. (2021) analyzed that the number of tRNA genes in jumbo phages ranges from 4.5 to 22 per genome. The abundance of transcription and translation-related genes enables the post-infection development of jumbo phages, which implies a high level of independence from the host molecular machinery (Ceyssens et al., 2014; Lavysh et al., 2016). This independence appears to confer broad host ranges to jumbo phages, as in the case of *Xanthomonas citri* jumbo phage XacN1 (Yoshikawa et al., 2018).

In this study, we isolated and characterized *E. coli* jumbo phage EJP2, which has a genome size of 349,185 bp (accession no. OQ411014) with low sequence homology to the other jumbo phages currently known. We investigated its biological and genomic features of EJP2, and revealed that EJP2 recognized LPS as a phage receptor. EJP2 presented a broad host range, biofilm removal activity and exhibited synergistic antimicrobial efficacy against AMR *E. coli* when used in combination with cefotaxime (CTX) treatment. These results would help to expand our knowledge of jumbo phages and suggest the potential of EJP2 as alternative antimicrobial agents for biocontrol of AMR *E. coli*.

# Materials and methods

## Bacterial strains, plasmids and growth conditions

The bacterial strains used in this study are listed in Table 1. All bacteria were grown in Luria-Bertani (LB) broth and agar plates at 30°C and/or 37°C supplemented with appropriate antibiotics:

TABLE 1 Bacterial strains and plasmids used in this study.

| Strain and plasmid | Genotype and main characteristics[a] | Reference |
|---|---|---|
| *Escherichia coli* | | |
| DH5α *λpir* | ΦΔM15 Δ(*lacZYA-argF*) U169 *recA1 hsdR17 thi-1 supE44 gyrA96 relA1/λpir* | Platt et al. (2000) |
| FORC82 | Host for phages | This study |
| PS01 | *β*-lactam antibiotics sensitive mutant of FORC82 | This study |
| PS01 + pUHE | PS01 + pUHE21-2 *lacI*$^{q}$ | This study |
| PRS07 | PS01 *waaR*::Tn5 | This study |
| PRS07 + pUHE | PRS07 + pUHE21-2 *lacI*$^{q}$ | This study |
| PRS07 + pFORC82_*waaR* | PRS07 + pUHE21-2 *lacI*$^{q}$::*waaR* | This study |
| AMR *E. coli* isolate 62 | | Kim et al. (2021) |
| Plasmids | | |
| pUHE21-2*lacI*$^{q}$ | rep$_{pMB1}$ *lacI*$^{q}$; inducible Lac promoter; AmpR | Soncini et al. (1995) |
| pFORC82_*waaR* | pUHE21-2 *lacI*$^{q}$::*waaR* | This study |
| pKD13 | *oriRγR6k bla FRT::kan::FRT*;KanR | Datsenko and Wanner (2000) |

[a]Amp$^{R}$, ampicillin resistant; Kan$^{R}$, kanamycin resistant.

ampicillin (Amp), 50 μg/ml; carbenicillin (Car), 100 μg/ml; kanamycin (Kan), 50 μg/ml; acridine orange (AO), 100 μg/ml, while isopropyl β-D-1-thiogalactopyranoside (IPTG) was added at a concentration of 50 or 100 μM.

## Bacteriophage isolation and propagation

Animal fecal samples were collected in Seoul, South Korea. Phage isolation was conducted as previously described with some modifications (Saad et al., 2019). Sample was homogenized with 50 ml of sodium chloride-magnesium sulfate (SM) buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl and 8 mM $MgSO_4 \cdot 7H_2O$). Large particles were excluded by centrifuge (5,000 × *g*, 10 min, 4°C) and chloroform was added to supernatant for removal of residual bacteria. After centrifugation, supernatants were discarded to remove small phages and the pellet was suspended with 5 ml of SM buffer. This process was repeated three times. The resultant lysate was spotted on the bacterial lawn containing host cell. Briefly, 100 μl of cultured host cell (*E. coli* FORC82) was inoculated into 5 ml of LB soft agar [0.3% (supplemented with appropriate antibiotic and IPTG, if necessary)]. Mixture was poured on LB agar plates and solidified for 30 min. Ten microliters of serially diluted (10-fold) phage lysates were spotted on the bacterial lawn and dried for 20 min at room temperature. The plates were incubated at 30°C for at least 12 h to obtain single plaques. Small single plaques were picked with the sterile tip and eluted in 250 μl of SM buffer for further purification. This purification step was repeated at least three times. For phage propagation, host strain was incubated at 30°C for 2 h 30 min. The phage lysate was added into bacterial culture at a multiplicity of infection (MOI) of 1 and incubated for 4 h. The propagated phages were precipitated with polyethylene glycol (PEG) 6,000 and concentrated by CsCl density gradient ultracentrifugation (78,500 × *g*, 2 h, 4°C) (Kim H. et al., 2019).

## Morphological analysis by TEM

Each purified phage stock dilutions (4 μl, approximately $10^9$ PFU/mL) were placed on carbon-coated copper grids for 60 s and the excess phage was removed with filter paper. Equal volume of 2% aqueous uranyl acetate (pH 4.0) were added for 90 s to negatively stain the phage particles. Phages were examined by transmission electron microscopy (TEM; LEO 912AB transmission electron microscope; Carl Zeiss, Wezlar, Germany) at a 120-kV accelerating voltage, and images were scanned at the National Instrumentation Center for Environmental Management (Seoul, South Korea). Phage were morphologically classified according to the guidelines of the International Committee on Taxonomy of Viruses (ICTV) (Walker et al., 2022).

## Bacteriophage spot assay

The bacterial lawn was prepared as described by (Kim and Ryu, 2011). Briefly, 200 μl of cultured host cells was inoculated into 5 ml of LB soft agar [0.3% agar (supplemented with the appropriate antibiotic and IPTG, if necessary)]. This mixture was poured onto LB agar plates and solidified for 30 min. Ten microliters of serially diluted (10-fold) phage lysates were spotted on the bacterial lawn and dried at room temperature for 20 min. The plates were incubated for 12 h at 30°C, and the phage plaques were monitored.

## Bacterial challenge assays

One milliliter of LB broth was inoculated with overnight culture of *E. coli* FORC82. Bacterial cultures were incubated at 30 and 37°C for 2 h and infected with phage EJP2 at a MOI of 1. Optical density at 600 nm was measured every 1 h after phage infection until 12 h. SM buffer was added as a negative control. The experiments were conducted in triplicate.

## Sequencing of phage DNA and bioinformatics analysis

Phage genomic DNA was extracted by phenol-chloroform method as previously described (Sambrook and Russell, 2001). The purified phage DNA was sequenced using the Illumina Miseq platform (Illumina, San Diego, CA, United States) and assembled with the SPAdeS v.3.13.0 (Bankevich et al., 2012) at Sanigen Inc., South Korea. The ORFs were predicted by using Glimmer3 (Delcher et al., 2007), GeneMarkS (Besemer et al., 2001), and RAST annotation server[1] (Aziz et al., 2008; Overbeek et al., 2014). The annotated data were assorted and arranged by using Artemis (Carver et al., 2008). The tRNA sequence in the phage genome were analyzed by tRNAscan-SE program (Lowe and Eddy, 1997). The functions of phage proteins were predicted by using NCBI BLASTp and InterProscan program (Altschul et al., 1990; Jones et al., 2014). Sequence alignment among EJP2 and other phages was performed using ClustalW (Thompson et al., 1994) using amino acid sequences of phage terminase large subunit (TerL), major capsid protein (MCP) and portal vertex protein. Relationships among the phage genome sequences were inferred using neighbor-joining method (Saitou and Nei, 1987) and the phylogenetic tree was constructed using MEGA-X v10.0.5 (Kumar et al., 2018). The bootstrap value of 5,000 replicates represented the evolutionary history of the analyzed taxa (Felsenstein, 1985). The evolutionary distances were represented using p-distance method (Nei and Kumar, 2000). Dot plot analysis was conducted using Gepard v1.40 (Krumsiek et al., 2007).

## Acridine orange and PNA treatment for curing the plasmids

Acridine orange (AO) was treated to *E. coli* FORC82 in order to cure the plasmids of *E. coli* FORC82 (Hirota, 1960). An overnight culture of *E. coli* FORC82 was sub-cultured to 3 ml of fresh LB broth with AO (100 μg/ml) and incubated at 37°C for 24 h. This process was repeated five times and serially diluted (10-fold) bacterial cells were streaked on LB agar plate. Plasmid curing was confirmed by colony PCR.

1 https://rast.nmpdr.org/

To cure pFORC82_1 which harbors a gene encoding ESBL from *E. coli* FORC82 cells, we used two peptide nucleic acids (PNAs, RepE-PNA1 and RepE-PNA2), which are composed of complementary sequences including predicted ribosome binding site and start codon, respectively, against replication initiation protein E of pFORC82_1 (Panagene, Daejeon, South Korea) (Table 2). All PNAs were covalently conjugated with peptide KFFKFFKFFK to improve cell-penetrating efficiency (Good et al., 2001). *E. coli* FORC82 cells were harvested at early log phase and diluted to $10^5$ CFU/ml. The 20 μM RepE-PNA2 or the combination of PNA2 and Amp (50 μg/ml) were added to bacterial cell culture. The optical density at 600 nm of bacterial cell culture was measured every 15 min up to 7 h. The PNAs-treated cells were plated on the LB/Amp plates. Several colonies resuspended with 20 μl of PBS were spotted on LB and LB/Amp agar plate to distinguish the sensitivity against Amp. We selected clone which was sensitive to Amp and named the strain as *E. coli* PS01.

## Construction of Tn5 transposon mutant library and screening phage resistance mutants

A Tn5 transposon mutant library was generated using the EZ-Tn5 kit as described by the manufacturer (Lucigen®, Middleton, WI, United States) with some modifications. Briefly, kanamycin resistance gene in plasmid pKD13 was amplified by polymerase chain reaction (PCR), wherein DNA fragment had inverted repeat sequence on both end (CTGTCTCTTATACACATCT). Purified DNA fragment (approximately 250 ng/μL) was mixed with 100% glycerol and EZ-Tn5 transposase and the mixture was incubated for 30 min at room temperature to construct transposome. One microliter of transposome was transformed by electroporation to *E. coli* PS01. To screen the phage-resistant clone, pools of transformants were mixed with EJP2 (approximately $10^9$ PFU/mL) and incubated at 30°C for 30 min and the mixture was spread on LB/Kan agar plate. Surviving colonies were isolated by streaking three times on LB/Kan agar to remove the effect of remaining phages. Phage resistance against EJP2 was confirmed by spot assay. The locus of transposon insertion was identified by whole genome sequencing (WGS) using Illumina NextSeq platform (Illumina, San Diego, CA, United States). The sequenced DNA fragments were assembled using CLC Genomics Workbench 9.0.

## Plasmid construction

The plasmids and oligonucleotides used in this study are listed in Table 1 and Table 3. Plasmid pFORC82_*waaR* (pUHE21-2 *lacI*$^q$::*waaR*), which expresses *FORC82_0109* gene (putative LPS α-1,2 glycosyltransferase), was constructed using isothermal assembly with the two DNA fragments; a PCR amplified linearized pUHE21-2 *lacI*$^q$ plasmid, and a PCR amplified *FORC82_0109* gene. The two DNA fragments had overlapped sequences (20 bp) with each other (Gibson et al., 2009) and were inserted into the pFORC82_0109 by incubating at 50°C for 1 h in reaction buffer (25% PEG-8000, 500 nM Tris–HCl [pH 7.5], 50 mM MgCl2, 50 mM DTT, 1 mM of dNTPs, and 5 mM Nicotinamide adenine dinucleotide). The assembled plasmid was transformed to *E. coli* PS01 strain for complementation test.

## Biofilm inhibition assay

Overnight cultures of *E. coli* FORC82 and other AMR *E. coli* strains were diluted 1:100 in 2 ml of fresh LB broth. EJP2 was added at MOI 0.1 or 1 in each well of a 96-well polystyrene plate containing *E. coli* FORC82 and incubated at 25°C without shaking for 48 h. Two hundred microliter of LB broth was used as a negative control. After incubation, biofilm staining was conducted as described by Cha et al. (2019) with some modifications. All wells were washed with PBS three times to remove vegetable cells. Biofilm was fixed with 95% methanol for 15 min and stained by 0.1% crystal violet (CV) for 30 min. Each well was washed with PBS to remove the residual CV. All wells were filled with 200 μl of 33% glacial acetic acid and incubated for 45 min at RT for dissolution of biofilm. The optical density at 570 nm of each well was measured.

## Phage-antibiotic synergy

EJP2 phage was treated with cefotaxime (CTX) to investigate synergistic effect with antibiotics. Overnight-cultured *E. coli* FORC82 cells was sub-cultured 1:100 into fresh LB broth. EJP2 was treated to bacterial culture ($5 \times 10^6$ CFU/ml) at a MOI of 1 with or without sublethal concentration of CTX (64 μg/ml). Mixtures were incubated at 30°C for 12 h. *E. coli* FORC82 cells were obtained every 1 h up to 3 h and diluted (10-fold) bacterial cells were plated on LB plates. The

TABLE 2 Oligonucleotides used in this study.

| Oligonucleotide | Sequence (5′ to 3′) | Purpose |
|---|---|---|
| Tn_pKD13_F | CTG TCT CTT ATA CAC ATC TTG TAG GCT GGA GCT TCG | Gene (*kanR*) amplification |
| Tn_pKD13_R | CTG TCT CTT ATA CAC ATC TCT GTC AAA CAT GAG AAT TAA TTC C | Gene (*kanR*) amplification |
| PRS07_confirm_F | TGG CAT GAA GCA AAT TTG ACA C | Sequence confirmation |
| PRS07_confirm_R | GAA GTT ATG CCT TTT ATA TAC TCA C | Sequence confirmation |
| pUHE21-2_F | GGA TCC TCT CAT AGT TAA TTT CT | Plasmid construction |
| pUHE21-2_R | AAG CTT AAT TAG CTG AGC TTG G | Plasmid construction |
| *waaR*_comple_F | AGA AAT TAA CTA TGA GAG GAT CCA TGA ATG AAT TTA TAA AAG AAC GGT TTT | Plasmid construction |
| *waaR*_comple_R | CCA AGC TCA GCT AAT TAA GCT TTT ATT TCT TAA GCT TGT ACT TAA TTA ATG | Plasmid construction |

TABLE 3 Peptide nucleic acids used in this study.

| PNA | Sequence (N->C) |
|---|---|
| RepE-PNA1 | KFFKFFKFFK-TCT GCT TAC CAG |
| RepE-PNA2 | KFFKFFKFFK-CAA AGG CCT TAC |

number of *E. coli* FORC82 cells was determined by counting the number of colonies on LB plates. The bacterial culture treated with LB broth was used as negative control.

## Statistical analysis

GraphPad Prism 5 software was used for statistical analysis. Statistical analysis was conducted by one-way analysis of variance (ANOVA) with Tukey's multiple comparison tests among the experimental groups (95% confidence interval). The significant differences among the experimental groups are marked with asterisks. $p < 0.05$ (*), or $p < 0.001$ (***).

# Results and discussion

## Isolation and morphological analysis of jumbo phage EJP2

The novel *E. coli* phage EJP2 was isolated from animal feces without filtration (Supplementary Figure S1). EJP2 could infect AMR *E. coli* FORC82 strain that contains *mcr-1*-harboring plasmid (Kim J. et al., 2019) and formed small clear plaques on LB soft agar (0.7%) over the bacterial lawn of *E. coli* FORC82. EJP2 plaques were rarely visible on a high concentration of LB soft agar (0.7%) (Figure 1A), indicating that EJP2 exhibits characteristic in common with *Escherichia* jumbo phages, in which plaque size decreased as the agar concentration increased (Saad et al., 2019).

Of over 220 jumbo phages reported to date, more than over hundred phages were classified as *Myoviridae* family (Yuan and Gao, 2017). The morphological analysis using transmission electron microscopy (TEM) demonstrated that phage EJP2 belongs to the *Myoviridae* family. Both the diameter of icosahedral head (120.9 ± 2.9 nm) and the length of contractile tail (111.1 ± 0.6 nm) were over 100 nm (Figure 1B), suggesting that EJP2 has a similar morphological feature compared to large virions classified as jumbo phages.

## Bacterial growth inhibition efficacy of EJP2

Sixty-seven AMR *E. coli* isolates, pathogenic *E. coli* strains, and other Gram-negative strains were used to determine the host range of EJP2. Among 67 AMR *E. coli* strains, which were classified by the Clermont phylotyping (Clermont et al., 2013), EJP2 inhibited the growth of 33 AMR *E. coli* strains in phylogroup A (19/38), B1 (9/13), B2 (2/4), D (2/4), and E (1/6) (Figure 2A). This result indicates that EJP2 showed different host spectrum compared to those of five JEP coliphages reported by Kim et al. (2021). EJP2 showed a broader host range than those of JEP1, JEP6, JEP7 and JEP8 phages. JEP4 phage could infect AMR *E. coli* strain in phylogroup A (28/38) and D (3/4) (Kim et al., 2021), but EJP2 showed a broader inhibition spectrum against major phylogroups of AMR *E. coli* (A, B1, B2, D, and E) than JEP4 phage. EJP2 was also capable of forming plaques against Enterohemorrhagic *E. coli* (EHEC), Enterotoxigenic *E. coli* (ETEC), Enteroaggregative *E. coli* (EAEC), and *Shigella flexneri* (Figure 2B). In bacterial challenge assay, EJP2 could retard the growth of *E. coli* FORC82 4 h after infection at 30°C and 2 h after infection at 37°C (Supplementary Figure S2). The broad host spectrum of EJP2 implies its potential utility as biocontrol agents in clinical or food applications.

## Genomic and phylogenetic analysis of EJP2

Genomic characteristics of phage EJP2 were identified through whole-genome analysis. EJP2 possesses a 349,185 bp circular double-stranded DNA genome with average G + C content 37% and was thus

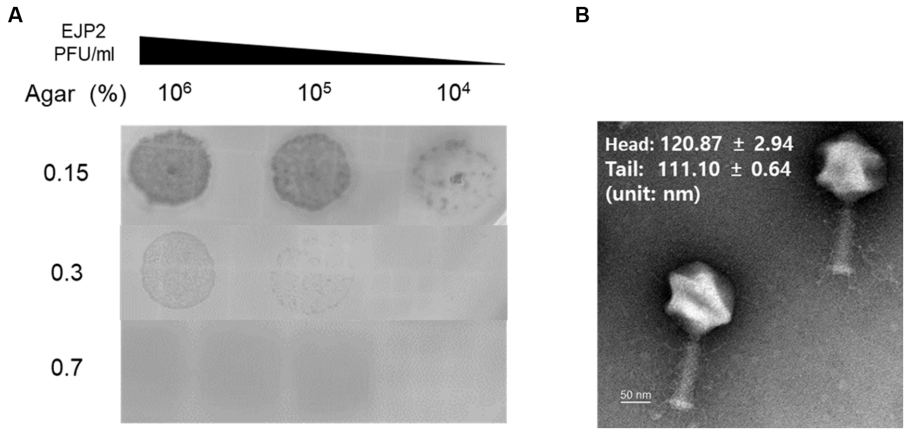


FIGURE 1
**(A)** Comparison of plaque size of EJP2 depending on the concentration of agar in LB soft agar. The concentrations of agar are shown on the left side of **(A)**. The larger plaques of EJP2 were observed at low agar concentration. **(B)** Transmission electron micrographs of phage EJP2. Phage EJP2 particles were negatively stained with 2% aqueous uranyl acetate (pH 4.0). EJP2 belongs to the *Myoviridae* family. Bar, 50nm.

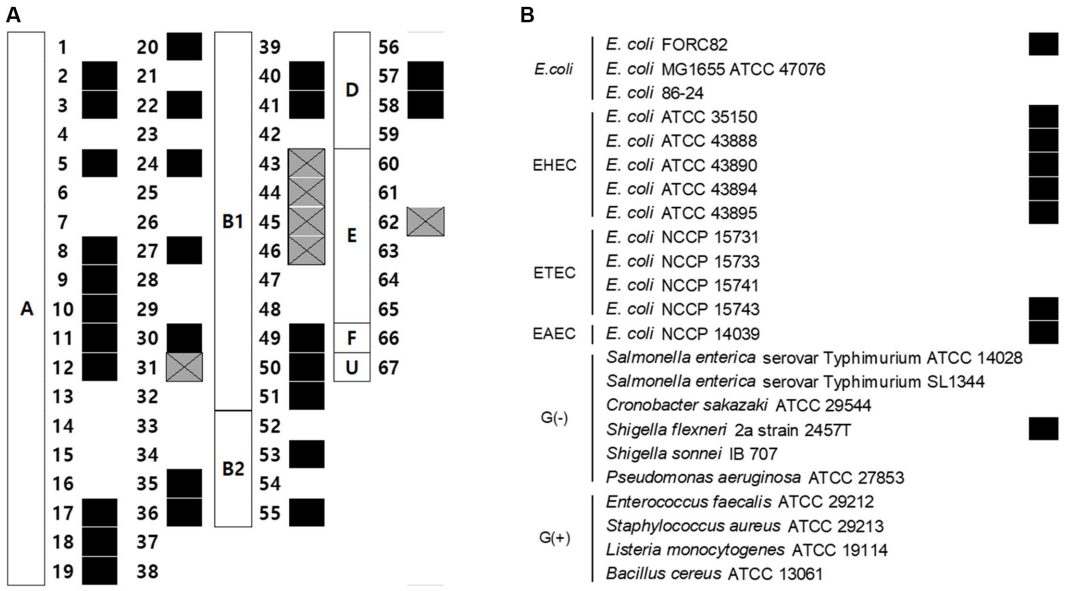


FIGURE 2
Antimicrobial spectrum of phage EJP2. **(A)** The susceptibility of 67 AMR *E. coli* against EJP2 was examined by using the spot assay. Alphabet in the white box indicates the phylogenetic groups of AMR *E. coli*. **(B)** Host range of EJP2 against pathogenic *E. coli,* other Gram-negative bacteria, and Gram-positive bacteria was determined by spot assay. Black squares indicate that EJP2 can form a plaque on the lawn of indicated bacterial strains. Gray squares with X mark present the growth inhibition zone. FORC, Food-borne pathogen Omics Research Center; ATCC, American Type Culture Collection; NCCP, National Culture Collection for Pathogens; EHEC, Enterohemorrhagic *E. coli*; ETEC, Enterotoxigenic *E. coli*; EAEC, Enteroaggregative *E. coli*; G(–), Gram-negative bacteria; G(+), Gram-positive bacteria.

classified as jumbo phage. EJP2 contains 540 putative ORFs and 6 genes encoding tRNAs (Figure 3A). Most of predicted genes (471 ORFs) encode hypothetical proteins with unknown functions. Of the 540 putative ORFs, only a small subset (12.8%, 69/540) were assigned putative functions (Supplementary Table S1). The analysis of phage life cycle is essential for developing phage therapeutic agent because temperate phages have the inherent capacity to transfer genes associated with bacterial virulence or antibiotic resistance by transduction (Howard-Varona et al., 2017). No genes associated with lysogenization, such as integrase, excisionase, transposase, superinfection immunity, repressor, and genome attachment site (attP) were predicted in the EJP2 genome, suggesting that EJP2 may be a virulent phage (Fogg et al., 2011; Davies et al., 2016). In addition, antibiotics resistance and virulence-associated genes were not identified in EJP2. BlastN analysis revealed that the whole genome of phage EJP2 shares less than 14% nucleotide identity with registered *E. coli* jumbo phages. Dot plot analysis presented low sequence homology with five *Escherichia* jumbo phages and one *Salmonella* jumbo phage at both the nucleotide and amino acid level (Supplementary Figure S3).

As different from the small phage genomes, the putative functional genes of EJP2 were scattered throughout its genome. The function of ORFs were categorized into 6 groups; nucleotide metabolism, DNA replication, DNA recombination, DNA packaging, structure proteins, and lysis (Figure 3A). One noticeable features of jumbo phages is that they possess their own enzymes for DNA replication, recombination, and transcription, capable of self-replication independent of host machinery (Iyer et al., 2021). In EJP2 genome, four ORFs (EJP02_150, EJP02_151, EJP02_301, and EJP02_303) were annotated as ribonucleotide reductase subunit whose composes enzyme for synthesis of deoxyribonucleotides from ribonucleotides with the help of glutaredoxin 1 (EJP02_192) and thioredoxin (EJP02_485) (Dwivedi et al., 2013; Sengupta and Holmgren, 2014). Two ORFs were predicted as DNA polymerase (EJP02_261) and DNA polymerase I (EJP02_270) (Supplementary Table S1). EJP2 also encoded other 9 genes associated with DNA replication and 3 genes associated with DNA recombination, respectively. In addition to various genes involved in DNA metabolism, EJP2 possesses two proteins (RNA polymerase sigma factor D and RNA ligase) for its own transcription (Supplementary Table S1). Overall, the results of genome annotation of EJP2 suggest the evolution of EJP2 toward reduced dependency on the host bacterium. This finding would be helpful to explain the broad host spectrum of phage EJP2.

The MCP, TerL protein and portal vertex protein are major conserved proteins in phage genomes and are thus used as phylogenetic markers to organize the phage families through single gene analysis (Smith et al., 2013; Prevelige and Cortines, 2018). Analysis of phylogenetic trees based on these three genes revealed that EJP2 is grouped with Rak2-like phage family (Šimoliūnas et al., 2013), which includes *E. coli* phage PBECO4 (Kim et al., 2013), *E. coli* phage 121Q (accession number: NC_025447), *Cronobacter* phage vB_CsaM_GAP32 (Abbasifar et al., 2014), *Enterobacteria* phage vB_PcaM_CBB (Buttimer et al., 2017), and *Salmonella* phage Munch (accession number: MK268344.1). EJP2 appeared to be evolutionally closest to *Salmonella* phage Munch for three proteins (Figure 3B).

## Phage EJP2 receptor analysis using Tn5 insertion mutant library

The host of EJP2, *E. coli* FORC82, harbors three F+ plasmids encoding various antibiotics resistance genes (Kim J. et al., 2019).

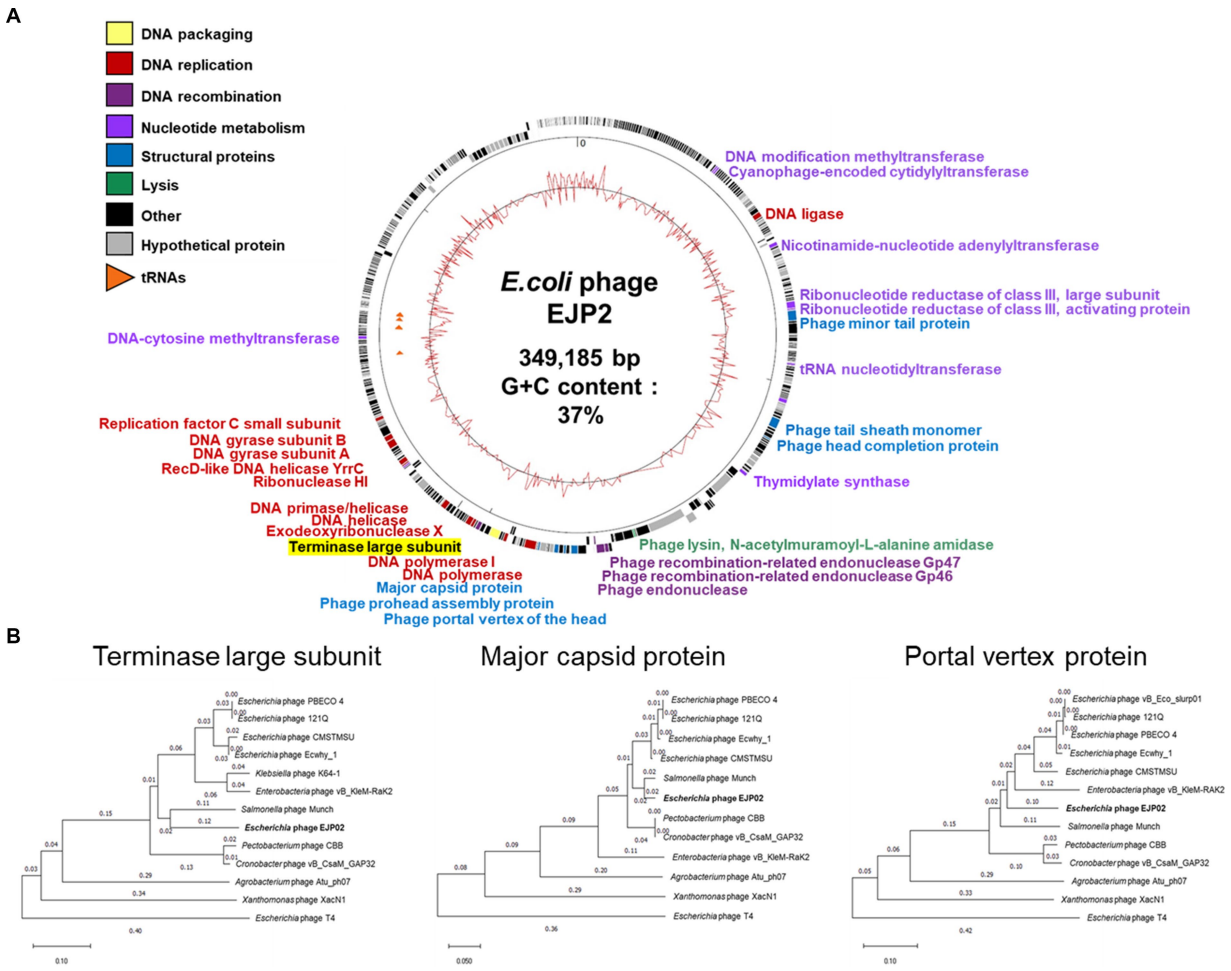


FIGURE 3
**(A)** Whole-genome map of phage EJP2. Predicted ORFs with the corresponding gene products are arranged on the EJP2 genome. Functional groups are categorized into colors. The GC content is presented as the inner track. **(B)** Phylogenetic tree comparing the terminase large subunit, major capsid protein, and portal vertex protein of phage EJP2 among other jumbo phages. Jumbo phages were selected as they share homology with the amino acid sequences of indicated protein using BlastP. Phage T4 was selected as an outgroup phage that is not closely related to the corresponding sequence of EJP2. Sequence relationships were inferred using the neighbor-joining method and evolutionary distances were computed using *p*-distance method by MegaX software.

We conducted plasmid curing by using acridine orange and PNA treatments to remove pFORC82_1, which carries most AMR genes. Unexpectedly, acridine orange treatment cured the pFORC82_2 (data not shown). Treatments of PNAs targeting RBS or start codon of plasmid replication protein (RepE) resulted in *E. coli* PS01 that was sensitive to β-lactam antibiotics (Supplementary Figure S4A). *E. coli* PS01 showed no difference in phage sensitivity compared to the *E. coli* FORC82 (Supplementary Figure S4B). We confirmed the deletion of about 6.3 kb region containing class A extended-spectrum β-lactamase ($bla_{CTX\text{-}M\text{-}65}$) gene between IS26 family transposase genes (Tnp26), which is known to form transposons carrying antibiotic resistance genes (Harmer and Hall, 2016; Supplementary Figure S4C). A Tn5 insertional mutant library of *E. coli* PS01 was constructed to screen for phage EJP2 resistance. Two EJP2-resistant colonies were obtained and Tn5 insertion sites were identified by WGS as putative *waaO* (*FORC82_RS00495*, LPS 3-α galactosyltransferase) and putative *waaR* (*FORC82_RS00500,* LPS α-1,2 glucosyltransferase), both of which are associated with biosynthesis of LPS (Figure 4A). LPS 3-α galactosyltransferase is an enzyme that adds the galactose (GalI) to the first glucose (GlcI) to form branches of LPS (Pradel et al., 1992). Other reports describe that *waaO* gene encodes LPS α-1,3-glucosyltransferase or LPS α-1,3-galactosyltransferase, which adds hexose II residue to glucose I of outer core of LPS (Shibayama et al., 1998; Qian et al., 2014). LPS α-1,2 glucosyltransferase is an enzyme that adds the third glucose (GlcIII) to the second glucose (GlcII) in the outer core of *E. coli* LPS (Shibayama et al., 1999). Phage resistant mutant with Tn5 insertion in putative *waaR* gene was designated as *E. coli* PRS07. The susceptibility of *E. coli* PS01 and *E. coli* PRS07 to EJP2 was examined by spot assay. As expected, EJP2 could not form plaques on the lawn of *E. coli* PRS07 (Figure 4B). When the putative *waaR* gene was complemented using inducible plasmid pUHE21-2 $lacI^q$, the sensitivity of EJP2 against PRS07 was completely restored, even without IPTG induction (Figure 4B). These results indicated that EJP2 recognizes LPS as a phage receptor and a third glucose of LPS outer core, or O-antigen would be required for EJP2 infection. LPS produced by *E. coli* varies depending on the pathogen type and this is due to the diversity in O-antigen structure (Liu et al., 2020). Moreover, the core region of LPS is known to have five different types and distribution of LPS core types

is diverse among different phylogroups (Leclercq et al., 2021). Because of these differences in LPS structure, the sensitivity against EJP2 was different among *E. coli* strains (Figure 2).

## Biofilm inhibition assay

Biofilm formation by AMR *E. coli* cells can pose a serious threat in human health and food industry because this structure is generally resistant to the human immune system and shows increased-tolerance against antibiotic treatments (Sharma et al., 2016; Zhou et al., 2022). Bacteriophages are known to efficiently remove biofilms (Pires et al., 2017), and the ability of EJP2 to eradicate biofilms formed by AMR *E. coli* was tested. Before phage treatment, we tried to screen AMR *E. coli* strain whose biofilm-forming ability was higher than *E. coli* FORC82. AMR *E. coli* isolate 62 showed significantly higher biofilm formation on polystyrene surfaces than *E. coli* FORC82 (Figure 5A), thus we used *E. coli* isolate 62 for biofilm inhibition assay. EJP2 was added to each well at MOI 1 and 0.1 and its treatment reduced biofilm formation by more than 80% compared to the positive control (Figure 5B). These results imply that anti-biofilm capacity of EJP2 would be helpful in reducing disease caused by AMR *E. coli* infection such as catheter-associated urinary tract infection.

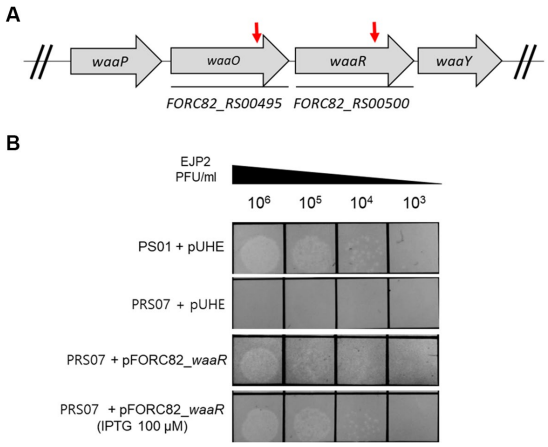


FIGURE 4
EJP2 receptor analysis. Schematic representation of the genes associated with LPS biosynthesis in *E. coli* FORC82 **(A)**. Red arrows indicate genes disrupted by transposon insertion. Locus tags of genes were presented below each arrow. Complementation of the LPS biosynthesis gene (putative *waaR*, *FORC82_RS00500*) to identify the restored susceptibility against EJP2 in *E. coli* PS01 **(B)**. The concentration of IPTG is presented in parentheses. LPS is a phage EJP2 receptor. One representative result of triplicate experiments is shown.

## EJP2 and CTX synergy against AMR *Escherichia coli* FORC82

CTX, one of third generation cephalosporins, was selected to investigate its synergistic antimicrobial effects. CTX is considered by WHO as the "highest priority critically important antimicrobials" for human medicine (World Health Organization, 2017) and the *E. coli* FORC82 was highly resistant to CTX (MIC ≥128 μg/ml, data not shown). The combination of EJP2 and sublethal concentration of CTX (64 μg/ml) significantly reduced *E. coli* FORC82 population after 3 h of treatment compared to the separate treatment of EJP2 or CTX (Figure 6). Two studies reported synergistic bacterial lysis by combining CTX treatment with T4 and two other T4-like phages, CTX treatment shortened the latent period of those phages (Comeau et al., 2007; Ryan et al., 2012). The present study revealed that EJP2 recognizes LPS as a phage receptor (Figure 4). LPS is considered to be a permeability barrier against hydrophilic and hydrophobic compounds (Lehman and Grabowicz, 2019) and it is well-known that LPS truncation or modification increases susceptibility of *E. coli* against antibiotics, and detergents. LPS truncation caused by T4

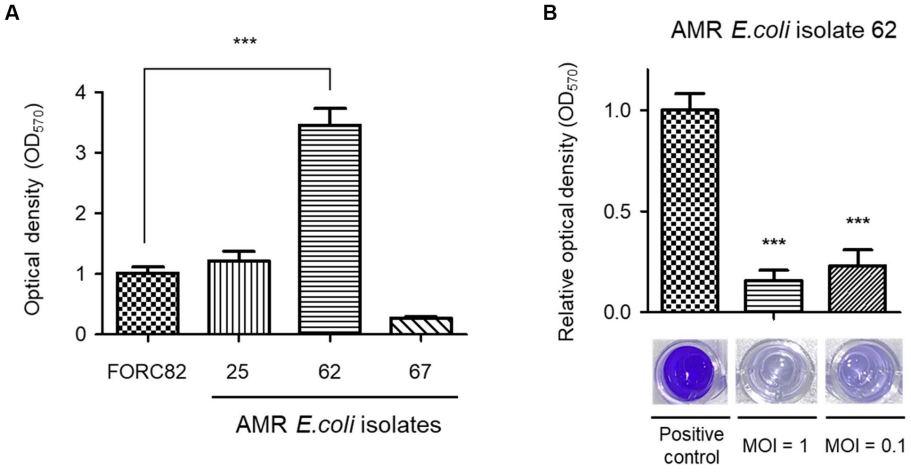


FIGURE 5
Comparison of biofilm formation of 4 AMR *E. coli* strains in 96-well polystyrene microplates **(A)**. Biofilm removal efficacy of phage EJP2 **(B)**. Biofilms of AMR *E. coli* isolate 62 were treated with EJP2 at a MOI of 1 and 0.1 and incubated for 48h. Each column represents the mean of triplicate experiments, and error bars indicate the standard deviation. One representative result of triplicate experiments is shown. ***$p<0.001$.

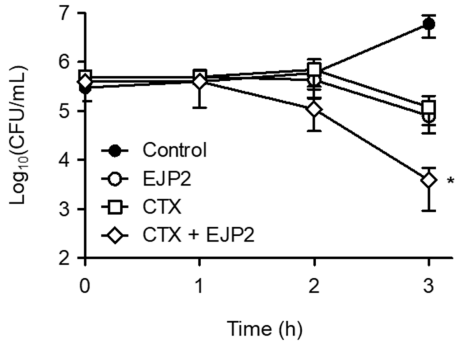


FIGURE 6
EJP2 and CTX synergy against *E. coli* FORC82. The antimicrobial activity of single treatment (EJP2, or CTX), and their combination were evaluated. Each Colony Forming Unit (CFU) was numerated after 3h inoculation. The means with standard deviation of triplicate experiments are shown. *$p$<0.05.

phage infection led to hypersensitivity against food grade surfactant (Zhong et al., 2020) and rough type LPS by *rfa* gene knockouts increased sensitivity of *E. coli* to colistin (Burmeister et al., 2020). Similar to these findings, the increased susceptibility of *E. coli* FORC82 to CTX in combination treatment with EJP2 may be due to LPS modification caused by EJP2 infection, as bacteria generally modify their receptors to avoid the phage infection (Labrie et al., 2010). The synthesis of at least the third glucose in outer core of LPS is necessary for EJP2 infection (Figure 4), it is possible that EJP2-resistant *E. coli* FORC82 may have rough type LPS, potentially making CTX more accessible to outer membrane proteins, such as OmpC, and OmpF (Goltermann et al., 2022; Masi et al., 2022). The observed synergy between EJP2 and CTX suggests that EJP2 could be used as alternative and/or adjuvants to antibiotics, potentially reducing the use of antibiotics. For better understanding the mechanism of action about synergistic antimicrobial effects, further investigation is needed.

## Conclusion

In this work, we have described AMR *E. coli*-specific jumbo phage EJP2 which was isolated from animal feces. EJP2 belongs to the *Myoviridae* family with a head and tail size of over 100 nm. Its genome contains 349,185 bp with 540 ORFs encoding genes for DNA replication, DNA recombination and nucleotide metabolism, DNA packaging, structural proteins, lysis, 6 tRNAs, and many genes whose functions remain unknown. Phylogenetic analyses of TerL, MCP, and portal vertex protein places EJP2 in a clade similar to *E. coli* and *Salmonella* jumbo phages, but with low sequence homology, suggesting a novel lineage for EJP2. Phage EJP2 exhibits a wide host range, biofilm removal activity, and synergistic effect with CTX against AMR *E. coli*, making it potentially a good candidate for the development of a biocontrol agent against diseases caused by AMR *E. coli* strains.

## Data availability statement

The datasets presented in this study can be found in online repositories. The name of the repository and accession number can be found below: NCBI: OQ411014.

## Author contributions

DJ, HK, and SR conceived and designed the experiments. DJ and HK carried out the main body of research, performed the experiments and bioinformatics analysis, and wrote the manuscript. DJ and YL contributed in phage isolation. JK contributed in provide a source and an information about AMR *E. coli* FORC82. SR supervised the work progress and edited the manuscript. All authors have read and approved the final manuscript.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1194435/full#supplementary-material

## References

Abbasifar, R., Griffiths, M. W., Sabour, P. M., Ackermann, H.-W., Vandersteegen, K., Lavigne, R., et al. (2014). Supersize me: *Cronobacter sakazakii* phage GAP32. *Virology* 460-461, 138–146. doi: 10.1016/j.virol.2014.05.003

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215, 403–410. doi: 10.1016/S0022-2836(05)80360-2

Aziz, R. K., Bartels, D., Best, A. A., Dejongh, M., Disz, T., Edwards, R. A., et al. (2008). The RAST server: rapid annotations using subsystems technology. *BMC Genomics* 9. doi: 10.1186/1471-2164-9-75

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., et al. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* 19, 455–477. doi: 10.1089/cmb.2012.0021

Besemer, J., Lomsadze, A., and Borodovsky, M. (2001). GeneMarkS: a self-training method for prediction of gene starts in microbial genomes. Implications for finding sequence motifs in regulatory regions. *Nucleic Acids Res.* 29, 2607–2618. doi: 10.1093/nar/29.12.2607

Bragg, R., Van Der Westhuizen, W., Lee, J.-Y., Coetsee, E., and Boucher, C. (2014). "Bacteriophages as potential treatment option for antibiotic resistant bacteria" in *Infectious diseases and nanomedicine I* (Midtown Manhattan, New York City: Springer), 97–110.

Burmeister, A. R., Fortier, A., Roush, C., Lessing, A. J., Bender, R. G., Barahman, R., et al. (2020). Pleiotropy complicates a trade-off between phage resistance and antibiotic resistance. *Proc. Natl. Acad. Sci.* 117, 11207–11216. doi: 10.1073/pnas.1919888117

Buttimer, C., Hendrix, H., Oliveira, H., Casey, A., Neve, H., Mcauliffe, O., et al. (2017). Things are getting hairy: Enterobacteria bacteriophage vB_PcaM_CBB. *Front. Microbiol.* 8:44. doi: 10.3389/fmicb.2017.00044

Carver, T., Berriman, M., Tivey, A., Patel, C., Böhme, U., Barrell, B. G., et al. (2008). Artemis and ACT: viewing, annotating and comparing sequences stored in a relational database. *Bioinformatics* 24, 2672–2676. doi: 10.1093/bioinformatics/btn529

Ceyssens, P.-J., Minakhin, L., Van Den Bossche, A., Yakunina, M., Klimuk, E., Blasdel, B., et al. (2014). Development of giant bacteriophage ϕKZ is independent of the host transcription apparatus. *J. Virol.* 88, 10501–10510. doi: 10.1128/JVI.01347-14

Cha, Y., Chun, J., Son, B., and Ryu, S. (2019). Characterization and genome analysis of staphylococcus aureus podovirus CSA13 and its anti-biofilm capacity. *Viruses*, 11:54.

Clermont, O., Christenson, J. K., Denamur, E., and Gordon, D. M. (2013). The Clermont *Escherichia coli* phylo-typing method revisited: improvement of specificity and detection of new phylo-groups. *Environ. Microbiol. Rep.* 5, 58–65. doi: 10.1111/1758-2229.12019

Comeau, A. M., Tétart, F., Trojet, S. N., Prere, M.-F., and Krisch, H. (2007). Phage-antibiotic synergy (PAS): β-lactam and quinolone antibiotics stimulate virulent phage growth. *PLoS One* 2:e799. doi: 10.1371/journal.pone.0000799

Coque, T., Baquero, F., and Canton, R. (2008). Increasing prevalence of ESBL-producing *Enterobacteriaceae* in Europe. *Eur. Secur.* 13:19044. doi: 10.2807/ese.13.47.19044-en

Datsenko, K. A., and Wanner, B. L. (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci.* 97, 6640–6645. doi: 10.1073/pnas.120163297

Davies, E. V., Winstanley, C., Fothergill, J. L., and James, C. E. (2016). The role of temperate bacteriophages in bacterial infection. *FEMS Microbiol. Lett.* 363. doi: 10.1093/femsle/fnw015

Dd Pitout, J. (2013). *Enterobacteriaceae* that produce extended-spectrum β-lactamases and AmpC β-lactamases in the community: the tip of the iceberg? *Curr. Pharm. Des.* 19, 257–263. doi: 10.2174/138161213804070348

Delcher, A. L., Bratke, K. A., Powers, E. C., and Salzberg, S. L. (2007). Identifying bacterial genes and endosymbiont DNA with glimmer. *Bioinformatics* 23, 673–679. doi: 10.1093/bioinformatics/btm009

Dwivedi, B., Xue, B., Lundin, D., Edwards, R. A., and Breitbart, M. (2013). A bioinformatic analysis of ribonucleotide reductase genes in phage genomes and metagenomes. *BMC Evol. Biol.* 13, 1–17. doi: 10.1186/1471-2148-13-33

Felsenstein, J. (1985). Phylogenies and the comparative method. *Am. Nat.* 125, 1–15. doi: 10.1086/284325

Ferrieres, L., Hemery, G., Nham, T., Guerout, A. M., Mazel, D., Beloin, C., et al. (2010). Silent mischief: bacteriophage mu insertions contaminate products of *Escherichia coli* random mutagenesis performed using suicidal transposon delivery plasmids mobilized by broad- host- range RP4 conjugative machinery. *J. Bacteriol.* 192, 6418–6427. doi: 10.1128/JB.00621-10

Fogg, P., Rigden, D., Saunders, J., Mccarthy, A., and Allison, H. (2011). Characterization of the relationship between integrase, excisionase and antirepressor activities associated with a superinfecting Shiga toxin encoding bacteriophage. *Nucleic Acids Res.* 39, 2116–2129. doi: 10.1093/nar/gkq923

Gibson, D. G., Young, L., Chuang, R.-Y., Venter, J. C., Hutchison, C. A., and Smith, H. O. (2009). Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat. Methods* 6, 343–345. doi: 10.1038/nmeth.1318

Goltermann, L., Zhang, M., Ebbensgaard, A. E., Fiodorovaite, M., Yavari, N., Løbner-Olesen, A., et al. (2022). Effects of LPS composition in *Escherichia coli* on antibacterial activity and bacterial uptake of antisense peptide-PNA conjugates. *Front. Microbiol.* 13. doi: 10.3389/fmicb.2022.877377

Good, L., Awasthi, S. K., Dryselius, R., Larsson, O., and Nielsen, P. E. (2001). Bactericidal antisense effects of peptide–PNA conjugates. *Nat. Biotechnol.* 19, 360–364. doi: 10.1038/86753

Guan, J., and Bondy-Denomy, J. (2020). Intracellular organization by jumbo bacteriophages. *J. Bacteriol.* 203, e00362–e00320. doi: 10.1128/JB.00362-20

Harmer, C. J., and Hall, R. M. (2016). IS26-mediated formation of transposons carrying antibiotic resistance genes. *MSphere* 1. doi: 10.1128/mSphere.00038-16

Hassan, A. Y., Lin, J. T., Ricker, N., and Anany, H. (2021). The age of phage: friend or foe in the new dawn of therapeutic and biocontrol applications? *Pharmaceuticals* 14:199. doi: 10.3390/ph14030199

Hirota, Y. (1960). The effect of acridine dyes on mating type factors in *Escherichia coli*. *Proc. Natl. Acad. Sci. U. S. A.* 46, 57–64. doi: 10.1073/pnas.46.1.57

Howard-Varona, C., Hargreaves, K. R., Abedon, S. T., and Sullivan, M. B. (2017). Lysogeny in nature: mechanisms, impact and ecology of temperate phages. *ISME J.* 11, 1511–1520. doi: 10.1038/ismej.2017.16

Huang, Y., Wang, W., Zhang, Z., Gu, Y., Huang, A., Wang, J., et al. (2022). Phage products for fighting antimicrobial resistance. *Microorganisms* 10:1324. doi: 10.3390/microorganisms10071324

Imam, M., Alrashid, B., Patel, F., Dowah, A. S., Brown, N., Millard, A., et al. (2019). vB_PaeM_MIJ3, a novel jumbo phage infecting *Pseudomonas aeruginosa*, possesses unusual genomic features. *Front. Microbiol.* 10:2772. doi: 10.3389/fmicb.2019.02772

Iyer, L. M., Anantharaman, V., Krishnan, A., Burroughs, A. M., and Aravind, L. (2021). Jumbo phages: a comparative genomic overview of core functions and adaptions for biological conflicts. *Viruses* 13:63. doi: 10.3390/v13010063

Jones, P., Binns, D., Chang, H.-Y., Fraser, M., Li, W., Mcanulla, C., et al. (2014). InterProScan 5: genome-scale protein function classification. *Bioinformatics* 30, 1236–1240. doi: 10.1093/bioinformatics/btu031

Kim, M. S., Hong, S. S., Park, K., and Myung, H. (2013). Genomic analysis of bacteriophage PBECO4 infecting *Escherichia coli* O157: H7. *Arch. Virol.* 158, 2399–2403. doi: 10.1007/s00705-013-1718-3

Kim, J., Hwang, B. K., Choi, H., Wang, Y., Choi, S. H., Ryu, S., et al. (2019). Characterization of mcr-1-harboring plasmids from pan drug-resistant *Escherichia coli* strains isolated from retail raw chicken in South Korea. *Microorganisms* 7:344. doi: 10.3390/microorganisms7090344

Kim, H., Kim, M., Bai, J., Lim, J.-A., Heu, S., and Ryu, S. (2019). Colanic acid is a novel phage receptor of *Pectobacterium carotovorum* subsp. *carotovorum* phage POP72. *Front. Microbiol.* 10. doi: 10.3389/fmicb.2019.00143

Kim, J., Park, H., Ryu, S., and Jeon, B. (2021). Inhibition of antimicrobial-resistant *Escherichia coli* using a broad host range phage cocktail targeting various bacterial phylogenetic groups. *Front. Microbiol.* 12:699630. doi: 10.3389/fmicb.2021.699630

Kim, M., and Ryu, S. (2011). Characterization of a T5-like coliphage, SPC35, and differential development of resistance to SPC35 in *Salmonella enterica* serovar typhimurium and *Escherichia coli*. *Appl. Environ. Microbiol.* 77, 2042–2050. doi: 10.1128/AEM.02504-10

Krumsiek, J., Arnold, R., and Rattei, T. (2007). Gepard: a rapid and sensitive tool for creating dotplots on genome scale. *Bioinformatics* 23, 1026–1028. doi: 10.1093/bioinformatics/btm039

Kumar, S., Stecher, G., Li, M., Knyaz, C., and Tamura, K. (2018). MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* 35, 1547–1549. doi: 10.1093/molbev/msy096

Kutter, E., and Sulakvelidze, A. (2004). *Bacteriophages: Biology and applications* Boca Raton, Florida, United States: CRC press.

Labrie, S. J., Samson, J. E., and Moineau, S. (2010). Bacteriophage resistance mechanisms. *Nat. Rev. Microbiol.* 8, 317–327. doi: 10.1038/nrmicro2315

Lavysh, D., Sokolova, M., Minakhin, L., Yakunina, M., Artamonova, T., Kozyavkin, S., et al. (2016). The genome of AR9, a giant transducing *Bacillus* phage encoding two multisubunit RNA polymerases. *Virology* 495, 185–196. doi: 10.1016/j.virol.2016.04.030

Leclercq, S. O., Branger, M., Smith, D. G., and Germon, P. (2021). Lipopolysaccharide core type diversity in the *Escherichia coli* species in association with phylogeny, virulence gene repertoire and distribution of type VI secretion systems. *Microbial. Genomics* 7. doi: 10.1099/mgen.0.000652

Lehman, K. M., and Grabowicz, M. (2019). Countering gram-negative antibiotic resistance: recent progress in disrupting the outer membrane with novel therapeutics. *Antibiotics* 8:163. doi: 10.3390/antibiotics8040163

Leskinen, K., Blasdel, B. G., Lavigne, R., and Skurnik, M. (2016). RNA-sequencing reveals the progression of phage-host interactions between φR1-37 and *Yersinia enterocolitica*. *Viruses* 8:111. doi: 10.3390/v8040111

Liu, B., Furevi, A., Perepelov, A. V., Guo, X., Cao, H., Wang, Q., et al. (2020). Structure and genetics of *Escherichia coli* O antigens. *FEMS Microbiol. Rev.* 44, 655–683. doi: 10.1093/femsre/fuz028

Loc-Carrillo, C., and Abedon, S. T. (2011). Pros and cons of phage therapy. *Bacteriophage* 1, 111–114. doi: 10.4161/bact.1.2.14590

Lowe, T. M., and Eddy, S. R. (1997). tRNAscan-SE: A program for improved detection of transfer RNA genes in genomic sequence. *Nucleic Acids Res.* 25, 955–964. doi: 10.1093/nar/25.5.955

Martak, D., Guther, J., Verschuuren, T. D., Valot, B., Conzelmann, N., Bunk, S., et al. (2022). Populations of extended-spectrum β-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* are different in human-polluted environment and food items: A multicentre European study. *Clin. Microbiol. Infect.* 28, 447.e7. e414–447.e14. doi: 10.1016/j.cmi.2021.07.022

Masi, M., Vergalli, J., Ghai, I., Barba-Bon, A., Schembri, T., Nau, W. M., et al. (2022). Cephalosporin translocation across enterobacterial OmpF and OmpC channels, a filter across the outer membrane. *Commun. Biol.* 5:1059. doi: 10.1038/s42003-022-04035-y

Mesyanzhinov, V. V., Robben, J., Grymonprez, B., Kostyuchenko, V. A., Bourkaltseva, M. V., Sykilinda, N. N., et al. (2002). The genome of bacteriophage φKZ of *Pseudomonas aeruginosa*. *J. Mol. Biol.* 317, 1–19. doi: 10.1006/jmbi.2001.5396

Nazir, A., Ali, A., Qing, H., and Tong, Y. (2021). Emerging aspects of jumbo bacteriophages. *Infect. Drug Resist.* 14, 5041–5055. doi: 10.2147/IDR.S330560

Nei, M., and Kumar, S. (2000). *Molecular evolution and phylogenetics*. Oxford: Oxford University Press.

Orekhova, M., Koreshova, A., Artamonova, T., Khodorkovskii, M., and Yakunina, M. (2019). The study of the phiKZ phage non-canonical non-virion RNA polymerase. *Biochem. Biophys. Res. Commun.* 511, 759–764. doi: 10.1016/j.bbrc.2019.02.132

Overbeek, R., Olson, R., Pusch, G. D., Olsen, G. J., Davis, J. J., Disz, T., et al. (2014). The SEED and the rapid annotation of microbial genomes using subsystems technology (RAST). *Nucleic Acids Res.* 42, D206–D214. doi: 10.1093/nar/gkt1226

Park, H., Kim, J., Ryu, S., and Jeon, B. (2019). Predominance of blaCTX-M-65 and blaCTX-M-55 in extended-spectrum β-lactamase-producing *Escherichia coli* from raw retail chicken in South Korea. *J. Global Antimicrob. Resist.* 17, 216–220. doi: 10.1016/j.jgar.2019.01.005

Parvez, S., and Khan, A. U. (2018). Hospital sewage water: a reservoir for variants of New Delhi metallo-β-lactamase (NDM)-and extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae*. *Int. J. Antimicrob. Agents* 51, 82–88. doi: 10.1016/j.ijantimicag.2017.08.032

Pires, D. P., Melo, L. D., Boas, D. V., Sillankorva, S., and Azeredo, J. (2017). Phage therapy as an alternative or complementary strategy to prevent and control biofilm-related infections. *Curr. Opin. Microbiol.* 39, 48–56. doi: 10.1016/j.mib.2017.09.004

Platt, R., Drescher, C., Park, S.-K., and Phillips, G. J. (2000). Genetic system for reversible integration of DNA constructs and *lacZ* gene fusions into the *Escherichia coli* chromosome. *Plasmid* 43, 12–23. doi: 10.1006/plas.1999.1433

Poirel, L., Madec, J.-Y., Lupo, A., Schink, A.-K., Kieffer, N., Nordmann, P., et al. (2018). Antimicrobial resistance in *Escherichia coli*. *Microbiology*. *Spectrum* 6:6.4. 14. doi: 10.1128/microbiolspec.ARBA-0026-2017

Pradel, E., Parker, C. T., and Schnaitman, C. A. (1992). Structures of the *rfaB, rfaI, rfaJ*, and *rfaS* genes of *Escherichia coli* K-12 and their roles in assembly of the lipopolysaccharide core. *J. Bacteriol.* 174, 4736–4745. doi: 10.1128/jb.174.14.4736-4745.1992

Prevelige, P. E. Jr., and Cortines, J. R. (2018). Phage assembly and the special role of the portal protein. *Curr. Opin. Virol.* 31, 66–73. doi: 10.1016/j.coviro.2018.09.004

Qian, J., Garrett, T. A., and Raetz, C. R. (2014). In vitro assembly of the outer core of the lipopolysaccharide from *Escherichia coli* K-12 and *Salmonella typhimurium*. *Biochemistry* 53, 1250–1262. doi: 10.1021/bi4015665

Roca, I., Akova, M., Baquero, F., Carlet, J., Cavaleri, M., Coenen, S., et al. (2015). The global threat of antimicrobial resistance: science for intervention. *New Microbes New Infect.* 6, 22–29. doi: 10.1016/j.nmni.2015.02.007

Rupp, M. E., and Fey, P. D. (2003). Extended spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae*. *Drugs* 63, 353–365. doi: 10.2165/00003495-200363040-00002

Ryan, E. M., Alkawareek, M. Y., Donnelly, R. F., and Gilmore, B. F. (2012). Synergistic phage-antibiotic combinations for the control of *Escherichia coli* biofilms in vitro. *FEMS Immunol. Med. Microbiol.* 65, 395–398. doi: 10.1111/j.1574-695X.2012.00977.x

Saad, A. M., Soliman, A. M., Kawasaki, T., Fujie, M., Nariya, H., Shimamoto, T., et al. (2019). Systemic method to isolate large bacteriophages for use in biocontrol of a wide-range of pathogenic bacteria. *J. Biosci. Bioeng.* 127, 73–78. doi: 10.1016/j.jbiosc.2018.07.001

Saitou, N., and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4, 406–425.

Sambrook, J., and Russell, D. (2001). *Molecular cloning: a laboratory manual 1. 3rd* Cold Spring Harbor. New York, NY: 6.4–6.12

Sengupta, R., and Holmgren, A. (2014). Thioredoxin and glutaredoxin-mediated redox regulation of ribonucleotide reductase. *World J. Biol. Chem.* 5, 68–74. doi: 10.4331/wjbc.v5.i1.68

Sharma, G., Sharma, S., Sharma, P., Chandola, D., Dang, S., Gupta, S., et al. (2016). *Escherichia coli* biofilm: development and therapeutic strategies. *J. Appl. Microbiol.* 121, 309–319. doi: 10.1111/jam.13078

Shibayama, K., Ohsuka, S., Sato, K., Yokoyama, K., Horii, T., and Ohta, M. (1999). Four critical aspartic acid residues potentially involved in the catalytic mechanism of *Escherichia coli* K-12 WaaR. *FEMS Microbiol. Lett.* 174, 105–109. doi: 10.1111/j.1574-6968.1999.tb13555.x

Shibayama, K., Ohsuka, S., Tanaka, T., Arakawa, Y., and Ohta, M. (1998). Conserved structural regions involved in the catalytic mechanism of *Escherichia coli* K-12 WaaO (RfaI). *J. Bacteriol.* 180, 5313–5318. doi: 10.1128/JB.180.20.5313-5318.1998

Shrivastava, S. R., Shrivastava, P. S., and Ramasamy, J. (2018). World health organization releases global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. *J. Med. Society* 32:76. doi: 10.4103/jms.jms_25_17

Šimoliūnas, E., Kaliniene, L., Truncaitė, L., Zajančkauskaitė, A., Staniulis, J., Kaupinis, A., et al. (2013). *Klebsiella* phage vB_KleM-RaK2—A giant singleton virus of the family *Myoviridae*. *PLoS One* 8. doi: 10.1371/journal.pone.0060717

Smith, K. C., Castro-Nallar, E., Fisher, J. N., Breakwell, D. P., Grose, J. H., and Burnett, S. H. (2013). Phage cluster relationships identified through single gene analysis. *BMC Genom.* 14, 1–16. doi: 10.1186/1471-2164-14-410

Soncini, F. C., Véscovi, E. G., and Groisman, E. A. (1995). Transcriptional autoregulation of the *Salmonella typhimurium* phoPQ operon. *J. Bacteriol.* 177, 4364–4371. doi: 10.1128/jb.177.15.4364-4371.1995

Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22, 4673–4680. doi: 10.1093/nar/22.22.4673

Walker, P. J., Siddell, S. G., Lefkowitz, E. J., Mushegian, A. R., Adriaenssens, E. M., Alfenas-Zerbini, P., et al. (2022). Recent changes to virus taxonomy ratified by the international committee on taxonomy of viruses (2022). *Arch. Virol.* 167, 2429–2440. doi: 10.1007/s00705-022-05516-5

World Health Organization. (2017). *Critically important antimicrobials for human medicine: ranking of antimicrobial agents for risk management of antimicrobial resistance due to non-human use*.

Wu, C., Wang, Y., Shi, X., Wang, S., Ren, H., Shen, Z., et al. (2018). Rapid rise of the ESBL and mcr-1 genes in *Escherichia coli* of chicken origin in China, 2008–2014. *Emerg. Microbes Infect.* 7, 1–10. doi: 10.1038/s41426-018-0033-1

Yoshikawa, G., Askora, A., Blanc-Mathieu, R., Kawasaki, T., Li, Y., Nakano, M., et al. (2018). *Xanthomonas citri* jumbo phage XacN1 exhibits a wide host range and high complement of tRNA genes. *Sci. Rep.* 8:4486. doi: 10.1038/s41598-018-22239-3

Yuan, Y., and Gao, M. (2017). Jumbo bacteriophages: an overview. *Front. Microbiol.* 8:403. doi: 10.3389/fmicb.2017.00403

Zhong, Z., Emond-Rheault, J.-G., Bhandare, S., Lévesque, R., and Goodridge, L. (2020). Bacteriophage-induced lipopolysaccharide mutations in *Escherichia coli* lead to hypersensitivity to food grade surfactant sodium dodecyl sulfate. *Antibiotics* 9:552. doi: 10.3390/antibiotics9090552

Zhou, F., Wang, D., Hu, J., Zhang, Y., Tan, B. K., and Lin, S. (2022). Control measurements of *Escherichia coli* biofilm: A review. *Foods* 11:2469. doi: 10.3390/foods11162469

Zurfuh, K., Poirel, L., Nordmann, P., Nüesch-Inderbinen, M., Hächler, H., and Stephan, R. (2016). Occurrence of the plasmid-borne mcr-1 colistin resistance gene in ESBL-producing *Enterobacteriacae* in river water and imported vegetable samples in Switzerland. *Antimicrob Agents Chemother* 60, 2594–2595. doi: 10.1128/AAC.00066-16

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# Pulmonary bacteriophage and cystic fibrosis airway mucus: friends or foes?

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For those born with cystic fibrosis (CF), hyper-concentrated mucus with a dysfunctional structure significantly impacts CF airways, providing a perfect environment for bacterial colonization and subsequent chronic infection. Early treatment with antibiotics limits the prevalence of bacterial pathogens but permanently alters the CF airway microenvironment, resulting in antibiotic resistance and other long-term consequences. With little investment into new traditional antibiotics, safe and effective alternative therapeutic options are urgently needed. One gathering significant traction is bacteriophage (phage) therapy. However, little is known about which phages are effective for respiratory infections, the dynamics involved between phage(s) and the host airway, and associated by-products, including mucus. Work utilizing gut cell models suggest that phages adhere to mucus components, reducing microbial colonization and providing non-host-derived immune protection. Thus, phages retained in the CF mucus layer result from the positive selection that enables them to remain in the mucus layer. Phages bind weakly to mucus components, slowing down the diffusion motion and increasing their chance of encountering bacterial species for subsequent infection. Adherence of phage to mucus could also facilitate phage enrichment and persistence within the microenvironment, resulting in a potent phage phenotype or vice versa. However, how the CF microenvironment responds to phage and impacts phage functionality remains unknown. This review discusses CF associated lung diseases, the impact of CF mucus, and chronic bacterial infection. It then discusses the therapeutic potential of phages, their dynamic relationship with mucus and whether this may enhance or hinder airway bacterial infections in CF.

KEYWORDS

**bacteriophage, cystic fibrosis, mucus, airway epithelium, antimicrobial resistance**

## Introduction

Antimicrobial resistance (AMR) has a tremendous healthcare burden. It is estimated to account for over 10 million deaths (1) at an annual healthcare cost of $400 million in Australia alone by 2050 (2). Unfortunately, individuals with chronic conditions, including cystic fibrosis (CF), are likely to experience more severe consequences of AMR. Therapies such as antibiotics are essential for chronic bacterial eradication; however, their repeated and semi-continuous

consumption is likely a key driver of AMR, especially in the CF population. Disappointingly, the discovery pipeline into the production of novel antibiotics is waning, and current innovation has not kept up with evolving resistance. The arrival of CFTR modulators has substantially improved the lung function of individuals with CF, but the benefit for inflammation and infection remains inconclusive (3). Therefore, there is still a great need for therapeutics that can complement CFTR modulators and reduce chronic bacterial infection.

Bacteriophage (phage) therapy has been proposed to tackle chronic bacterial infections in CF (4–6). Phages are found ubiquitously in the body and the natural environment and show strong potential for clinical use (7–9). Those applicable for therapy are typically lytic phages that recognize and bind to specific bacterial cell surface receptors (10, 11). Several clinical cases have demonstrated a reduction in bacterial density and clinical improvement when bacterial infections have been treated with phage (12–16). However, the full impact of phage therapy on pulmonary infection in CF is yet to be fully appreciated. Many unanswered questions include determining the best administration route, concomitant use of antibiotics or mucolytic agents, length of the treatment period, and phage formulation. Furthermore, additional basic research is needed to predict these parameters and accurately measure host immune responses.

In young children with CF, mucus flakes present very early in life (17) and are associated with inflammation and airway luminal hypoxia without bacterial infection and structural lung disease (18). The resultant mucus flakes create a microenvironment favorable for bacterial colonization (17, 19). The mucus layer is an essential entity facilitating phage diffusion across the mucosal surface, and recent evidence in the gut suggests that they can bind to parts of mucus, improving their bactericidal activity (11). Knowing that mucus and its structure in CF are distinctly unique, little is known about how this would impact phage functionality. In this review, we explore the clinical features of CF disease, antibiotic choice, and the underlying drivers of MDR. We summarize treatment options and provide evidence as to why phage therapy may be crucial for infection control in this population. We explore the feasibility and benefit of phage therapy in treating chronic infection in CF, including investigating phage behavior and efficacy in the CF lung microenvironment.

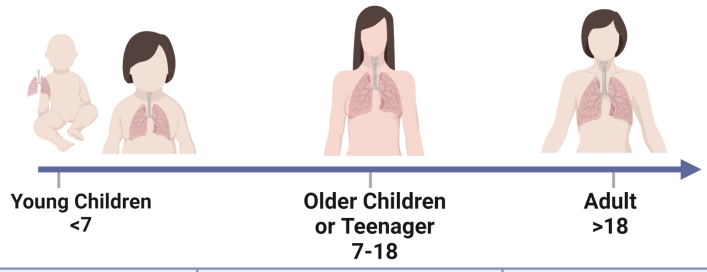
# Cystic fibrosis

## CF lung disease and what do we know about CF mucus?

Cystic Fibrosis is an autosomal recessive genetic disease that markedly impacts multiple mucosal surfaces, particularly the pancreatic ducts, intestinal mucosa and airway epithelium. The mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) gene on chromosome 7 (7q31.2) results in defective chloride transport across the apical surface of the epithelial (20–22). Impaired ion transport, imbalanced water flow in the CF airway surface liquid volume, and airway dehydration prevent adequate cough clearance via the mucociliary escalator (23). There are also higher concentrations of mucins in the lungs of those with CF and elevated osmotic pressure of the mucus layers, which subsequently triggers thick, dense mucus production and drives muco-inflammatory airway obstruction (24–27). Recently, mucin content analysis in bronchoalveolar lavage fluid (BALf) of children with CF revealed both elevated mucin concentration and increased mucus burden typified by "mucus flakes" (17) which were associated with inflammation and airway luminal hypoxia (17, 18). The impact of dysfunctional mucus in CF on inhaled therapeutic agents such as antibiotics and chemical compounds has also been investigated (28, 29). Specifically, macromolecules (mucins and DNA) in CF mucus increased its viscosity and impeded the diffusion of therapeutic agents such as antibiotics (30). Furthermore, high salt concentrations and low oxygen levels have been shown to reduce antibiotic effectiveness (31, 32). Knowing this effect raises the question of whether the CF mucus could affect other treatment approaches, including phage.

## Chronic bacterial infections and associated treatment regimens in CF

A hallmark of CF lung disease includes hyper-inflammatory responses to early-life colonization and infection events that continue over life to develop into chronic inflammation (33–35). The acquisition of bacterial pathogens in CF airways appears to be an age-dependent sequence (Figure 1) (36). Bacterial-induced pulmonary exacerbations in infants and children with CF are often associated with *Haemophilus influenzae*, *Staphylococcus aureus*, and *Streptococcus pneumoniae* (37). As disease progresses with age, the CF airway becomes more susceptible to gram-negative bacteria, particularly *Pseudomonas aeruginosa*, which is highly associated with chronic airway inflammation (Figure 1) (38–41). In adults with CF, most pulmonary exacerbations are independent of new bacterial strain acquisition (38) or increases in the airway density of *P. aeruginosa* (40). Other less common pathogens include *Streptococcus pneumoniae*, *Stenotrophomonas maltophilia*, *Moraxella catarrhalis*, *methicillin-resistant Staphylococcus aureus* (MRSA), *Burkholderia cepacia complex* (BCC), *Achromobacter xylosoxidans*, and nontuberculous mycobacteria (NTM) (42–46). Selection criteria, including the delivery method (e.g., intravenous or inhalation) and treatment duration, also influence which type of antibiotic could be chosen for CF pulmonary infections. For example, inhalation therapy is advantageous since it can directly target the lower respiratory tract and site of infection with higher doses of antibiotics and exhibits reduced systemic toxicity and side effects (32, 47). Administration of inhaled antibiotics, including tobramycin, colistin, aztreonam lysine and levofloxacin are now commonly used to manage infections in CF (48–50) and others, such as ceftaroline and vancomycin, have been assessed for their ability to eradicate specific infections including MRSA (51). In Australia, CF physicians typically select inhaled tobramycin or colistin as a secondary antibiotic in combination with a primary intravenous antibiotic such as ceftazidime (Figure 1) (52). Although prolonged use of inhaled antibiotics has not been reported for newly acquired multidrug-resistant bacterial strains, the long-term clinical impacts of high-dose antibiotics on the perturbation of airway microbes are still unknown. Antibiotic susceptibility profiles of various CF pathogens are also summarized in Figure 1 (53–58). The extensive consumption of antibiotics has resulted in increased isolated MDR pathogens. Indeed, polymicrobial interaction between *P. aeruginosa* and *S. aureus* has resulted in tobramycin resistance in isolates recovered from children with CF (59). The development of resistance towards the last-line option, colistin, in isolates derived from individuals with CF is particularly concerning (60–63).




| | Young Children <7 | Older Children or Teenager 7-18 | Adult >18 |
|---|---|---|---|
| **Pathogen Acquisition** | ***S. aureus (41%)***<br>*H. influenzae (20%)*<br>*P. aeruginosa (12%)*<br>*S. maltophilia (4%)*<br>*MRSA (3%)*<br>*Serratia marcescens (2%)*<br>*Achromobacter spp (1%)*<br>BCC *(1%)*<br>NTM (1%) | ***S. aureus (53-57%)***<br>*H. influenzae (12-15%)*<br>*P. aeruginosa (16-28%)*<br>*S. maltophilia (5%)*<br>*MRSA (4-5%)*<br>*S. marcescens (2-4%)*<br>*Achromobacter spp (2%)*<br>BCC *(2%)*<br>NTM (4%) | *S. aureus (31-59%)*<br>*H. influenzae (2-8%)*<br>***P. aeruginosa (50-76%)***<br>*S. maltophilia (4-10%)*<br>*MRSA (5-6%)*<br>*S. marcescens (0-2%)*<br>*Achromobacter spp (2-7%)*<br>BCC *(3-4%)*<br>NTM (3-14%) |
| **Physician Selection of Primary Antibiotic** | **IV ceftazidime (CAZ)**<br>IV ceftriaxone (CEFT)<br>IV piperacillin-tazobactam (PIP/TAZ)<br>IV flucloxacillin (FLU)<br>IV cefepime (CEF) | | |
| **Physician Selection of Secondary Antibiotic** | **IV tobramycin (TOB)**<br>IV colistin (COL)<br>IV gentamicin (GEN)<br>Inhaled tobramycin (IH TOB)<br>Inhaled colistin (IH COL) | | **IV tobramycin (TOB)**<br>Inhaled tobramycin (IH TOB)<br>Inhaled colistin (IH COL)<br>IV ciprofloxacin (CIP) |

**Typical susceptibilities of CF path to commonly used antibiotic**

| | CAZ | PIP/TAZ | TOB | COL | MER | CIP | VAN |
|---|---|---|---|---|---|---|---|
| *S. aureus* | +/- | -- | +/- | NR | ✓ | +/- | ✓ |
| *H. influenzae* | ✓ | ✓ | ✓ | ✓ | ✓ | +/- | NR |
| *P. aeruginosa* | +/- | +/- | +/- | ✓ | +/- | +/- | -- |
| *S. maltophilia* | +/- | -- | -- | +/- | -- | +/- | -- |
| *MRSA* | -- | -- | -- | -- | ✓ | -- | ✓ |
| *S. marcescens* | -- | +/- | -- | -- | -- | -- | -- |
| *Achromobacter spp* | +/- | ✓ | NR | ✓ | +/- | +/- | NR |
| BCC | +/- | +/- | NR | -- | +/- | NR | NR |
| NTM | NR | NR | NR | NR | NR | NR | NR |

FIGURE 1
According to the Australian Cystic Fibrosis Data Registry 2020 (ACFDR 2020) (64), common microorganisms found in the respiratory tracts of individuals with CF, including the most common pathogen in early childhood *S. aureus*, and the most common pathogens in adulthood *P. aeruginosa*. Currie and colleagues reported the selection of intravenous primary and secondary inhaled or intravenous antibiotics by pediatrics or adult physicians across multiple CF centers in Australia (52). The susceptibility of a few common antibiotics (CAZ=ceftazimide; PIP/TAZ=piperacillin-tazobactam; TOB=tobramycin; COL=colistin; MER=meropenem; CIP=ciprofloxacin; VAN=vancomycin) of the common pathogens recovered from CF lung (60–63). √ indicates susceptibility, +/– indicates partial susceptibility, – indicates resistant and NR indicates not reported. For example, *S. aureus* typically exhibits multi-drug resistance, across most antibiotics including macrolides (55, 65). Furthermore, the MRSA strain confers resistance to β-lactams, quinolones and aminoglycosides (55, 65). The prominent CF pathogen, *P. aeruginosa*, also is developing broad spectrum resistance and other less common pathogens, including BCC, are resistant to most of the β-lactams, aminoglycosides, and cationic antimicrobial peptides (66). Created with BioRender.com.

## Alternative treatments and phage therapy

Antibiotic resistance has become a global issue, and the prescription of antibiotics by physicians has increased by 10 times in response to the expectation of patients (67). Long-term use of antibiotics and dosing of these drugs have become a major concern in CF, leading to the emergence of resistance over time. Alternative treatment methods have been and are currently being explored to address this, including targeting bacterial virulence and resistance (66). Anti-virulence compounds such as quorum sensing inhibitors (67, 68) and iron chelation (71, 72) successfully prevented bacterial aggregate, inhibited biofilm formation, reduced pathogenicity, and increased susceptibility to traditional antimicrobials. Strategies targeting resistance have included investigating efflux pump inhibitors (73, 74), anti-sense oligomers (75, 76), immunotherapy (77), host defence peptides (78, 79) and bacteriophages (4). Many of these strategies are still in the exploration and validation phases and are years off from translating to clinical care practice. The vital need for swift translation of alternative therapy into clinical use has identified phage therapy as the top candidate due to its successful use in humans when approved on compassionate grounds.

Phage therapy is highly applicable in CF, where most healthcare costs are associated with recurrent hospital visits due to chronic

bacterial infections (80). It holds many advantages over conventional antibiotic treatments used in CF, including shorter treatment periods (81–83), bacterial specificity (84), potent efficacy (85) and low toxicity (86). In the last 5 years, phage therapy has successfully been used to treat more than 30 individuals with CF (Supplementary Table S1) against various infections, including *P. aeruginosa*, *S. aureus*, *B. dolosa*, and *M. abscessus*. Phages were used singularly or in combination with other phages, typically called phage cocktails (Supplementary Table S1). Various treatment administration routes were also observed, including intravenous injection, oral or inhalation. Of significance were the reported improved clinical benefits (including a reduction in sputum and cough and improved lung function) but, more importantly, infection eradication. Furthermore, phage therapy was well tolerated, with no adverse events reported. Of these cases, the largest involved 20 patients (primarily individuals with CF), where mycobacterial infections were successfully treated with phage (87). Patients were well-tolerated with treatment, and phage resistance was not observed (87). Recently, a multi-center clinical trial was successfully conducted assessing the safety, tolerability, pharmacokinetics, and pharmacodynamics of a multi-phage candidate in people with CF suffering chronic *P. aeruginosa* pulmonary infections (88). Encouragingly, results showed that the formulation was well tolerated, was effectively delivered to the site of infection, and reduced bacterial load in participants (88).

## Airway mucus and CF

Normal airway mucus is a soft hydrogel composed of 90-95% water, mucus lipids and mucus proteins (such as glycoproteins) and ~1–5% high-molecular-weight mucins (89). Mucus forms a protective layer above the airway epithelium to trap airborne particles, including pathogens. Under normal circumstances, cell surface glycosylation is attributed to the gene expression of glycosyltransferases, as these enzymes are involved in the biosynthesis of glycan products (90, 91). The O-linked glycosylation is when glycans (carbohydrates/oligosaccharides) are added to mucins. A previous study by our group has demonstrated distinct mucins and glycosyltransferase profiles before and after rhinovirus infection (92). Not only did the expression of mucins vary between primary cells from CF and non-CF individuals, but the glycosyltransferases which form the eight core types of mucin O-glycans were also differentially expressed. These glycosyltransferases, including galactose, N-acetylglucosamine, fucose and terminal sialic acid or sulphate, form the various glycan moieties on the mucosal surface (93). The dysregulated mucin production suggests that other regulatory mechanisms, such as appropriate mucin packaging and secretion, might compromise CF cells (92). These variations are also linked to increased bacterial infection and inflammation due to the altered biomolecular properties of CF mucus (17, 94). Whether applying phage therapy to treat chronic infection in CF could further alter the glycosyltransferases profile of CF mucus requires a thorough understanding of the phage behavior and efficacy in the CF airway.

Before clinical translation, safety and efficacy assessment *in vivo* needs to be addressed, and small animal models have been typically used in this setting (95, 96). A systematic review assessing phage efficacy in various *in vivo* infection models showed significantly improved survival with treatment and reduced bacterial tissue burden (97). In addition, animal models have been used to effectively assess phage-antibiotic interactions (98) and the development of phage resistance (99). Furthermore, when combined with mathematical modelling, they have been used to quantify the dose and route of phage administration to fully capture phage efficacy, bacterial kinetics, and animal outcomes. The authors suggested that when accounting for host immune responses (mucins, cytokine, immune cells), this model could characterize the synergism between phages and the host innate immune system on bacteria elimination (100). In the setting of CF, animal models, including βENaC-Tg mice, CF pigs, CFTR rats, and ferrets, have all shed light on the early onset and progression of lung disease (101–105). However, these models have several limitations, including that CF pigs lack effective host defence mechanisms against bacterial pathogens. The CF ferrets model develop severe CF-like lung disease rapidly despite antibiotic prophylaxis and exhibits spontaneous lung infection which requires ongoing high levels of care (102, 103). With no single animal model completely recapitulating progressive CF lung disease, assessing phage efficacy in this setting may prove challenging (106).

One alternative is (107) primary airway epithelial cells obtained from paediatric CF airways that can be established *in vitro* and subsequently infected with pathogens and/or phages. Work conducted has shown little impact on to host airway, with no viability loss or significant inflammatory responses to phage reported (108). However, primary monolayer cultures do not fully reflect the CF mucosal epithelium. In order to more accurately recapitulate the *in vivo* CF airway, a more sophisticated proxy should be employed. Specifically, airway cells established at air-liquid interface (ALI) undergo differentiation and polarization, forming a mixed population of epithelial cells (ciliated, basal, goblet cells) to mimic the *in vivo* condition (109, 110). Importantly, since these models also contain goblet cells that produce mucus, it enables researchers to investigate the interplay between phage, mucus and pathogens and how the unique mucus properties seen in CF affect these relationships.

## The interplay between CF mucus, pathogens and phage

The defective airway physiology of CF impairs mucociliary clearance, triggers thick, dense mucus production, promotes the establishment of microbes and affects host immune responses to infection and inflammation. In these environments, including the CF airway, bacteria live in aggregates forming clusters of communities suspended within the airway mucus and its self-secreted protein, called biofilms (53, 111). Although an ideal phage formulation to treat CF lung infection and penetrate biofilms is yet to be identified, phage cocktails targeting different receptors have effectively reduced the emergence of bacteriophage insensitive mutants (BIMs) (112). Others have also shown that concomitant treatment of phages and antibiotics (also termed phage-antibiotic synergy; PAS) results in synergistic efficacy and reduced antibiotic resistance (56, 57, 113). Several case studies have reported favorable outcomes of phage-antibiotic treatments in various scenarios (13, 14, 98, 114–116) Specifically and pertinent to this review, an adult with CF suffering a multi-drug resistance *P. aeruginosa* infection was successfully treated with a combination of a multi-phage cocktail and ciprofloxacin andpiperacillin–tazobactam (14).

Despite this, phage activity is often limited by biofilm formation due to the impermeability of the biofilm matrix, preventing phages from reaching their receptors on the bacteria cell membrane. However, certain phages encode capsular depolymerase, which binds and breaks down the polysaccharide layer of bacteria for binding of phages to the receptors on bacteria surface (117) or degradation of exopolysaccharides which enable biofilm penetration, and hence is subsequently bactericidal (117–119). However, the effects of CF mucus on phage-bacteria/biofilm activity in the CF airway are still largely unknown and require investigation.

In response to phage, several bacterial defence mechanisms are activated, including modification of surface receptors (120, 121), prevention of phage DNA injection into bacteria cells (122), cleavage of phage DNA (123, 124), and finally, suicidal induction of infected bacterial cells (125, 126). However, whether CF mucus facilitates phage resistance while treating a chronic bacterial infection in CF lung is currently unknown. A recent study has provided some insight into this (130). Specifically, the authors demonstrated that the co-existence of a fish pathogen *Flavobacterium columnare* and their virulent phage in the presence of mucin lead to a significant increase in phage resistance, particularly CRISPR-Cas (an adaptive immune system that recognizes phage genomes) (127). The presence of static CF mucus flakes in the human lung creates a low-oxygen environment with an accompanying anaerobic layer (128). Although this may facilitate bacterial attachment, it could stimulate biofilm growth with the generation of dormant persister cells in its deeper layers, inhibiting phage propagation since nutrient resources are scarce.

## The potential impact of CF mucus on phage therapy

The type of phages within CF lungs appears more similar, mainly derived from the pathogens that persist in the CF airways for longer, compared to a more diverse phage population in healthy airways (129) (Figure 2). However, very limited studies have reported the phage-mucus interactions in the airway and the potential impacts of phage efficacy on respiratory bacterial infection. Recent evidence suggests that phages can bind to aspects of normal mucus that improve their bactericidal activity (11). However, little is known about how the CF mucus impacts phage functionality (Figure 2). Phage glycan-binding proteins bind to the bacterial surface for infection, while glycan ligands

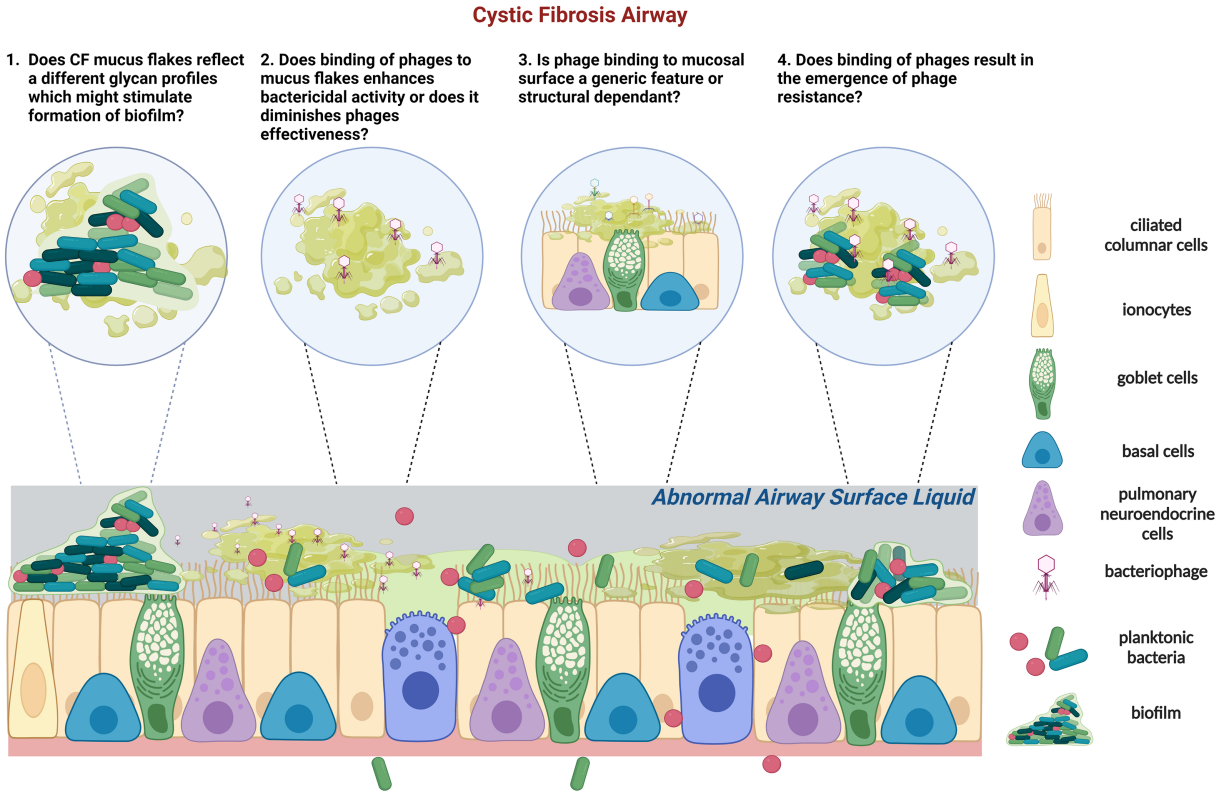



FIGURE 2
Schematic representation of the tripartite (phage-bacteria-epithelial cells) of CF epithelium is characterized by low airway surface liquid and thick and dense mucus containing mucus flakes and hyperconcentrated mucins. In CF, commensal bacteria and phage communities are dysregulated following long-term pathogenic chronic bacterial infection with dominant species and limited phages. (1) In addition, glycans that form mucins can also regulate the adhesion of *P. aeruginosa*. It is unknown if CF mucus flakes constitute a different glycan profile which could stimulate biofilm formation. (2) Phages can adhere to mucin directly; it is unknown how or if phages bind to CF mucus flakes directly and if their capsid proteins are subseqeuntly altered. Adherence of phage to CF mucus may facilitate phage enrichment and persistence within the microenvironment, resulting in a potent phage phenotype or vice versa. (3) Previous studies identified that certain phages, but not all, bind to mucins and diminish bacterial killing activity. Of concern, it is unknown if the binding activity of phages to mucin is a generic feature or size and structure dependent. (4) Importantly, if CF mucus results in the emergence of phage resistance which contradicts the theory of virulence enhancement by mucus, other therapeutic strategies might need to be applied in conjunction with the proposed phage therapy in CF. Created with BioRender.com.

on the surrounding environment, including those on mucins, are modified to promote phage retention in the gastrointestinal tracts (130). Barr et al. (131) have demonstrated the binding of a coliphage to mucin through the Ig-like domains in its capsid proteins called T4 Hoc protein (131). This binding increased the retention of T4 in the environment, allowing more interaction between phage and *E. coli* and serving as a critical regulator for phage-mediated bacterial lysis (132). Other work in non-respiratory research has shown that phages adhering to mucin have enhanced virulence toward bacteria (11, 133), empowering the chances of phage encountering bacterial hosts and providing additional mucosal immunity protection. Almeida et al. (133) demonstrated using a natural infection system that tailed phages with Ig-like domains in the phage capsids preferentially bind to mucin-containing agar. Phage concentration was previously found to be 4.4-fold higher within the mucus layer and associated with phage enrichment (134). Phages adhere to mucus glycans through weak binding interactions with the capsid proteins (127). This binding mechanism enables the subdiffusive motion of phages within mucosal surfaces, providing notably enhanced encounter rates with bacterial hosts (131, 135). These benefits allow mucus-adherent phage to propagate throughout the mucus layer, forming a non-host-derived layer of immunity. In addition, the direct evolutionary benefits of phage binding to the mucosal surface are the increased cost of bacterial virulence and modifications of bacterial phenotype to be more susceptible to phage infections in *Flavobacterium columnare* and *Aeromonas* sp. (133).

In contrast, Green et al. (136) found that incubating certain phages with porcine intestinal mucins reduced or inhibited bacterial killing. The bactericidal activity was restored after adding the mucolytic agent N-acetyl cysteine (NAC). Nevertheless, the same study identified a novel phage ES17, whose bactericidal activity was enhanced by binding to human heparan sulfated proteoglycans in mucus, forming a protective layer on the intestinal epithelium (136). In the CF airway, it is unknown how or if phages bind to CF mucus or the airway glycans directly and if phage binding enhances the bactericidal activity and virulence or vice versa, as observed in the gut lines previously.

In addition to regulating phages, glycans that form mucins can also regulate the physiology *P. aeruginosa*, including virulence and adhesion (137). A recent *in vitro* study found that *P. aeruginosa* induces contractions of luminal mucus, which accelerates bacterial aggregation and biofilm formation. This study showed that the host mucus production protects epithelium from acute virulence yet provides a breeding ground for biofilm and chronic infections (138). In the scenario of CF, dysregulated mucin production may enhance biofilm formation on the mucosal surface, however, it may counteract the positive interaction of mucus and phages on bactericidal activity. Future research addressing whether mucus increases or decreases the chance of phages and bacterial interaction, virulence, and enhancing phage resistance within the mucus layers may be key to successful phage therapy in CF.

The mucin content assessment in BALf of young children with CF has shown both elevated mucin concentration and the presence of mucus flakes evident very early in life (17). Mucus and mucin polymers, including MUC5AC, MUC2 and MUC5B, have been found to substantially diminish the activity of polymyxin and fluoroquinolone antibiotics against *P. aeruginosa* (139). However, what remains unknown is whether phages bind to CF mucus directly and if their capsid proteins are resultingly altered. The consideration here is whether this mucus will alter the glycan residues on mucin, affecting bacteria binding and modifying the efficacy of phage therapy. Would it be possible for this inhibition to also allow for increased predation as the bacteria move within the mucin microenvironment? Future work should assess if CF mucus can create an antimicrobial layer that reduces bacterial attachment and lessens epithelial cell death, as observed in a gut cell model (11). Furthermore, investigations are warranted to investigate whether phages retained in the CF mucus layer facilitate phage enrichment and persistence within the microenvironment, resulting in a potent phage phenotype or vice versa.

## Conclusion

In summary, AMR in CF has long-term clinical consequences, and the hyper-concentrated mucus with a dysfunctional structure strikingly impacts CF airways, providing the right environment for chronic bacterial infections. With little investment in discovering new antibiotics, assessing the implementation of phage therapy as an alternative therapeutic strategy for AMR pulmonary infections is critical. This may include using 3D airway cultures to examine phage tropism for CF pathogens and determine the impact of phage therapy on bacterial biofilm penetration. In addition, research is needed to elucidate the interactive relationships between phage, CF pathogens and the host airway epithelium, including impacts of the dehydrated mucus typical of the CF airway. Furthermore, a recent study suggests that phage may be able to infect a much broader repertoire of bacteria beyond a single species (140). This raises the question of whether they also cause microbiome dysbiosis in the lung by infecting the resident commensal population. All these necessary research pieces must be conducted to understand the translational implications of such a therapy in CF. Many unanswered questions include determining the best administration route, concomitant use of antibiotics or mucolytic agents, length of the treatment period, and phage formulation. Furthermore, additional basic research is needed to predict these parameters and accurately measure host immune responses.

## Author contributions

K-ML, SS, and AK conceptualized the contents of the manuscript, critically reviewed, and edited the manuscript. K-ML wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

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## Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2023.1088494/full#supplementary-material

## References


1. O'Neil J. (2014). Antimicrobial resistance: Tackling a crisis for the health and wealth of nations. Available at: https://www.who.int/news/item/29-04-2019-new-report-calls-for-urgent-action-to-avert-antimicrobial-resistance-crisis [Accessed August 31, 2021]

2. Anitha Kopinathan, Halili Maria A., Begona Heras, Scanlon Martin J., Makrina Totsika, JL Martin AC. (2021). The next pandemic: antimicrobial resistance - Curious. Available at: https://www.science.org.au/curious/policy-features/next-pandemic-antimicrobial-resistance [Accessed November 23, 2021]

3. Flume PA, Suthoff ED, Kosinski M, Marigowda G, Quittner AL. Measuring recovery in health-related quality of life during and after pulmonary exacerbations in patients with cystic fibrosis. *J Cyst Fibros*. (2019) 18:737–42. doi: 10.1016/j.jcf.2018.12.004

4. Chan BK, Abedon ST. Phage therapy pharmacology. Phage cocktails In Allen I Laskin, Sima Sariaslani, Geoffrey M Gadd, editors. *Advances in applied microbiology. 78*: Taylor & Francis (2012). 1–23.

5. Howard-Jones AR, Iredell JR, Khatami A. Phage therapy in pediatrics: the way forward for difficult-to-treat infections? *Expert Rev Anti-Infect Ther*. (2022) 20:487–91. doi: 10.1080/14787210.2022.1990755

6. Chan BK, Stanley G, Modak M, Koff JL, Turner PE. Bacteriophage therapy for infections in CF. *Pediatr Pulmonol*. (2021) 56:S4–9. doi: 10.1002/ppul.25190

7. Trend S, Chang BJ, O'Dea M, Stick SM, Kicic A. Use of a primary epithelial cell screening tool to investigate phage therapy in cystic fibrosis. *Front Pharmacol*. (2018) 9:1330. doi: 10.3389/fphar.2018.01330

8. Shan J, Korbsrisate S, Withatanung P, Lazar Adler NJ, Clokie MR, Galyov EE, et al. Temperature dependent bacteriophages of a tropical bacterial pathogen. *Front Microbiol*. (2014) 5:599. doi: 10.3389/fmicb.2014.00599

9. Erez Z, Steinberger-Levy I, Shamir M, Doron S, Stokar-Avihail A, Peleg Y, et al. Communication between viruses guides lysis–lysogeny decisions. *Nature*. (2017) 541:488–93. doi: 10.1038/nature21049

10. Clokie MRJ, Millard AD, Letarov AV, Heaphy S. Phages in nature. *Bacteriophage*. (2011) 1:31–45. doi: 10.4161/bact.1.1.14942

11. Chin WH, Kett C, Cooper O, Müseler D, Zhang Y, Bamert RS, et al. Bacteriophages evolve enhanced persistence to a mucosal surface. *Proc Natl Acad Sci U S A*. (2021) 119:e2116197119. doi: 10.2139/ssrn.3871902

12. Kutateladze M, Adamia R. Phage therapy experience at the Eliava institute. *Med Mal Infect*. (2008) 38:426–30. doi: 10.1016/j.medmal.2008.06.023

13. Aslam S, Courtwright AM, Koval C, Lehman SM, Morales S, Furr CLL, et al. Early clinical experience of bacteriophage therapy in 3 lung transplant recipients. *Am J Transplant*. (2019) 19:2631–9. doi: 10.1111/ajt.15503

14. Law N, Logan C, Yung G, Furr CLL, Lehman SM, Morales S, et al. Successful adjunctive use of bacteriophage therapy for treatment of multidrug-resistant *Pseudomonas aeruginosa* infection in a cystic fibrosis patient. *Infection*. (2019) 47:665–8. doi: 10.1007/S15010-019-01319-0

15. Dedrick RM, Guerrero-Bustamante CA, Garlena RA, Russell DA, Ford K, Harris K, et al. Engineered bacteriophages for treatment of a patient with a disseminated drug-resistant *Mycobacterium abscessus*. *Nat Med*. (2019) 25:730–3. doi: 10.1038/s41591-019-0437-z

16. Kvachadze L, Balarjishvili N, Meskhi T, Tevdoradze E, Skhirtladze N, Pataridze T, et al. Evaluation of lytic activity of staphylococcal bacteriophage Sb-1 against freshly isolated clinical pathogens. *Microb Biotechnol*. (2011) 4:643–50. doi: 10.1111/j.1751-7915.2011.00259.x

17. Esther CR, Muhlebach MS, Ehre C, Hill DB, Wolfgang MC, Kesimer M, et al. Mucus accumulation in the lungs precedes structural changes and infection in children with cystic fibrosis. *Sci Transl Med*. (2019) 11:eaav3488. doi: 10.1126/scitranslmed.aav3488

18. Muhlebach MS, Zorn BT, Esther CR, Hatch JE, Murray CP, Turkovic L, et al. Initial acquisition and succession of the cystic fibrosis lung microbiome is associated with disease progression in infants and preschool children. *PLoS Pathog*. (2018) 14:e1006798. doi: 10.1371/journal.ppat.1006798

19. Tang AC, Turvey SE, Alves MP, Regamey N, Tümmler B, Hartl D. Current concepts: host-pathogen interactions in cystic fibrosis airways disease. *Eur Respir Rev*. (2014) 23:320–32. doi: 10.1183/09059180.00006113

20. Stutts MJ, Canessa CM, Olsen JC, Hamrick M, Cohn JA, Rossier BC, et al. CFTR as a cAMP-dependent regulator of sodium channels. *Science*. (1995) 269:847–50. doi: 10.1126/science.7543698

21. Guggino WB, Banks-Schlegel SP. Macromolecular interactions and ion transport in cystic fibrosis. *Am J Respir Crit Care Med*. (2004) 170:815–20. doi: 10.1164/rccm.200403-381WS

22. Gentzsch M, Dang H, Dang Y, Garcia-Caballero A, Suchindran H, Boucher RC, et al. The cystic fibrosis transmembrane conductance regulator impedes proteolytic stimulation of the epithelial $Na^+$ channel. *J Biol Chem*. (2010) 285:32227–32. doi: 10.1074/jbc.M110.155259

23. Foster WMF. Mucociliary function In: RA Parent, editor. *Comparative biology of the Normal lung. Second* ed. Amsterdam: Academic Press (2015). 561–79.

24. Knowles M, Gatzy J, Boucher R. Increased bioelectric potential difference across respiratory epithelia in cystic fibrosis. *N Engl J Med*. (1981) 305:1489–95. doi: 10.1056/nejm198112173052502

25. Matsui H, Grubb BR, Tarran R, Randell SH, Gatzy JT, Davis CW, et al. Evidence for periciliary liquid layer depletion, not abnormal ion composition, in the pathogenesis of cystic fibrosis airways disease. *Cells*. (1998) 95:1005–15. doi: 10.1016/S0092-8674(00)81724-9

26. Proesmans M, Vermeulen F, De Boeck K. What's new in cystic fibrosis? From treating symptoms to correction of the basic defect. *Eur J Pediatr*. (2008) 167:839–49. doi: 10.1007/s00431-008-0693-2

27. Button B, Cai LH, Ehre C, Kesimer M, Hill DB, Sheehan JK, et al. A periciliary brush promotes the lung health by separating the mucus layer from airway epithelia. *Science*. (2012) 337:937–41. doi: 10.1126/science.1223012

28. Drew KRP, Sanders LK, Culumber ZW, Zribi O, Wong GCL. Cationic amphiphiles increase activity of aminoglycoside antibiotic tobramycin in the presence of airway polyelectrolytes. *J Am Chem Soc*. (2009) 131:486–93. doi: 10.1021/ja803925n

29. Leal J, Smyth HDC, Ghosh D. Physicochemical properties of mucus and their impact on transmucosal drug delivery. *Int J Pharm*. (2017) 532:555–72. doi: 10.1016/j.ijpharm.2017.09.018

30. Alipour M, Suntres ZE, Omri A. Importance of DNase and alginate lyase for enhancing free and liposome encapsulated aminoglycoside activity against Pseudomonas aeruginosa. *J Antimicrob Chemother*. (2009) 64:317–25. doi: 10.1093/jac/dkp165

31. Mendelman PM, Smith AL, Levy J, Weber A, Ramsey B, Davis RL. Aminoglycoside penetration, inactivation, and efficacy in cystic fibrosis sputum. *Am Rev Respir Dis*. (1985) 132:761–5. doi: 10.1164/ARRD.1985.132.4.761

32. Akkerman-Nijland AM, Akkerman OW, Grasmeijer F, Hagedoorn P, Frijlink HW, Rottier BL, et al. The pharmacokinetics of antibiotics in cystic fibrosis. *Expert Opin Drug Metab Toxicol*. (2021) 17:53–68. doi: 10.1080/17425255.2021.1836157

33. Downey DG, Bell SC, Elborn JS. Neutrophils in cystic fibrosis. *Thorax*. (2009) 64:81–8. doi: 10.1136/thx.2007.082388

34. Pillarisetti N, Williamson E, Linnane B, Skoric B, Robertson CF, Robinson P, et al. Infection, inflammation, and lung function decline in infants with cystic fibrosis. *Am J Respir Crit Care Med*. (2011) 184:75–81. doi: 10.1164/rccm.201011-1892OC

35. Sly PD, Gangell CL, Chen L, Ware RS, Ranganathan S, Mott LS, et al. Risk factors for bronchiectasis in children with cystic fibrosis. *N Engl J Med*. (2013) 368:1963–70. doi: 10.1056/NEJMoa1301725

36. Breuer O, Schultz A, Turkovic L, de Klerk N, Keil AD, Brennan S, et al. Changing prevalence of lower airway infections in young children with cystic fibrosis. *Am J Respir Crit Care Med*. (2019) 200:590–9. doi: 10.1164/rccm.201810-1919OC

37. Bhatt JM. Treatment of pulmonary exacerbations in cystic fibrosis. *Eur Respir Rev*. (2013) 22:205–16. doi: 10.1183/09059180.00006512

38. Aaron SD, Ramotar K, Ferris W, Vandemheen K, Saginur R, Tullis E, et al. Adult cystic fibrosis exacerbations and new strains of Pseudomonas aeruginosa. *Am J Respir Crit Care Med*. (2004) 169:811–5. doi: 10.1164/rccm.200309-1306oc

39. VanDevanter DR, van Dalfsen JM. How much do *Pseudomonas* biofilms contribute to symptoms of pulmonary exacerbation in cystic fibrosis? *Pediatr Pulmonol*. (2005) 39:504–6. doi: 10.1002/ppul.20220

40. Stressmann FA, Rogers GB, Marsh P, Lilley AK, Daniels TWV, Carroll MP, et al. Does bacterial density in cystic fibrosis sputum increase prior to pulmonary exacerbation? *J Cyst Fibros*. (2011) 10:357–65. doi: 10.1016/j.jcf.2011.05.002

41. Martínez-Alemán SR, Campos-García L, Palma-Nicolas JP, Hernández-Bello R, González GM, Sánchez-González A. Understanding the entanglement: neutrophil extracellular traps (NETs) in cystic fibrosis. *Front Cell Infect Microbiol*. (2017) 7:104. doi: 10.3389/fcimb.2017.00104

42. Miall LS, McGinley NT, Brownlee KG, Conway SP. Methicillin resistant Staphylococcus aureus (MRSA) infection in cystic fibrosis. *Arch Dis Child*. (2001) 84:160–2. doi: 10.1136/adc.84.2.160

43. Sherrard LJ, Tunney MM, Elborn JS. Antimicrobial resistance in the respiratory microbiota of people with cystic fibrosis. *Lancet*. (2014) 384:703–13. doi: 10.1016/S0140-6736(14)61137-5

44. Firmida MC, Pereira RHV, Silva EASR, Marques EA, Lopes AJ. Clinical impact of *Achromobacter xylosoxidans* colonization/infection in patients with cystic fibrosis. *Braz J Med Biol Res*. (2016) 49. doi: 10.1590/1414-431X20155097

45. Skolnik K, Kirkpatrick G, Quon BS. Nontuberculous mycobacteria in cystic fibrosis. *Curr Treat Options Infect Dis*. (2016) 8:259–74. doi: 10.1007/s40506-016-0092-6

46. Barsky EE, Williams KA, Priebe GP, Sawicki GS. Incident *Stenotrophomonas maltophilia* infection and lung function decline in cystic fibrosis. *Pediatr Pulmonol*. (2017) 52:1276–82. doi: 10.1002/ppul.23781

47. Wang X, Koehne-Voss S, Anumolu SNS, Yu J. Population pharmacokinetics of tobramycin inhalation solution in pediatric patients with cystic fibrosis. *J Pharm Sci*. (2017) 106:3402–9. doi: 10.1016/j.xphs.2017.06.010

48. Taccetti G, Francalanci M, Pizzamiglio G, Messore B, Carnovale V, Cimino G, et al. Cystic fibrosis: recent insights into inhaled antibiotic treatment and future perspectives. *Antibiotics*. (2021) 10:338. doi: 10.3390/antibiotics10030338

49. Van de Kerkhove C, Goeminne PC, Kicinski M, Nawrot TS, Lorent N, Van Bleyenbergh P, et al. Continuous alternating inhaled antibiotic therapy in CF: a single center retrospective analysis. *J Cyst Fibros*. (2016) 15:802–8. doi: 10.1016/j.jcf.2016.09.002

50. Castellani C, Duff AJA, Bell SC, Heijerman HGM, Munck A, Ratjen F, et al. ECFS best practice guidelines: the 2018 revision. *J Cyst Fibros*. (2018) 17:153–78. doi: 10.1016/j.jcf.2018.02.006

51. Esquivel MD, Monogue ML, Smith GS, Finklea JD, Sanders JM. Ceftaroline versus vancomycin for treatment of acute pulmonary exacerbations of cystic fibrosis in adults. *J Glob Antimicrob Resist*. (2022) 28:67–70. doi: 10.1016/j.jgar.2021.12.008

52. Currie G, Tai A, Snelling T, Schultz A. Variation in treatment preferences of pulmonary exacerbations among Australian and New Zealand cystic fibrosis physicians. *BMJ Open Respir Res*. (2021) 8:e000956. doi: 10.1136/bmjresp-2021-000956

53. Matsui H, Wagner VE, Hill DB, Schwab UE, Rogers TD, Button B, et al. A physical linkage between cystic fibrosis airway surface dehydration and *Pseudomonas aeruginosa* biofilms. *Proc Natl Acad Sci U S A*. (2006) 103:18131–6. doi: 10.1073/pnas.0606428103

54. Samonis G, Karageorgopoulos DE, Maraki S, Levis P, Dimopoulou D, Spernovasilis NA, et al. *Stenotrophomonas maltophilia* infections in a general hospital: patient characteristics, antimicrobial susceptibility, and treatment outcome. *PLoS One*. (2012) 7:e37375. doi: 10.1371/journal.pone.0037375

55. López-Causapé C, Rojo-Molinero E, Maclà MD, Oliver A. The problems of antibiotic resistance in cystic fibrosis and solutions. *Expert Rev Respir Med*. (2015) 9:73–88. doi: 10.1586/17476348.2015.995640

56. Kamal F, Dennis JJ. Burkholderia cepacia complex phage-antibiotic synergy (PAS): antibiotics stimulate lytic phage activity. *Appl Environ Microbiol*. (2015) 81:1132–8. doi: 10.1128/AEM.02850-14

57. Uchiyama J, Shigehisa R, Nasukawa T, Mizukami K, Takemura-Uchiyama I, Ujihara T, et al. Piperacillin and ceftazidime produce the strongest synergistic phage–antibiotic effect in *Pseudomonas aeruginosa*. *Arch Virol*. (2018) 163:1941–8. doi: 10.1007/s00705-018-3811-0

58. Isler B, Kidd TJ, Stewart AG, Harris P, Paterson DL. Achromobacter infections and treatment options. *Antimicrob Agents Chemother*. (2020) 64. doi: 10.1128/AAC.01025-20

59. Beaudoin T, Yau YCW, Stapleton PJ, Gong Y, Wang PW, Guttman DS, et al. *Staphylococcus aureus* interaction with *Pseudomonas aeruginosa* biofilm enhances tobramycin resistance. *NPJ Biofilms Microbio*. (2017) 3:25. doi: 10.1038/S41522-017-0035-0

60. Li J, Turnidge J, Milne R, Nation RL, Coulthard K. In vitro pharmacodynamic properties of colistin and colistin methanesulfonate against *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis. *Antimicrob Agents Chemother*. (2001) 45:781–5. doi: 10.1128/AAC.45.3.781-785.2001

61. Johansen HK, Moskowitz SM, Ciofu O, Pressler T, Høiby N. Spread of colistin resistant non-mucoid *Pseudomonas aeruginosa* among chronically infected Danish cystic fibrosis patients. *J Cyst Fibros*. (2008) 7:391–7. doi: 10.1016/j.jcf.2008.02.003

62. Lim LM, Ly N, Anderson D, Yang JC, Macander L, Jarkowski A, et al. Resurgence of colistin: a review of resistance, toxicity, pharmacodynamics, and dosing. *Pharmacotherapy*. (2010) 30:1279–91. doi: 10.1592/phco.30.12.1279

63. Moskowitz SM, Brannon MK, Dasgupta N, Pier M, Sgambati N, Miller AK, et al. PmrB mutations promote polymyxin resistance of *Pseudomonas aeruginosa* isolated from colistin-treated cystic fibrosis patients. *Antimicrob Agents Chemother*. (2012) 56:1019–30. doi: 10.1128/AAC.05829-11

64. Ahern S, Salimi FE, Caruso M, Ruseckaite R, Bell S, Burke N. Registry on behalf of the ACFD. The Australian Cystic Fibrosis Data Registry Annual Report 2020. (2021) https://research.monash.edu/en/publications/the-australian-cystic-fibrosis-data-registry-annual-report-2020 [Accessed April 19, 2023].

65. Chmiel JF, Aksamit TR, Chotirmall SH, Dasenbrook EC, Elborn JS, LiPuma JJ, et al. Antibiotic management of lung infections in cystic fibrosis: I. The microbiome, methicillin-resistant Staphylococcus aureus, gram-negative bacteria, and multiple infections. *Ann Am Thorac Soc*. (2014) 11:1120–1129. doi: 10.1513/ANNALSATS.201402-050AS/SUPPL_FILE/DISCLOSURES.PDF

66. Lauman P, Dennis JJ. Advances in Phage Therapy: Targeting the Burkholderia cepacia Complex. *Viruses*. (2021) 13. doi: 10.3390/V13071331

67. Miller BJ, Carson KA, Keller S. Educating patients on unnecessary antibiotics: personalizing potential harm aids patient understanding. *J Am Board Fam Med*. (2020) 33:969–77. doi: 10.3122/jabfm.2020.06.200210

68. Manos J. Current and emerging therapies to combat cystic fibrosis lung infections. *Microorganisms*. (2021) 9. doi: 10.3390/microorganisms9091874

69. Brackman G, Cos P, Maes L, Nelis HJ, Coenye T. Quorum sensing inhibitors increase the susceptibility of bacterial biofilms to antibiotics in vitro and in vivo. *Antimicrob Agents Chemother*. (2011) 55:2655–61. doi: 10.1128/AAC.00045-11

70. O'Loughlin CT, Miller LC, Siryaporn A, Drescher K, Semmelhack MF, Bassler BL. A quorum-sensing inhibitor blocks Pseudomonas aeruginosa virulence and biofilm formation. *Proc Natl Acad Sci U S A*. (2013) 110:17981–6. doi: 10.1073/pnas.1316981110

71. Moreau-Marquis S, O'Toole GA, Stanton BA. Tobramycin and FDA-approved iron chelators eliminate Pseudomonas aeruginosa biofilms on cystic fibrosis cells. *Am J Respir Cell Mol Biol*. (2009) 41:305–13. doi: 10.1165/rcmb.2008-0299OC

72. Kirienko NV, Haag AF, Nairz M, Holbein BE, del Carmen PM, Savage KA, et al. Novel iron-chelator DIBI inhibits Staphylococcus aureus growth, suppresses experimental MRSA infection in mice and enhances the activities of diverse antibiotics *in vitro*. *Front Microbiol*. (2018) 9:1811. doi: 10.3389/fmicb.2018.01811

73. Sabatini S, Gosetto F, Iraci N, Barreca ML, Massari S, Sancineto L, et al. Re-evolution of the 2-phenylquinolines: ligand-based design, synthesis, and biological evaluation of a potent new class of staphylococcus aureus NorA efflux pump inhibitors to combat antimicrobial resistance. *J Med Chem*. (2013) 56:4975–89. doi: 10.1021/jm400262a

74. Shriram V, Khare T, Bhagwat R, Shukla R, Kumar V. Inhibiting bacterial drug efflux pumps via phyto-therapeutics to combat threatening antimicrobial resistance. *Front Microbiol*. (2018) 9:2990. doi: 10.3389/fmicb.2018.02990

75. Sawyer AJ, Wesolowski D, Gandotra N, Stojadinovic A, Izadjoo M, Altman S, et al. A peptide-morpholino oligomer conjugate targeting *Staphylococcus aureus* gyrA mRNA improves healing in an infected mouse cutaneous wound model. *Int J Pharm*. (2013) 453:651–5. doi: 10.1016/j.ijpharm.2013.05.041

76. Geller BL, Marshall-Batty K, Schnell FJ, McKnight MM, Iversen PL, Greenberg DE. Gene-silencing antisense oligomers inhibit acinetobacter growth *in vitro* and *in vivo*. *J Infect Dis*. (2013) 208:1553–60. doi: 10.1093/infdis/jit460

77. Feigman MS, Kim S, Pidgeon SE, Yu Y, Ongwae GM, Patel DS, et al. Synthetic immunotherapeutics against Gram-negative pathogens. *Cell Chem Biol*. (2018) 25:1185–1194.e5. doi: 10.1016/j.chembiol.2018.05.019

78. Overhage J, Campisano A, Bains M, Torfs ECW, Rehm BHA, Hancock REW. Human host defense peptide LL-37 prevents bacterial biofilm formation. *Infect Immun*. (2008) 76:4176–82. doi: 10.1128/IAI.00318-08

79. Rázquin-Olazarán I, Shahrour H, Martínez-De-Tejada G. A synthetic peptide sensitizes multi-drug resistant *Pseudomonas aeruginosa* to antibiotics for more than two hours and permeabilizes its envelope for twenty hours. *J Biomed Sci*. (2020) 27:85. doi: 10.1186/s12929-020-00678-3

80. Sansgiry SS, Joish VN, Boklage S, Goyal RK, Chopra P, Sethi S. Economic burden of *Pseudomonas aeruginosa* infection in patients with cystic fibrosis. *J Med Econ*. (2011) 15:219–24. doi: 10.3111/13696998.2011.638954

81. Capparelli R, Nocerino N, Iannaccone M, Ercolini D, Parlato M, Chiara M, et al. Bacteriophage therapy of *Salmonella enterica*: a fresh appraisal of bacteriophage therapy. *J Infect Dis*. (2010) 201:52–61. doi: 10.1086/648478

82. Sarker SA, McCallin S, Barretto C, Berger B, Pittet A-C, Sultana S, et al. Oral T4-like phage cocktail application to healthy adult volunteers from Bangladesh. *Virology*. (2012) 434:222–32. doi: 10.1016/j.virol.2012.09.002

83. Pouillot F, Chomton M, Blois H, Courroux C, Noelig J, Bidet P, et al. Efficacy of bacteriophage therapy in experimental sepsis and meningitis caused by a clone O25b: H4-ST131 *Escherichia coli* strain producing CTX-M-15. *Antimicrob Agents Chemother*. (2012) 56:3568–75. doi: 10.1128/AAC.06330-11

84. Hyman P, Abedon ST. "Bacteriophage host range and bacterial resistance" In Allen I Laskin, Sima Sariaslani, Geoffrey M Gadd, editors. *Advances in applied microbiology*: Academic Press (2010). 217–48.

85. Shan J, Ramachandran A, Thanki AM, Vukusic FBI, Barylski J, Clokie MRJ. Bacteriophages are more virulent to bacteria with human cells than they are in bacterial culture; insights from HT-29 cells. *Sci Rep*. (2018) 8:1–8. doi: 10.1038/s41598-018-23418-y

86. Petrovic Fabijan A, Lin RCY, Ho J, Maddocks S, Ben Zakour NL, Iredell JR. Safety of bacteriophage therapy in severe *Staphylococcus aureus* infection. *Nat Microbiol*. (2020) 5:465–72. doi: 10.1038/s41564-019-0634-z

87. Dedrick RM, Smith BE, Cristinziano M, Freeman KG, Jacobs-Sera D, Belessis Y, et al. Phage therapy of mycobacterium infections: compassionate use of phages in 20 patients with drug-resistant mycobacterial disease. *Clin Infect Dis*. (2023) 76:103–12. doi: 10.1093/cid/ciac453

88. Armata Pharmaceuticals Announces Positive Topline Data from Phase 1b/2a SWARM-P.a (2023). Clinical trial of inhaled AP-PA02 in patients with cystic fibrosis. Available at: https://www.prnewswire.com/news-releases/armata-pharmaceuticals-announces-positive-topline-data-from-phase-1b2a-swarm-pa-clinical-trial-of-inhaled-ap-pa02-in-patients-with-cystic-fibrosis-301762961.html [Accessed March 15, 2023].

89. Bansil R, Turner BS. The biology of mucus: composition, synthesis and organization. *Adv Drug Deliv Rev*. (2018) 124:3–15. doi: 10.1016/j.addr.2017.09.023

90. Ohtsubo K, Marth JD. Glycosylation in cellular mechanisms of health and disease. *Cells*. (2006) 126:855–67. doi: 10.1016/j.cell.2006.08.019

91. Neelamegham S, Mahal LK. Multi-level regulation of cellular glycosylation: from genes to transcript to enzyme to structure. *Curr Opin Struct Biol*. (2016) 40:145–52. doi: 10.1016/j.sbi.2016.09.013

92. Ling K. (2021). Innate immune response of airway epithelial cells from children with cystic fibrosis to rhinovirus infection Kak Ming Ling.

93. Lillehoj EP, Kato K, Lu W, Kim KC. "Cellular and molecular biology of airway mucins" In: Jeon Kwang W editor. *International review of cell and molecular biology*: Elsevier Inc (2013). 139–202.

94. Markovetz MR, Garbarine IC, Morrison CB, Kissner WJ, Seim I, Forest MG, et al. Mucus and mucus flake composition and abundance reflect inflammatory and infection status in cystic fibrosis. *J Cyst Fibros*. (2022) 21:959–66. doi: 10.1016/j.jcf.2022.04.008

95. Debarbieux L, Leduc D, Maura D, Morello E, Criscuolo A, Grossi O, et al. Bacteriophages can treat and prevent *Pseudomonas aeruginosa* lung infections. *J Infect Dis*. (2010) 201:1096–104. doi: 10.1086/651135

96. Waters EM, Neill DR, Kaman B, Sahota JS, Clokie MRJ, Winstanley C, et al. Phage therapy is highly effective against chronic lung infections with *Pseudomonas aeruginosa*. *Thorax*. (2017) 72:666–7. doi: 10.1136/thoraxjnl-2016-209265

97. Gómez-Ochoa SA, Pitton M, Valente LG, Sosa Vesga CD, Largo J, Quiroga-Centeno AC, et al. Efficacy of phage therapy in preclinical models of bacterial infection: a systematic review and meta-analysis. *Lancet Microbe*. (2022) 3:e956–68. doi: 10.1016/S2666-5247(22)00288-9

98. Lin Y, Quan D, Chang RYK, Chow MYT, Wang Y, Li M, et al. Synergistic activity of phage PEV20-ciprofloxacin combination powder formulation—a proof-of-principle study in a *P. aeruginosa* lung infection model. *Eur J Pharm Biopharm*. (2021) 158:166–71. doi: 10.1016/j.ejpb.2020.11.019

99. Gaborieau B, Debarbieux L. The role of the animal host in the management of bacteriophage resistance during phage therapy. *Curr Opin Virol*. (2023) 58:101290. doi: 10.1016/j.coviro.2022.101290

100. Delattre R, Seurat J, Haddad F, Nguyen TT, Gaborieau B, Kane R, et al. Combination of *in vivo* phage therapy data with in silico model highlights key parameters for pneumonia treatment efficacy. *Cell Rep*. (2022) 39. doi: 10.1016/j.celrep.2022.110825

101. Mall M, Grubb BR, Harkema JR, O'Neal WK, Boucher RC. Increased airway epithelial $Na^+$ absorption produces cystic fibrosis-like lung disease in mice. *Nat Med*. (2004) 10:487–93. doi: 10.1038/nm1028

102. Stoltz DA, Meyerholz DK, Pezzulo AA, Ramachandran S, Rogan MP, Davis GJ, et al. Cystic fibrosis pigs develop lung disease and exhibit defective bacterial eradication at birth. *Sci Transl Med*. (2010) 2:29ra31. doi: 10.1126/scitranslmed.3000928

103. Sun X, Olivier AK, Liang B, Yi Y, Sui H, Evans TIA, et al. Lung phenotype of juvenile and adult cystic fibrosis transmembrane conductance regulator–knockout ferrets. *Am J Respir Cell Mol Biol*. (2014) 50:502–12. doi: 10.1165/rcmb.2013-0261OC

104. Tuggle KL, Birket SE, Cui X, Hong J, Warren J, Reid L, et al. Characterization of defects in ion transport and tissue development in cystic fibrosis transmembrane conductance regulator (CFTR)-knockout rats. *PLoS One*. (2014) 9:e91253. doi: 10.1371/journal.pone.0091253

105. McCarron A, Cmielewski P, Reyne N, McIntyre C, Finnie J, Craig F, et al. Phenotypic characterization and comparison of cystic fibrosis rat models generated using CRISPR/Cas9 gene editing. *Am J Pathol*. (2020) 190:977–93. doi: 10.1016/j.ajpath.2020.01.009

106. Semaniakou A, Croll RP, Chappe V. Animal models in the pathophysiology of cystic fibrosis. *Front Pharmacol*. (2019) 9:1475. doi: 10.3389/fphar.2018.01475

107. Ng RN, Tai AS, Chang BJ, Stick SM, Kicic A. Overcoming challenges to make bacteriophage therapy standard clinical treatment practice for cystic fibrosis. *Front Microbiol*. (2021) 11:3389. doi: 10.3389/fmicb.2020.593988

108. Trend S, Chang BJ, O'Dea M, Stick SM, Kicic A. Use of a primary epithelial cell screening tool to investigate phage therapy in cystic fibrosis. *Front Pharmacol*. (2018) 9:1330. doi: 10.3389/fphar.2018.01330

109. BéruBé K, Prytherch Z, Job C, Hughes T. Human primary bronchial lung cell constructs: the new respiratory models. *Toxicology*. (2010) 278:311–8. doi: 10.1016/j.tox.2010.04.004

110. Pezzulo AA, Starner TD, Scheetz TE, Traver GL, Tilley AE, Harvey BG, et al. The air-liquid interface and use of primary cell cultures are important to recapitulate the transcriptional profile of in vivo airway epithelia. *Am J Phys Lung Cell Mol Phys*. (2011) 300. doi: 10.1152/ajplung.00256.2010

111. Ciofu O, Tolker-Nielsen T, Jensen PØ, Wang H, Høiby N. Antimicrobial resistance, respiratory tract infections and role of biofilms in lung infections in cystic fibrosis patients. *Adv Drug Deliv Rev*. (2015) 85:7–23. doi: 10.1016/j.addr.2014.11.017

112. O'Flynn G, Ross RP, Fitzgerald GF, Coffey A. Evaluation of a cocktail of three bacteriophages for biocontrol of *Escherichia coli* O157:H7. *Appl Environ Microbiol*. (2004) 70:3417–24. doi: 10.1128/AEM.70.6.3417-3424.2004

113. Liu CG, Green SI, Min L, Clark JR, Salazar KC, Terwilliger AL, et al. Phage-antibiotic synergy is driven by a unique combination of antibacterial mechanism of action and stoichiometry. *MBio*. (2020) 11:1–19. doi: 10.1128/mBio.01462-20

114. Chan BK, Turner PE, Kim S, Mojibian HR, Elefteriades JA, Narayan D. Phage treatment of an aortic graft infected with Pseudomonas aeruginosa. *Evol Med Public Health*. (2018) 2018:60–6. doi: 10.1093/emph/eoy005

115. Eskenazi A, Lood C, Wubbolts J, Hites M, Balarjishvili N, Leshkasheli L, et al. Combination of pre-adapted bacteriophage therapy and antibiotics for treatment of fracture-related infection due to pandrug-resistant Klebsiella pneumoniae. *Nat Commun*. (2022) 13:1–14. doi: 10.1038/s41467-021-27656-z

116. Nir-Paz R, Gelman D, Khouri A, Sisson BM, Fackler J, Alkalay-Oren S, et al. Successful treatment of antibiotic-resistant, poly-microbial bone infection with bacteriophages and antibiotics combination. *Clin Infect Dis*. (2019) 69:2015–8. doi: 10.1093/cid/ciz222

117. Sutherland IW, Hughes KA, Skillman LC, Tait K. The interaction of phage and biofilms. *FEMS Microbiol Lett*. (2004) 232:1–6. doi: 10.1016/S0378-1097(04)00041-2

118. Harper DR, Parracho HMRT, Walker J, Sharp R, Hughes G, Werthén M, et al. Bacteriophages and biofilms. *Antibiotics*. (2014) 3:270–84. doi: 10.3390/antibiotics3030270

119. Malik S, Nehra K, Rana JS. Bacteriophage cocktail and phage antibiotic synergism as promising alternatives to conventional antibiotics for the control of multi-drug-resistant uropathogenic *Escherichia coli*. *Virus Res*. (2021) 302:198496. doi: 10.1016/j.virusres.2021.198496

120. Harvey H, Bondy-Denomy J, Marquis H, Sztanko KM, Davidson AR, Burrows LL. *Pseudomonas aeruginosa* defends against phages through type IV pilus glycosylation. *Nat Microbiol*. (2017) 3:47–52. doi: 10.1038/s41564-017-0061-y

121. Burmeister AR, Fortier A, Roush C, Lessing AJ, Bender RG, Barahman R, et al. Pleiotropy complicates a trade-off between phage resistance and antibiotic resistance. *Proc Natl Acad Sci U S A*. (2020) 117:11207–16. doi: 10.1073/pnas.1919888117

122. Hampton HG, Watson BNJ, Fineran PC. The arms race between bacteria and their phage foes. *Nature*. (2020) 577:327–36. doi: 10.1038/s41586-019-1894-8

123. Ofir G, Melamed S, Sberro H, Mukamel Z, Silverman S, Yaakov G, et al. DISARM is a widespread bacterial defence system with broad anti-phage activities. *Nat Microbiol*. (2017) 3:90–8. doi: 10.1038/s41564-017-0051-0

124. Liu D, Van Belleghem JD, de Vries CR, Burgener E, Chen Q, Manasherob R, et al. The safety and toxicity of phage therapy: a review of animal and clinical studies. *Viruses*. (2021) 13:1268. doi: 10.3390/v13071268

125. Dy RL, Przybilski R, Semeijn K, Salmond GPC, Fineran PC. A widespread bacteriophage abortive infection system functions through a type IV toxin-antitoxin mechanism. *Nucleic Acids Res*. (2014) 42:4590–605. doi: 10.1093/NAR/GKT1419

126. Lopatina A, Tal N, Sorek R. Abortive infection: bacterial suicide as an antiviral immune strategy. *Annu Rev Virol*. (2020) 7:371–84. doi: 10.1146/annurev-virology-011620-040628

127. de Freitas Almeida GM, Hoikkala V, Ravantti J, Rantanen N, Sundberg LR. Mucin induces CRISPR-Cas defense in an opportunistic pathogen. *Nat Commun*. (2022) 13:1–12. doi: 10.1038/s41467-022-31330-3

128. King P, Citron DM, Griffith DC, Lomovskaya O, Dudley MN. Effect of oxygen limitation on the in vitro activity of levofloxacin and other antibiotics administered by the aerosol route against *Pseudomonas aeruginosa* from cystic

fibrosis patients. *Diagn Microbiol Infect Dis*. (2010) 66:181–6. doi: 10.1016/j.diagmicrobio.2009.09.009

129. Willner D, Furlan M, Haynes M, Schmieder R, Angly FE, Silva J, et al. Metagenomic analysis of respiratory tract DNA viral communities in cystic fibrosis and non-cystic fibrosis individuals. *PLoS One*. (2009) 4:e7370. doi: 10.1371/journal.pone.0007370

130. Simpson DJ, Sacher JC, Szymanski CM. Exploring the interactions between bacteriophage-encoded glycan binding proteins and carbohydrates. *Curr Opin Struct Biol*. (2015) 34:69–77. doi: 10.1016/j.sbi.2015.07.006

131. Barr JJ, Auro R, Furlan M, Whiteson KL, Erb ML, Pogliano J, et al. Bacteriophage adhering to mucus provide a non-host-derived immunity. *Proc Natl Acad Sci U S A*. (2013) 110:10771–6. doi: 10.1073/pnas.1305923110

132. Green SI, Gu Liu C, Yu X, Gibson S, Salmen W, Rajan A, et al. Targeting of mammalian glycans enhances phage predation in the gastrointestinal tract. *MBio*. (2021) 12:1–18. doi: 10.1128/mBio.03474-20

133. Almeida GMF, Laanto E, Ashrafi R, Sundberg LR. Bacteriophage adherence to mucus mediates preventive protection against pathogenic bacteria. *MBio*. (2019) 10. doi: 10.1128/mBio.01984-19

134. Nguyen-Kim H, Bettarel Y, Bouvier T, Bouvier C, Doan-Nhu H, Nguyen-Ngoc L, et al. Coral mucus is a hot spot for viral infections. *Appl Environ Microbiol*. (2015) 81:5773–83. doi: 10.1128/AEM.00542-15

135. Barr JJ, Auro R, Sam-Soon N, Kassegne S, Peters G, Bonilla N, et al. Subdiffusive motion of bacteriophage in mucosal surfaces increases the frequency of bacterial encounters. *Proc Natl Acad Sci U S A*. (2015) 112:13675–80. doi: 10.1073/pnas.1508355112

136. Green SI, Gu Liu C, Yu X, Gibson S, Salmen W, Rajan A, et al. Targeting of mammalian Glycans enhances phage predation in the gastrointestinal tract. *MBio*. (2021) 12:1–18. doi: 10.1128/mBio.03474-20

137. Wheeler KM, Cárcamo-Oyarce G, Turner BS, Dellos-Nolan S, Co JY, Lehoux S, et al. Mucin glycans attenuate the virulence of *Pseudomonas aeruginosa* in infection introductory paragraph. *Nat Microbiol*. (2019) 4:2146–54. doi: 10.1038/s41564-019-0581-8

138. Rossy T, Distler T, Pezoldt J, Kim J, Talà L, Bouklas N, et al. Pseudomonas aeruginosa contracts mucus to rapidly form biofilms in tissue-engineered human airways. *bioRxiv*. (2022):2022.05.26.493615. doi: 10.1101/2022.05.26.493615

139. Samad T, Co JY, Witten J, Ribbeck K. Mucus and mucin environments reduce the efficacy of polymyxin and fluoroquinolone antibiotics against *Pseudomonas aeruginosa*. *ACS Biomater Sci Eng*. (2019) 5:1189–94. doi: 10.1021/acsbiomaterials.8b01054

140. Cazares D, Cazares A, Fiueroa W, Guarneros G, Edwards RA, Vinuesa P. A novel group of promiscuous podophages infecting diverse gammaproteobacteria from river communities exhibits dynamic intergenus host adaptation. *mSystems*. (2021) 6. doi: 10.1128/mSystems.00773-20

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# Case report: Analysis of phage therapy failure in a patient with a *Pseudomonas aeruginosa* prosthetic vascular graft infection

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Clinical case of a patient with a *Pseudomonas aeruginosa* multidrug-resistant prosthetic vascular graft infection which was treated with a cocktail of phages (PT07, 14/01, and PNM) in combination with ceftazidime-avibactam (CZA). After the application of the phage treatment and in absence of antimicrobial therapy, a new *P. aeruginosa* bloodstream infection (BSI) with a septic residual limb metastasis occurred, now involving a wild-type strain being susceptible to ß-lactams and quinolones. Clinical strains were analyzed by microbiology and whole genome sequencing techniques. In relation with phage administration, the clinical isolates of *P. aeruginosa* before phage therapy (HE2011471) and post phage therapy (HE2105886) showed a clonal relationship but with important genomic changes which could be involved in the resistance to this therapy. Finally, phenotypic studies showed a decrease in Minimum Inhibitory Concentration (MIC) to ß-lactams and quinolones as well as an increase of the biofilm production and phage resistant mutants in the clinical isolate of *P. aeruginosa* post phage therapy.

KEYWORDS

**phage, phage therapy, antibiotic resistance, *Pseudomonas aeruginosa*, bypass, prosthetic vascular graft infection**

## Introduction

Prosthetic vascular graft infections (PVGI) are complicated events associated with high morbidity and mortality rates. PVGI incidence is between 1 and 6%, showing a death rate range between 15 to 75% with a rate of major amputation that may reach 70%, which is especially caused by aortic graft (1). PVGI are usually caused by the more

virulent microorganisms, such as *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella* spp, *Proteus* spp., and *Enterobacter* spp. (2). Carbapenem-resistant *Pseudomonas aeruginosa* is one the most critical pathogens according to the World Health Organization (WHO) and poses a particular threat in hospitals, nursing homes, and among patients whose care requires devices such as ventilators and blood catheters (3). Several mechanisms of resistance to carbapenems have been described in clinical isolates of *Pseudomonas aeruginosa*, among them, we can highlight ß-lactamases, mutations in porins, and overexpression of efflux pumps (4).

Phage therapy is a promising new treatment against infections produced by multi-drug resistant pathogens (5). To improve phage therapy application, it would be necessary to know more about the clinical response and bacterial host-phage interactions. The monitoring of these interactions can be done by massive sequencing, thus identifying the genes affected by mutations that occur during therapy, and therefore directing the way phage therapy should be applied.

In this work, we analyzed the clinical, microbiological, and molecular features of *P. aeruginosa* isolates in a case of prosthetic vascular graft infection (PVGI) after phage administration was deemed unsuccessful. This knowledge could allow the development of strategies to improve the use of clinical use of phage therapy.

## Case report

This work presents the clinical case of a man in his fifties who developed recurrent bloodstream infections (BSI) caused by ceftazidime and piperacillin-tazobactam resistant *Pseudomonas aeruginosa*. In August 2020, an axillo-bifemoral bypass was performed in this patient because of a severe infra-renal aorta atherosclerotic occlusion (Leriche syndrome). Shortly after, the patient first developed a BSI, which was interpreted as secondary to an infected sacral ulcer, and was treated with meropenem at dose of 1 g every 8 h for 17 days. From November to June 2021, the patient suffered several recurrences of BSI due to *P. aeruginosa*. After the second relapse, $^{18}$F-fluorodeoxyglucose ($^{18}$F-FDG) PET/CT was performed, showing radioactive-labeled glucose uptake along the pre-clavicular graft region (SUVmax value, 5.29), suggesting prosthetic vascular graft infection (PVGI). Graft replacement was rejected due to high risk of contralateral leg ischemia and bypass thrombosis if partial bypass replacement was done. The patient subsequently received different targeted antibiotherapies for 2 to 6 weeks (Figure 1). Two new *P. aeruginosa* BSI relapses occurred between March and May 2021. Ceftolozane-tazobactam was not used (not available), and off-label tebipenem use was denied; treatment was therefore as shown in Figure 1. Simultaneously, therapeutic phages were obtained from the Queen Astrid Military Hospital (Brussels, Belgium) in view of a possible compassionate use of phage therapy.

Phage susceptibility of the clinical isolate was determined in the A Coruña University Hospital, and three *P. aeruginosa* phages were selected. Two (14/01 and PT07) were myoviruses, and one (PNM) was a podovirus. Two of the phages had known receptors, being lipopolysaccharide (LPS) for phage 14/01, and the type IV pili for PNM (6, 7). In July 2021, 70 ml of the bacteriophage cocktail, consisting of the three phages, each at a concentration of $10^7$ plaque forming units (PFU)/mL for a total dose of $2.1 \times 10^9$ PFU/day, was administered intravenously, once a day in a 6-h infusion for 3 days in an inpatient regimen. Thereafter, and for four additional days, this cocktail of phages alongside ceftazidime-avibactam were administered by outpatient parenteral antimicrobial therapy (OPAT) at the patient's request. In summary, phage therapy was used for 1 week, with ceftazidime-avibactam being used for 8 weeks (from 6 weeks before, to 2 weeks after phage therapy). Importantly, no adverse events were observed.

Ceftazidime-avibactam was applied for 8 weeks (from 6 weeks before to 2 weeks after the phage therapy). In August 2021, in the absence of antimicrobial therapy, a new *P. aeruginosa* BSI with a septic residual limb metastasis occurred, now involving a wild-type strain being susceptible to ß-lactams and quinolones. Finally, upon a multidisciplinary discussion, a proximal vascular prosthesis replacement combined with antibiotherapy in OPAT was performed. As of this writing, 2023, after 10 months without treatment, the patient remains asymptomatic.

Eight blood culture isolates obtained between November 2020 and August 2021 were characterized by microbiological analysis. In August 2021, these isolates were identified by matrix assisted laser desorption/ionization—time of flight mass spectrometry (MALDI-TOF MS, Bruker Daltonics) as *P. aeruginosa*. Antimicrobial susceptibility testing was performed by broth microdilution (Microscan, Beckman Coulter) and interpreted using European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria. Pulsed-field gel electrophoresis (PFGE) revealed that four representative isolates, from 2020 and 2021, showed the same pattern and therefore were clonal (Supplementary Figure S1). Genomic sequence of four representative isolates showed that these isolates belonged to the high-risk clone ST308 with core genome MLST (MLST)cgST2675. High-risk clones are those with a wide dissemination and a global spread associated with a multidrug resistant (MDR) or extensively drug resistant (XDR) profiles including extended-spectrum β-lactamases (ESBLs) and carbapenemases (8). A search for antimicrobial resistance genes in the ResFinder database did not identify any acquired resistance genes such as ß-lactamases (9). Analysis of 40 chromosomal resistance genes revealed natural polymorphisms (SNPs) in numerous genes, including some changes with unknown effect (10). The profile of all isolates was identical for all genes studied, except for the *nfxB* gene, a transcriptional repressor that regulates the efflux pump MexCD-OprJ (11), in which multiple amino acid changes were observed in the last 3 isolates (Supplementary Table S1). Moreover, comparison of the genomes of *P. aeruginosa* isolates HE2011471 (previous to phage administration) and HE2105886 (1 month after phage treatment) revealed important mutations (Figure 2). Interestingly, several detected genomic changes could be involved in phage resistance (Supplementary Table S2, Figure 3). *P. aeruginosa* isolate HE2105886, recovered 1 month after phage treatment, exhibited a decrease in the Minimum Inhibitory Concentration (MICs) of ß-lactam and quinolone antibiotics (Figure 1) probably due to the

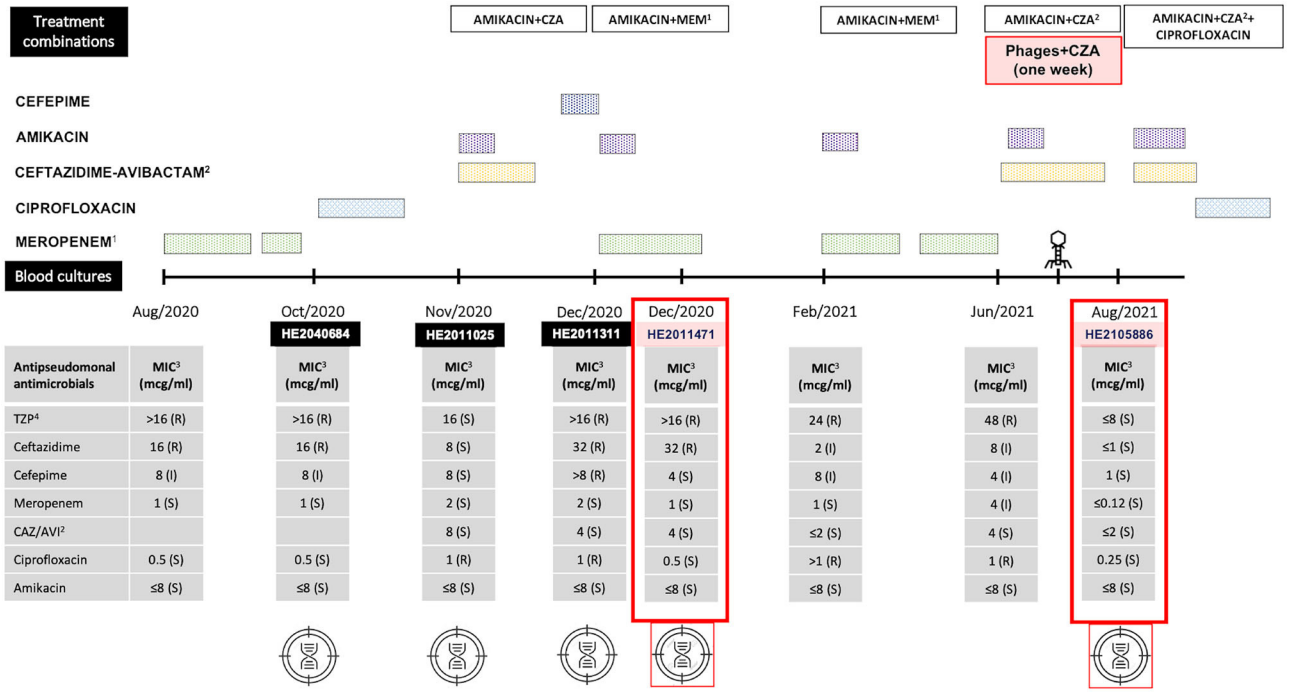


FIGURE 1
Timeline depicting the different antibiotic and phage therapies, the isolation of *Pseudomonas aeruginosa* strains from blood cultures and their antibiotic susceptibility and genomic analyses. PFGE of these strains in described in Supplementary Figure S1. Antimicrobial information: [1]MEM, Meropenem. [2]CZA, Ceftazidime-avibactam. [3]MIC, Minimum inhibitory concentration. [4]TZP, Piperacillin-tazobactam. The five isolates that underwent Whole Genome Sequencing (WGS) are shown with the "target" symbols; of those, the two isolates that are further analyzed in the manuscript, *P. aeruginosa* isolate HE2011471 (pre-phage therapy) and *P. aeruginosa* isolate HE2105886 (post-phage therapy) are highlighted with the red boxes.

mutations of the membrane receptors, regulators and efflux pumps. However, we did not test any of these isolates against the individual phages in the cocktail to track the sensitivity or resistance to each of them.

Finally, the post-phage therapy isolate exhibited increased production of biofilm and phage-resistant mutants. These two tests were deemed relevant, as bacteria can form biofilms as a response to environmental stress, such as the presence of antibiotics, and this, alongside the emergence of phage-resistance could explain the observed failure to eradicate the infection with phage therapy (Figures 4A, B) (16).

## Discussion

This case highlights three key issues. The first relates to the use of combined therapy (antibiotics and phages) vs. monotherapy (antibiotic or phages) as a definitive treatment for *P. aeruginosa* BSI. So far, combination therapy has not been associated with reduced mortality or any advantages in terms of clinical outcome or successful treatment of recurrent/persistent bacteraemia (17). The second issue concerns prosthetic vascular graft infection (PVGI) diagnosis and its best management. At the early stages of PVGI, a high degree of suspicion is essential. Indium-111-labeled white blood cell scintigraphy plus single-photon emission computed tomography (SPECT/CT) could reduce the false positive rates observed with PET/CT (18). In the present case, PET/CT was not performed at the early postoperative stage, because of the possibility of a false positive result. Regarding the treatment, graft excision is the preferred surgical approach. However, some patients are considered unacceptable surgical candidates due to underlying comorbidities or technically unfeasible surgery. When this occurs, lifelong suppressive antimicrobial therapy is an option, but is not free of side-effects or the risk of development of antimicrobial resistance. As the patient was initially denied surgical treatment, long-term suppressive treatment with quinolones was administered. However, ciprofloxacin-resistance developed, and surgery was finally deemed key to a favorable outcome. Furthermore, there is no evidence-based recommendation for either PVGI antimicrobial treatment or its optimal duration; minimum intravenous therapy for 6 weeks followed by oral antibiotherapy for up to 6 months has been proposed (19, 20). $^{18}$F-FDG PET/CT-guided treatment duration seems feasible and would allow treatment to be tailored to individual patients (21). The third highlighted issue is related to phage therapy. Phage therapy involves the targeted application of strictly virulent phages that can specifically infect and lyse the targeted pathogenic bacteria they encounter, hereby releasing virion progeny that continues the lytic cycle. A major advantage of phages is the minimal impact on non-target bacteria or body tissues (22). A recent systematic review suggested that phage therapy is safe and may be effective in different difficult-to-treat infections (23). Interestingly, a previously-reported case of ß-lactam resistance in an MDR *P. aeruginosa* isolate causing an aortic graft infection, showed the

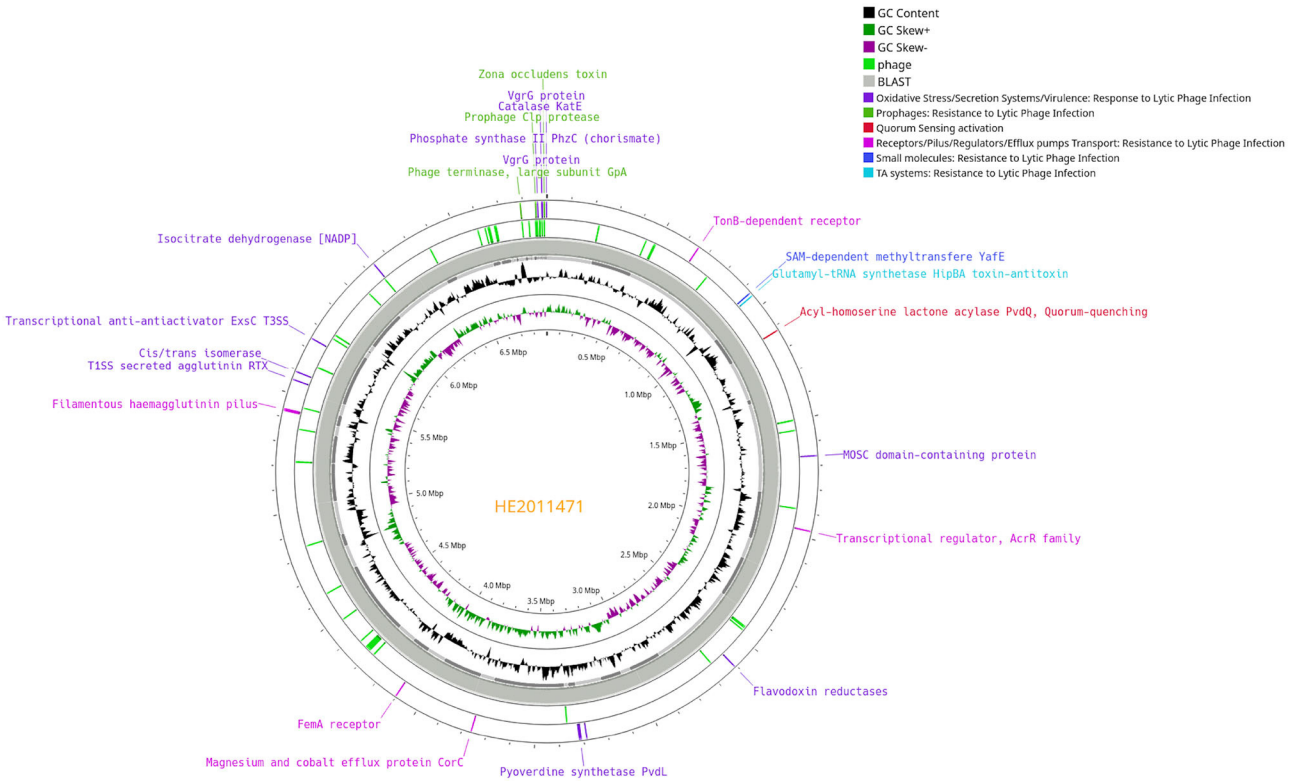


FIGURE 2
Circular chromosomic view of the bacterial genomes of two *Pseudomonas aeruginosa* isolates: *P. aeruginosa* isolate HE2105886 (after phage treatment) in comparison with *P. aeruginosa* isolate HE2011471 (prior to phage administration). The SNPs found in *P. aeruginosa* isolate HE2105886 respect to *P. aeruginosa* isolate HE2011471 are indicated.

reversion of the infection after treatment with a phage whose receptor was the outer membrane protein M (OprM) of the MexAB- and MexXY-multidrug efflux pumps, associated with antibiotic resistance and which was no longer expressed in the selected phage-resistant bacterial strain (24). In the present case, even though phage therapy did not cure the infection, the posterior infection recurrence was caused by an antibiotic susceptible isolate that belonged to the same lineage as the one that was causing the pre-phage treatment episodes of infection. However, the recurrent isolate was recovered 1 month after the phage therapy, and it is possible that the resensitization (to ß-lactams and quinolones) could have been due to phage action which produced genomic changes in the membrane receptors, regulators and efflux pumps (Supplementary Table S2) or to a spontaneous evolution. In order to confirm it, further studies could be carried out as to demonstrate that these mutations located in the membrane receptors, regulators and efflux pumps proteins indeed render a strain phage-resistant and susceptible to antimicrobials. These studies would involve constructing several plasmids encoding the wild-type genes with lack of the mutations and overexpressing them in the *P. aeruginosa* isolate HE2105886 to restore the phage susceptibility and the resistance to antimicrobials.

One strength of this study is that phage therapy was able to be safely administered in OPAT for greater patient convenience. To our knowledge, this has only been reported once before, in a series of six cases, without phage-related adverse events (25).

The main limitations of our study were: first, we did not perform *in vitro* studies of the effect of the phages in combination with antibiotics, especially ß-lactams; second, we could not study the *P. aeruginosa* clinical isolates during the seven days of application of phage therapy. Finally, it should be noticed that there are no national or local phage banks with characterized phages against *P. aeruginosa* in which specific phages could be selected to provide personalized approach in patients with complex infections caused by these bacteria.

In conclusion, in complex *P. aeruginosa* infections the choice of antibiotic therapy and its duration are crucial for minimizing antibiotic pressure and development of resistance. Although we have not demonstrated that phage treatment was effective in this case, studying the molecular mechanisms of resistance to phages and bacteria-phage interactions are key to improving phage therapy in the near future.

# Material/methods

## Microbiology studies

The isolates were identified by MALDI-TOF MS (Bruker Daltonics). Antimicrobial susceptibility testing

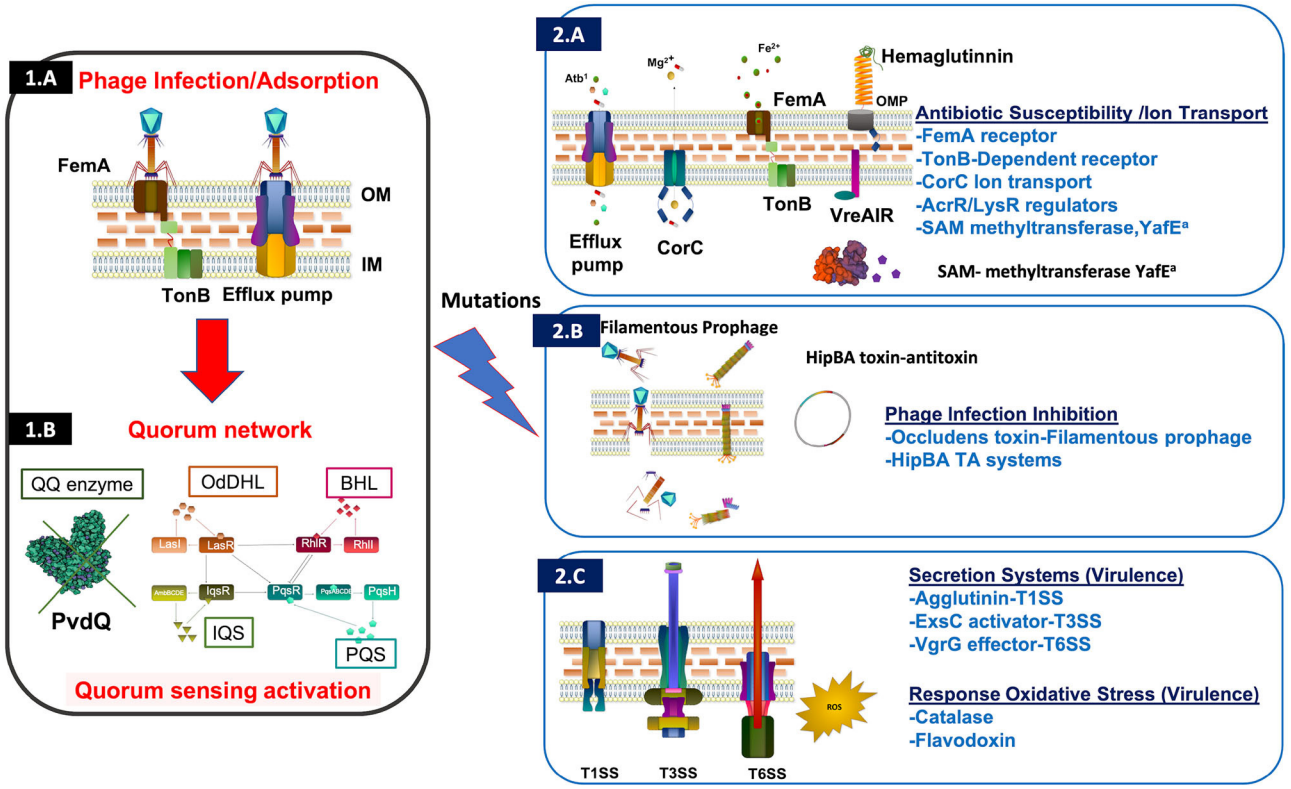


FIGURE 3
Graphic representation of the proteins coded by the *P. aeruginosa* HE2105886 genome (post-phage therapy isolate), that presented mutations when compared to pre-phage therapy isolate, with emphasis of changes associated with mechanisms of resistance and response to phage infection (12). Among them we highlight phage infection by adsorption as well as Quorum Sensing activation **(1A, 1B)** alteration of the antibiotic susceptibility (receptors and efflux pumps) **(2A)**, inhibition of the phage infection (filamentous prophages, TA (toxin-antitoxin) systems and small molecules) (13–15) **(2B)** and finally, virulence (oxidative stress and secretion systems) **(2C)**. [a]Small molecules such as aminoglycosides which participate in the inhibition of the phage infection well as bacterial antimicrobial profile.

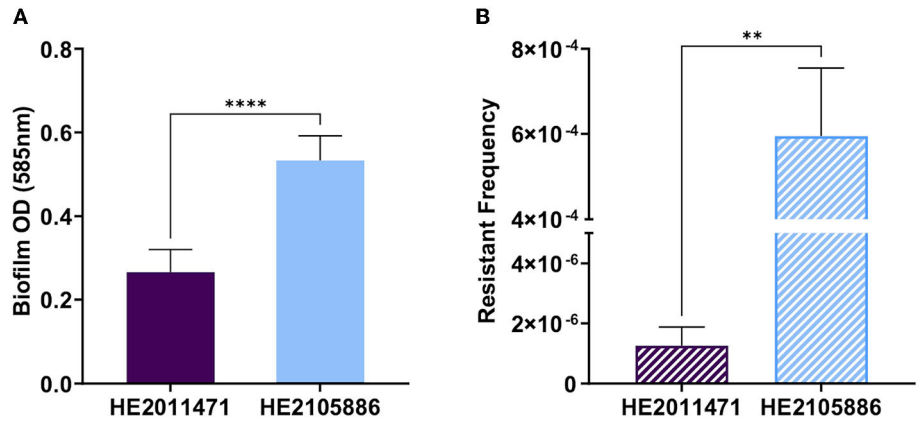


FIGURE 4
Comparison of the biofilm production **(A)** and the frequency of occurrence of phage resistant mutants **(B)** between the *Pseudomonas aeruginosa* isolates HE2011471 (pre-phage therapy) and HE2105886 (post-phage therapy). Statistical analysis is *t*-test with Graphpad 9.0.0 **** <0.0001; **0.0030.

was performed by broth microdilution with a Microscan system (Beckman Coulter), and the results were interpreted according to the clinical breakpoints defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). PFGE analysis was performed using *Spe*I.

## Genomic sequencing and bioinformatic tools

Whole genome sequencing of the five isolates was performed in a HiSeq 2500 sequencing system (Illumina), and sequences were assembled using SPAdes 3.10.1.

Clonality testing of genomes was carried out using MLST Finder (from CGE, available at https://bitbucket.org/genomicepidemiology/mlst.git) and Ridom SeqSphere+ v8.5, and the resistance mechanisms were analyzed using the ResFinder database. SNP calling was performed using snippy v4.6.0 against the NCBI PGAP annotation (https://www.ncbi.nlm.nih.gov/genome/annotation_prok/). The assemblies were visually inspected using Bandage v0.8.1. (https://rrwick.github.io/Bandage/). Further functional annotation genes and prophage elements were confirmed using Blastx (http://blast.ncbi.nlm.nih.gov), Hhmer (http://hmmer.org), and also the HHpred tool (https://toolkit.tuebingen.mpg.de/tools/hhpred), which predict functions through protein structure.

Assembled genomes of *P. aeruginosa* isolates were analyzed using Phaster (PHAge Search Tool Enhanced Release) software (https://phaster.ca/) and SourceFinder (https://cge.food.dtu.dk/services/SourceFinder/).

### Biofilm production

Overnight cultures of *P. aeruginosa* isolates HE2011471 and HE2105886 were diluted 1:100 and used to inoculate 100 μL of LB broth in a 96 multi-well plate. The plate was incubated for 24 h at 37°C in darkness. The supernatant was discarded and the wells were washed with PBS. One hundred μL of methanol was then added to each well and discarded after 10 min. When the methanol had completely evaporated, 100 μL of crystal violet (0.1%) was added and discarded after 15 min. Finally, the wells were washed with PBS before the addition of 150 μL of acetic acid (30%), and the absorbance was measured at OD 585 nm.

### Frequency of occurrence of phage resistant mutants

The frequency of occurrence of phage resistant mutants was determined as previously described, with some modifications (26). Overnight cultures of isolates HE2011471 and HE 2105886 were diluted 1:100 in LB and grown to an OD600 nm of 0.6–0.7. An aliquot of 100 μL of the culture containing $10^8$ colony forming units (CFU)/mL was serially diluted, and each dilution was mixed with 100 μL of $10^9$ PFU/mL phage cocktail and then plated by the agar overlay method (27). The plates were incubated at 37°C for 18 h and the number of CFUs was counted. The frequency of occurrence of phage resistant mutants and phage resistant mutants was calculated by dividing the number of resistant bacteria by the total number of sensitive bacteria.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

## Ethics statement

Written informed consent was obtained from the patient for the publication of any potentially identifiable images or data included in this article.

## Author contributions

LB, IL-H, MR-F, JP-F, and CC-S conducted the experiments, analyzed the results, and wrote the manuscript. SD, MM, and J-PP revised the results and phage therapy administration. JR-B, MT, and LL obtained the research funding, directed the clinical settings, and supervised the writing of the manuscript. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2023.1199657/full#supplementary-material

SUPPLEMENTARY FIGURE S1
PFGE (*Pulsed Field Gel Electrophoresis*) patterns of four sequential *Pseudomonas aeruginosa isolates*.

SUPPLEMENTARY TABLE S1
Mutations encountered in proteins known to be related to antimicrobial resistance in *Pseudomonas aeruginosa*. The mutations present in *P. aeruginosa* isolate HE2105886 in comparison to *P. aeruginosa* isolate HE2011471 are shown.

SUPPLEMENTARY TABLE S2
Mutations encountered in proteins probably to be related to phage resistance in response to lytic phage infection in *P. aeruginosa* isolates *P. aeruginosa* isolate HE2105886 in comparison to *P. aeruginosa* isolate HE2011471. [a] Proteins located in prophages as identified by Phaster and SourceFinder.

## References

1. Legout L, Sarraz-Bournet B, D'Elia PV, Devos P, Pasquet A, Caillaux M, et al. Characteristics and prognosis in patients with prosthetic vascular graft infection: a prospective observational cohort study. *Clin Microbiol Infect.* (2012) 18:352–8. doi: 10.1111/j.1469-0691.2011.03618.x

2. SR BMaK. Infections and Antibiotics in Vascular Surgery. In: *Basic Science and Clinical Correlations,* editor. White RA and Hollier LH, Philadelphia: J. B. Lippincott company. (1994).

3. Tacconelli E, Carrara E, Savoldi A, Harbarth S, Mendelson M, Monnet DL, et al. Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. *Lancet Infect Dis.* (2018) 18:318–27. doi: 10.1016/S1473-3099(17)30753-3

4. Cabot G, Ocampo-Sosa AA, Tubau F, Macia MD, Rodríguez C, Moya B, et al. Spanish Network for Research in Infectious Diseases (REIPI). Overexpression of AmpC and efflux pumps in Pseudomonas aeruginosa isolates from bloodstream infections: prevalence and impact on resistance in a Spanish multicenter study. *Antimicrob Agents Chemother.* (2011) 55:1906–11. doi: 10.1128/AAC.01645-10

5. Hatfull GF, Dedrick RM, Schooley RT. Phage Therapy for Antibiotic-Resistant Bacterial Infections. *Annu Rev Med.* (2022) 73:197–211. doi: 10.1146/annurev-med-080219-122208

6. Ceyssens PJ, Glonti T, Kropinski NM, Lavigne R, Chanishvili N, Kulakov L, et al. Phenotypic and genotypic variations within a single bacteriophage species. *Virol J.* (2011) 8:134. doi: 10.1186/1743-422X-8-134

7. Ceyssens PJ, Miroshnikov K, Mattheus W, Krylov V, Robben J, Noben JP, et al. Comparative analysis of the widespread and conserved PB1-like viruses infecting *Pseudomonas aeruginosa*. *Environ Microbiol.* (2009) 11:2874–83. doi: 10.1111/j.1462-2920.2009.02030.x

8. Del Barrio-Tofiño E, López-Causapé C, Oliver A. *Pseudomonas aeruginosa* epidemic high-risk clones and their association with horizontally-acquired β-lactamases: 2020 update. *Int J Antimicrob Agents.* (2020) 56:106196. doi: 10.1016/j.ijantimicag.2020.106196

9. Florensa AF, Kaas RS, Clausen PTLC, Aytan-Aktug D, Aarestrup FM. ResFinder - an open online resource for identification of antimicrobial resistance genes in next-generation sequencing data and prediction of phenotypes from genotypes. *Microb Genom.* (2022) 8:000748. doi: 10.1099/mgen.0.000748

10. Cortes-Lara S, Barrio-Tofiño ED, López-Causapé C, Oliver A, GEMARA-SEIMC/REIPI *Pseudomonas* study Group. Predicting Pseudomonas aeruginosa susceptibility phenotypes from whole genome sequence resistome analysis. *Clin Microbiol Infect.* (2021) 27:1631–7. doi: 10.1016/j.cmi.2021.05.011

11. Alcalde-Rico M, Olivares-Pacheco J, Alvarez-Ortega C, Cámara M, Martínez JL. Role of the Multidrug Resistance Efflux Pump MexCD-OprJ in the *Pseudomonas aeruginosa* Quorum Sensing Response. *Front Microbiol.* (2018) 9:2752. doi: 10.3389/fmicb.2018.02752

12. Luthe T, Kever L, Thormann K, Frunzke J. Bacterial multicellular behavior in antiviral defense. *Curr Opin Microbiol.* (2023) 74:102314. doi: 10.1016/j.mib.2023.102314

13. Bleriot I, Trastoy R, Blasco L, Fernández-Cuenca F, Ambroa A, Fernández-García L, et al. Genomic analysis of 40 prophages located in the genomes of 16 carbapenemase-producing clinical strains of *Klebsiella pneumoniae*. *Microb Genom.* (2020) 6:e000369. doi: 10.1099/mgen.0.000369

14. Bleriot I, Blasco L, Pacios O, Fernández-García L, Ambroa A, López M, et al. The role of PemIK (PemK/PemI) type II TA system from *Klebsiella pneumoniae* clinical strains in lytic phage infection. *Sci Rep.* (2022) 12:4488. doi: 10.1038/s41598-022-08111-5

15. Hardy A, Kever L, Frunzke J. Antiphage small molecules produced by bacteria-beyond protein-mediated defenses. *Trends Microbiol.* (2023) 31:92–106. doi: 10.1016/j.tim.2022.08.001

16. Castledine M, Padfield D, Sierocinski P, Soria Pascual J, Hughes A, Mäkinen L, et al. Parallel evolution of *Pseudomonas aeruginosa* phage resistance and virulence loss in response to phage treatment in vivo and in vitro. *Elife.* (2022) 11:e73679. doi: 10.7554/eLife.73679

17. Babich T, Naucler P, Valik JK, Giske CG, Benito N, Cardona R, et al. Combination versus monotherapy as definitive treatment for *Pseudomonas aeruginosa* bacteraemia: a multicentre retrospective observational cohort study. *J Antimicrob Chemother.* (2021) 76:2172–81. doi: 10.1093/jac/dkab134

18. Reinders Folmer EI, Von Meijenfeldt GCI, Van der Laan MJ, Glaudemans AWJM, Slart RHJA, Saleem BR, et al. Diagnostic Imaging in Vascular Graft Infection: A Systematic Review and Meta-Analysis. *Eur J Vasc Endovasc Surg.* (2018) 56:719–29. doi: 10.1016/j.ejvs.2018.07.010

19. Leroy O, Meybeck A, Sarraz-Bournet B, d'Elia P, Legout L. Vascular graft infections. *Curr Opin Infect Dis.* (2012) 25:154–8. doi: 10.1097/QCO.0b013e3283501853

20. Wilson WR, Bower TC, Creager MA, Amin-Hanjani S, O'Gara PT, Lockhart PB, et al. Vascular graft infections, mycotic aneurysms, and endovascular infections: a scientific statement from the american heart association. *Circulation.* (2016) 134:e412–60. doi: 10.1161/CIR.0000000000000457

21. Kung BT, Seraj SM, Zadeh MZ, Rojulpote C, Kothekar E, Ayubcha C, et al. An update on the role of $^{18}$F-FDG-PET/CT in major infectious and inflammatory diseases. *Am J Nucl Med Mol Imaging.* (2019) 9:255–73.

22. 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

23. Uyttebroek S, Chen B, Onsea J, Ruythooren F, Debaveye Y, Devolder D, et al. Safety and efficacy of phage therapy in difficult-to-treat infections: a systematic review. *Lancet Infect Dis.* (2022) 22:e208–20. doi: 10.1016/S1473-3099(21)00612-5

24. Chan BK, Turner PE, Kim S, Mojibian HR, Elefteriades JA, Narayan D. Phage treatment of an aortic graft infected with *Pseudomonas aeruginosa*. *Evol Med Public Health.* (2018) 2018:60–6. doi: 10.1093/emph/eoy005

25. Aslam S, Lampley E, Wooten D, Karris M, Benson C, Strathdee S, et al. Lessons learned from the first 10 consecutive cases of intravenous bacteriophage therapy to treat multidrug-resistant bacterial infections at a single center in the United States. *Open Forum Infect Dis.* (2020) 7:ofaa389. doi: 10.1093/ofid/ofaa389

26. Blasco L, Ambroa A, Lopez M, Fernandez-Garcia L, Bleriot I, Trastoy R, et al. Combined Use of the Ab105-2ϕΔCI Lytic Mutant Phage and Different Antibiotics in Clinical Isolates of Multi-Resistant *Acinetobacter baumannii*. *Microorganisms.* (2019) 7:556. doi: 10.3390/microorganisms7110556

27. Kropinski AM, Mazzocco A, Waddell TE, Lingohr E, Johnson RP. Enumeration of bacteriophages by double agar overlay plaque assay. *Methods Mol Biol.* (2009) 501:69–76. doi: 10.1007/978-1-60327-164-6_7

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# Successful use of a phage endolysin for treatment of chronic pelvic pain syndrome/chronic bacterial prostatitis

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Chronic prostatitis (CP) is a common inflammatory condition of the prostate that is estimated to effect 2%–10% of the world's male population. It can manifest as perineal, suprapubic, or lower back pain and urinary symptoms occurring with either recurrent bacterial infection [chronic bacterial prostatitis (CBP)] or in the absence of evidence of bacterial infection [chronic pelvic pain syndrome (CPPS)]. Here, in the case of a 39years-old CBP patient, we report the first successful use of a bacteriophage-derived muralytic enzyme (endolysin) to treat and resolve the disease. Bacteriological analysis of the patient's prostatic secretion and semen samples revealed a chronic *Enterococcus faecalis* prostate infection, supporting a diagnosis of CBP. The patient's *E. faecalis* strain was resistant to several antibiotics and developed resistance to others during the course of treatment. Previous treatment with multiple courses of antibiotics, bacteriophages, probiotics, and immunologic stimulation had failed to achieve long term eradication of the infection or lasting mitigation of the symptoms. A cloned endolysin gene, encoded by *E. faecalis* bacteriophage $\phi$Ef11, was expressed, and the resulting gene product was purified to electrophoretic homogeneity. A seven-day course of treatment with the endolysin resulted in the elimination of the *E. faecalis* infection to below culturally detectable levels, and the abatement of symptoms to near normal levels. Furthermore, during the endolysin treatment, the patient experienced no untoward reactions. The present report demonstrates the effectiveness of an endolysin as a novel modality in managing a recalcitrant infection that could not be controlled by conventional antibiotic therapy.

KEYWORDS

**chronic bacterial prostatitis, bacteriophage, endolysin therapy, antibiotic resistance, *Enterococcus faecalis***

## Introduction

Chronic prostatitis (CP) is an inflammatory condition of the prostate associated with pain and urinary symptoms occurring either with recurrent bacterial infection [chronic bacterial prostatitis (CBP)] or in the absence of evidence of bacterial infection [chronic pelvic pain syndrome (CPPS)] (1–5). CBP may manifest symptoms such as dysuria, localized pain in the

perineum, suprapubic region or lower back, and sexual dysfunction including erectile dysfunction and ejaculatory discomfort, along with a positive culture from expressed prostatic secretions (1, 5–8). It is estimated that chronic prostatitis (combined CBP and CPPS) effects approximately 2%–10% of the male population worldwide (1, 2, 9, 10), with a high rate (50%) of recurrence (1, 2, 9). Some studies suggest that 35% to 50% of men are affected by CP at some point in their lives (11). Although infection has not been reported to be associated with most cases of CP (10), when molecular methods (e.g., PCR) were applied diagnostically to cases that were previously determined (by cultural methods) to lack any evidence of bacteriuria or prostate-localized uropathogens, 16S rDNA was detected in prostate biopsies from 77% of these cases, indicating that in fact, most of the CP cases had evidence of bacterial infection, and therefore were in actuality CBP (10, 12).

A wide variety of bacterial species have been isolated from cases of CBP (13). Prominent among these are species of the Gram-negative family, *Enterobacteriaceae* (*Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, Acinetobacter* spp., *Citrobacter* spp.) (3, 13), and the Gram-positive Enterococcal genus (*Enterococcus faecalis, Enterococcus faecium*) (3, 7, 13, 14). Enterococcal infections (*E. faecalis* and *E. faecium*) present particularly challenging clinical management problems due to the remarkable hardiness of these species; surviving great extremes in temperature, pH, osmolality as well as starvation and desiccation (15–17). Furthermore, many strains of these species exhibit multidrug resistance (MDR) properties (18–20) such as resistance to $\beta$-lactams (21, 22), aminoglycosides (23), vancomycin (24), erythromycin (25), tetracycline (26), daptomycin (27), quinupristin-dalfopristin (28) and linezolid (29), complicating management of enterococcal infections.

The emergence and increasing prevalence of MDR bacteria has prompted the search for alternatives to antibiotics to treat these infections. One such promising alternative is the use of bacteriophage (phage) endolysins. Following the recognition that there was a "labile lytic factor in phage lysates" (30), it was proposed that the "lytic factor" (now known to be a muralytic enzyme/endolysin encoded by a phage) could be used to control bacterial infections (31, 32). Consequently, there have been numerous studies on endolysins, detailing their biochemical and biological characteristics, as well as, in some cases, their protective efficacy against infections in *in vivo* animal models [for reviews see references (33–36)]. Finally, one recent study reported the results of a clinical trial testing the efficacy of a phage endolysin (exebacase) in treating *Staphylococcus aureus* (including MRSA) bloodstream infections (BSIs) (37). The results of this clinical trial demonstrated that the combination of the endolysin plus an antibiotic (either semisynthetic penicillins or first-generation cephalosporins for methicillin-sensitive *S. aureus* infections or vancomycin or daptomycin for MRSA) was superior to the antibiotic alone in mitigating the morbidity and mortality of *S. aureus* BSIs.

Previously, the Stevens laboratory isolated a bacteriophage from an infected root canal that infects strains of *E. faecalis* (38). Sequencing and annotation of the phage genomic DNA permitted the identification of a gene that was predicted to code for the phage endolysin (39). Cloning and expression of the putative endolysin gene resulted in the production of a protein whose bacteriolytic activity confirmed the identity of the cloned gene (40). The purified protein [designated open reading frame (ORF) 28 endolysin] was shown to possess multifunctional muralytic activity, acting as an N-acetylmuramidase, an N-acetylglucosaminidase, and an endopeptidase, which could hydrolyze the *E. faecalis* cell wall peptidoglycan (40). The endolysin exhibited remarkably potent lytic activity against many strains of *E. faecalis* including many vancomycin-resistant strains (41). These *in vitro* data suggested the potential for the therapeutic use of the ORF28 endolysin for *E. faecalis* infections. Here we present a case of chronic bacterial prostatitis in which a bacteriophage endolysin was successfully used to treat and mitigate infection and clinical symptoms.

# Methods

## Phage cocktail preparation

Three phages (vB_Efa_VP14, vB_Efa_VP15, and vB_Efa_VP16) prepared in the Science Park Bratislava, were isolated from wastewater samples collected from different wastewater treatment plants in the Bratislava region on a bacterial strain isolated from the patient. Ten milliliters of wastewater, sterilized by passage through a 22 µm filter, was mixed with the same volume of twofold concentrated Trypticase Soy Broth medium and 200 µL of overnight bacterial culture. The inoculated mixture was cultivated overnight at 37°C by shaking. Single phage clones were obtained through three repeated isolations from single plaques on double agar (Supplementary Figure S1). Each of the three clones was purified by precipitation in 10% PEG6000 and 1 M NaCl and subsequent ultracentrifugation in a CsCl gradient. The visible phage band for each phage was collected (~1.5 mLs), and each phage sample was dialyzed against 1 liter of SM buffer (100 mM NaCl; 8 mM $MgSO_4$; 50 mM Tris-HCl, pH 7.5; 0.002% gelatin) four times for a minimum of 6 hours each. This yielded 2 mLs of phage with titers of $10^{11}$–$10^{12}$ PFU/mL. The antimicrobial activity of each of the three phages (as well as cocktails of all three together) against the patient's *E. faecalis* strain was evaluated by monitoring the optical density (600 nm) of broth cultures of the patient's *E. faecalis* strain inoculated with the phage. It was found that each of the three phages greatly depressed the growth of the patient's *E. faecalis* strain, and the cocktail of all three phages completely eliminated bacterial growth (Supplementary Figure S2). The host specificity of individual phages as well as a phage cocktail was determined using a plaque assay on double agar plates. Several bacterial species were tested, but only strains of *E. faecalis* were sensitive. The individual phages were lytic for 32% to 46% of the 28 *E. faecalis* strains tested. The cocktail of all three phages was active against 54% of the *E. faecalis* strains. Supplementary Table S1 contains an overview of the sensitivity of a panel of bacterial strains (including the patient's strains) to the individual phage isolates. The three phage isolates were sequenced on NextSeq (illumina) using the Nextera protocol. The average coverage was VP14 = 406, VP15 = 639 and VP16 = 542. Phage ends were screened with specific primers and Sanger sequencing. The three phage DNA sequences were deposited in GenBank as accession numbers OR237563 (for phage vB_Efa_VP14), OR237564 (for phage vB_Efa_VP15), and OR237565 (for phage vB_Efa_VP16). These data permitted the assignment of the three newly isolated phages to the genus *Efquatrovirus*. A tree diagram (Supplementary Figure S3) illustrates the relationship between phages vB_Efa_VP14, vB_Efa_VP15, and vB_Efa_VP16, and Efquatrovirus vB_EfaS_AL2 as well as other closely related phages. For cocktail preparation, 1 mL samples were combined to form a three-phage cocktail, each at $10^9$ PFU/mL. The isolated phages were preserved long-term in SM buffer at 6°C

for no more than 12 months, however the cocktail batches were prepared monthly.

## Phage endolysin production and purification

The production and purification of the phage endolysin was accomplished as described previously (40). In brief, the gene for the ORF28 endolysin was cloned into a pGEX4T2 expression vector in tandem with a glutathione S-transferase (GST) affinity tag. The recombinant plasmid, also featuring an isopropyl-$\beta$-D-thiogalactopyranoside (IPTG)-inducible *tac* promotor, was transformed into *E. coli* BL21/DE3. IPTG-induced expression of the linked ORF28 endolysin and GST genes produced an ORF28-GST fusion protein. A sonic extract (SE), made from the induced *E. coli* culture, was applied to a glutathione resin affinity column, and, after nonadsorbed SE material was eluted, the column was extensively washed with buffer to further remove any non-bound material. The ORF28 endolysin-GST fusion protein (bound to the glutathione of the column via the GST) was specifically desorbed from the column by the addition of a buffer containing glutathione. The process was repeated 4 times until only two protein bands (representing the ORF28-GST fusion protein and the GST protein alone) could be seen by SDS-PAGE analysis of the desorbed material. The ORF28 endolysin protein was recovered from the ORF28-GST fusion protein by reapplying the purified fusion protein to the affinity column and digesting the bound fusion protein with thrombin to cleave the thrombin-sensitive linkage between the ORF28 endolysin and the GST protein. The liberated ORF28 protein was then eluted from the column and collected. The homogeneity of the affinity-purified ORF28 protein was confirmed by SDS-PAGE analysis. As a final step in the purification process, the electrophoretically-homogeneous ORF28 endolysin preparation was passed through a 0.22 µ pore size sterilizing filter. The final purified endolysin preparation had a protein concentration of 0.8 mg/mL.

## Spot testing endolysin activity

Spot testing was used to examine the activity of the ORF28 endolysin against the *E. faecalis* strain isolated from the patient. 0.1 mL of an overnight culture of *E. faecalis* strain 587A, originally isolated by a clinical laboratory in Bratislava in June 2020, was grown in brain heart infusion (BHI) Broth and was inoculated into 3 mL of molten soft agar (BHI broth containing 0.7% agar). This was poured into plates over a layer of BHI agar (1.5%) and allowed to solidify and dry for approximately 15 min. Drops (3 µL) of dilutions of an ORF28 endolysin suspension were then applied to the surface of the solidified soft agar layer. The drops were allowed to dry into the soft agar layer, and the plates incubated overnight at 37°C. The plates were then examined for clear zones where the drops were originally placed, indicating the lytic activity of the endolysin against the *E. faecalis* strain.

## Whole exome sequencing analysis

(Department of Medical Genetics, Medical University of Warsaw, Poland). A library was prepared using the Human Core Exome Kit (Twist Bioscience, South San Francisco, CA, United States), according to manufacturer's instruction, and paired-end sequenced (2 × 100 bp) on a NovaSeq 6000 platform (Illumina, San Diego, CA, United States). Bioinformatic analysis of raw whole exome sequencing (WES) data and variants prioritization were performed as previously described (42).

# Results

## Case description

A 39 years-old Slovakian man was referred to a neurologist in October 2016 after 2 months of neuropathic pain in the perineum, which radiated to the scrotum and the entire anogenital area. The pain developed following repeated cold stimuli in the fall of 2016. The patient described several types of pain: pain in the perineum was dominant, radiating to the rectum, scrotum, and penis. Pain behind the pubic bone was also present. Initially, the pain was paroxysmal and neuralgiform, later it was continuous. Objective neurological findings included significant hyperalgesia of the entire anogenital area. The condition was concluded as chronic pelvic pain syndrome. The patient underwent a battery of examinations aimed at clarifying the origin of the pain. A urologist found an enlarged and painful prostate. Ultrasonography and subsequent magnetic resonance imaging confirmed prostatitis. Cultured ejaculate and expressed prostatic secretions repeatedly confirmed an infection by *Enterococcus faecalis* with high sensitivity to ampicillin/sulbactam (Figure 1, Micro 1). At that time, according to the NIH chronic prostatitis symptom index (NIH-CPSI) (43), the patient reported a pain score of 11 out of 21, a urinary symptom score of 5 out of 10, a quality-of-life impact score of 9 out of 12, and a total score 25, with higher scores indicative of worse outcomes. (In comparison, mean scores for pain, urinary symptoms and quality of life from a cohort of CBP patients were 8.7 ± 5.7, 4.1 ± 3.1, and 6.7 ± 3.6 respectively). The severity of the CBP can be classified as mild (0–9 points), moderate (10-18points) or severe (19–31 points) according to the NIH-CPSI score (43).

The patient's course of treatment is illustrated in the timeline shown in Figure 1. Initial treatment consisted of ampicillin/sulbactam for 2 weeks with satisfactory clinical improvement and bacteriological eradication of enterococcus (Figure 1, Micro 2). However, all the clinical symptoms previously described persisted but at milder intensity. The average NIH-CPSI score after this treatment was 16. Over the next 3 years, the patient had at least three exacerbations per year, when the NIH-CPSI score reached 26 or more, and the presence of *E. faecalis* was always confirmed during flares (Figure 1, Micro 3–7). After treatment with ampicillin/sulbactam or quinolones for 2 weeks, there was a clinical improvement and bacteriological negativity (Figure 1, Micro 8). The NIH-CPSI score averaged 16 during remission stages. In addition to the antibiotics, supplementary therapy consisting of silodosin 4 mg once a day, serenoa rapens, and quercetin 400 mg twice a day, was added to the patient's treatment, however, this produced no significant effect on relieving the symptoms.

In March 2019, prostatitis recurred, coincident with a positive culture of *E. faecalis*, which showed good sensitivity to ampicillin/sulbactam (MIC mg/L AMP 1, VAN 2, CIP 0.5 U, TET ≥16, GEN 128, Figure 1, Micro 9). The patient was re-treated with ampicillin/sulbactam in the usual dose, but after a week of therapy the symptoms

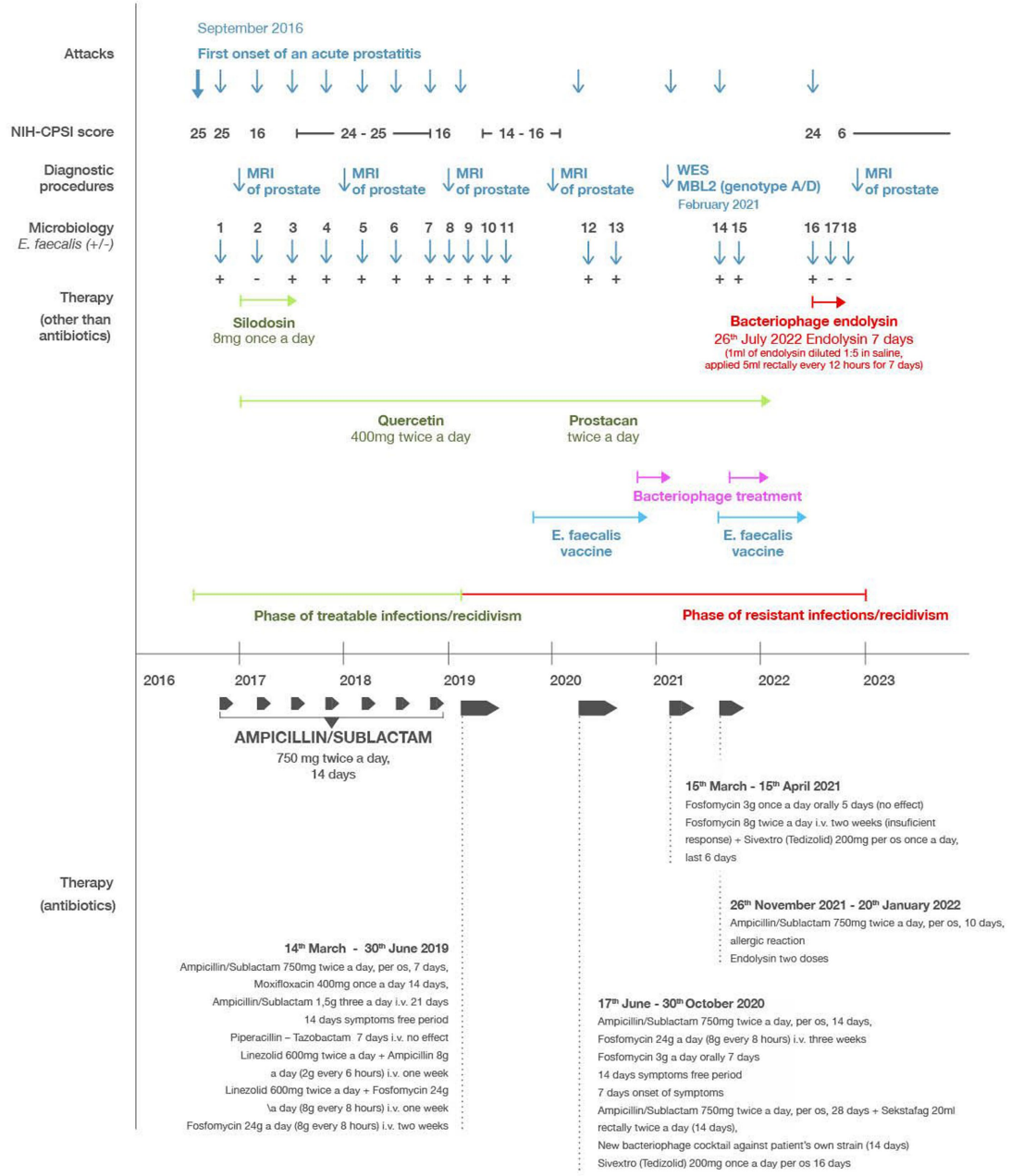


FIGURE 1
Timeline of the most relevant attacks, diagnostic procedures, microbiological results (dark blue), antibiotic therapies (dark grey), *Enterococcus faecalis* vaccination (light blue), phage therapy (magenta), endolysin therapy (red), and other supportive therapies (green). "Microbiology" refers to microbiological analysis determining the presence (*E. faecalis*$^{+}$) or absence (*E. faecalis*$^{-}$) of detectable *E. faecalis* infection.

did not subside. Ampicillin/sulbactam was changed to moxifloxacin, which showed equally good sensitivity *in vitro*. After 14 days of treatment, tachycardia and severe headaches occurred and moxifloxacin was discontinued. After 3 days of withdrawal, the difficulties returned completely. Re-culture of ejaculate and expressed prostatic secretion again showed *E. faecalis* with practically the same MIC values (Figure 1, Micro 10). A 21 days course of ampicillin/sulbactam (4.5 g/day, i.v.) was initiated, resulting in complete abatement of symptoms. After 3 weeks of withdrawal, the difficulties returned again. The culture of the ejaculate and prostatic secretion conducted for the third time after massage again showed *E. faecalis*, but with higher MIC values (AMP 2, VAN 4, CIP 1 U, TET ≥16, GEN 128, Figure 1, Micro 11).

Based on these results, the patient was placed on long-term treatment with high doses of antibiotics consisting of linezolid 600 mg twice a day in combination with ampicillin 2 g every 6 h for the first week. In the second week, the linezolid (600 mg twice a day) was combined with fosfomycin 8 g every 8 h. For the third and fourth weeks, he received monotherapy with fosfomycin 8 g every 8 h. After this treatment, the disease subsided and did not recur for a year. During this period the patient underwent treatment by enterococcal vaccine from his own strain of enterococcus.

In June 2020, there was another recurrence of the disease in the sense of urgency and pelvic pain. Culture of the ejaculate and prostatic secretion after the massage again showed *E. faecalis* with an MIC of AMP 2. Furthermore, the clinical microbiology laboratory reported that the *E. faecalis* isolate was resistant to a variety of antibiotics including oxacillin, cefoxitin, gentamicin and tetracycline (Figure 1, Micro 12). The patient began treatment with ampicillin/sulbactam 750 mg twice a day. After a temporary improvement, the condition worsened from the fifth day of treatment. Due to intolerance to quinolones and the previous eradication of the pathogen with fosfomycin, therapy continued with fosfomycin 8 g three times a day intravenously for 21 days, followed by fosfomycin 3 g per day orally for 7 days, i.e., a total of 28 days. After this treatment, the difficulties subsided. After 2 weeks, however, the difficulties recurred and again, enterococcus was cultivated from ejaculate (Figure 1, Micro 13).

## Application of phage therapy

Considering that the patient received the highest possible doses of fosfomycin for 21 days, and was intolerant to quinolones, we proceeded to a combination of bacteriophages (Sekstafag) 20 mL rectally twice a day and ampicillin/sulbactam 750 mg twice a day *per os* for 2 weeks, without any clinical effect. Furthermore, a new bacteriophage cocktail against patient's own strain in a concentration of $10^9$ pfu/mL, with convincing *in vitro* activity, had been prepared in the Science Park Bratislava. The cocktail contained three newly isolated phages (vB_Efa_VP14, vB_Efa_VP15, vB_Efa_VP16) belonging to the genus *Efquatrovirus*. Each phage had a unique host specificity and efficiently lysed the patient's strain. The patient used this cocktail for the next 2 weeks, 10 mL twice a day applied rectally, however no clinical effect was observed. In September 2020, the patient started taking linezolid 600 mg twice a day for 21 days.

## Application of phage endolysin eliminates enterococcal infection and mitigates CBP symptoms

The lack of improvement of the patient's condition prompted a search for an alternative therapy to the antibiotic treatments previously employed. In September 2020 a request was sent from Slovakia to the Stevens laboratory at Temple University, Philadelphia, for an *E. faecalis*-specific bacteriophage or a phage-based lytic enzyme that could be capable of degrading an *E. faecalis* biofilm. The laboratory had both: previously, a genetically engineered derivative of *E. faecalis* phage ($\phi$Ef11) had proven in *in vitro* testing to infect many strains of *E. faecalis*, reduce the populations of *E. faecalis* cultures, and drastically disrupt *E. faecalis* biofilms (44, 45). However, the genetically modified phage [$\phi$Ef11/FL1C(Δ36)$P^{nisA}$] possessed a nisin-dependent promoter ($P^{nisA}$) that required the presence of nisin, as a cofactor for activation. While this was advantageous for controlling phage activity in *in vitro* experimental conditions, it would not be suitable for *in vivo* clinical application. However, the $\phi$Ef11 phage endolysin (ORF28 endolysin) was available from the laboratory and had previously exhibited rapid and profound lysis of cells of most *E. faecalis* strains, including those that were antibiotic-resistant (40, 41). The patient's *E. faecalis* strain was isolated and sent to the Stevens laboratory for sensitivity testing against the $\phi$Ef11 phage ORF28 endolysin. Spot testing of dilutions of the purified endolysin on lawns of the patient's *E. faecalis* isolate revealed that this strain was indeed extremely sensitive to the lytic action of the endolysin (Figure 2).

Considering the pronounced endolysin-sensitivity of the patient's *E. faecalis* strain, it was not unreasonable to entertain the possibility that the endolysin might have a beneficial effect in controlling the patient's infection. In this regard, Temple University's Institutional Review Board was contacted to ensure that the contemplated endolysin therapy would be consistent with local (U.S.) regulatory requirements. Regulatory requirements in Slovakia consisted of the recognition that (1) bacteriophages and their products are considered as an alternative to antibiotics and (2) the clinical use of bacteriophages and their products is solely governed by the expert opinion of the attending physician or consultant. Consequently, the expert opinion of an infectious diseases specialist was obtained, concluding that the patient was suffering from "…a biofilm infection which can no longer be eradicated with current antibiotics", and that "…therapy with bacteriophage lysine (*sic*) as a last resort in the treatment of refractory bacterial prostatitis." Therefore, with no additional regulatory requirements to be satisfied, purification of the phage endolysin was completed, resulting in the isolation of an electrophoretically homogeneous protein with a molecular mass of 46.1 kDa, which is the predicted size of the phage ORF28 endolysin (Figure 3). The patient was fully informed about potential risks and benefits of the treatment and signed an informed consent and approved the publication of his course of treatment.

The sterile, purified endolysin preparation was sent to Slovakia in November of 2020 however at that time, the patient was already asymptomatic due to linezolid therapy. During the year 2021 the patient experienced several attacks of prostatitis usually treated with ampicillin/sulbactam or levofloxacin. Furthermore in 2021, the patient underwent whole exome sequencing (WES) analysis in the Department of Medical Genetics, Medical University of Warsaw, Poland. WES revealed a heterozygous variant in *MBL2* gene (hg38, chr10:g.052771482-G>A, NM_000242.3: c.154C>T/p.(Arg52Cys), rs5030737), which refers to D-allele and HYD haplotype (patient's genotype A/D and haplotype

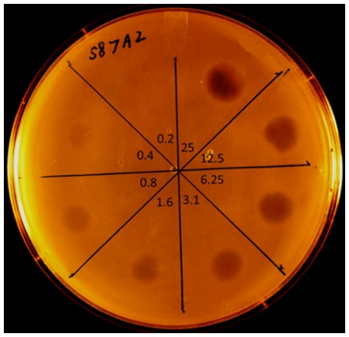


FIGURE 2
Spot testing sensitivity of *E. faecalis* strain (587A2) from the CBP patient to purified phage ORF28 endolysin. Dilutions of purified endolysin (original concentration = 800 μg/mL) spotted onto a soft-agar lawn of *E. faecalis* 587A2. Lytic zones observed after overnight incubation @ 37°C. Numbers towards center of plate indicate the concentration (μg/mL) of ORF28 applied in each section of the plate. Lytic zone observable down to endolysin concentration of 0.4 μg/mL.

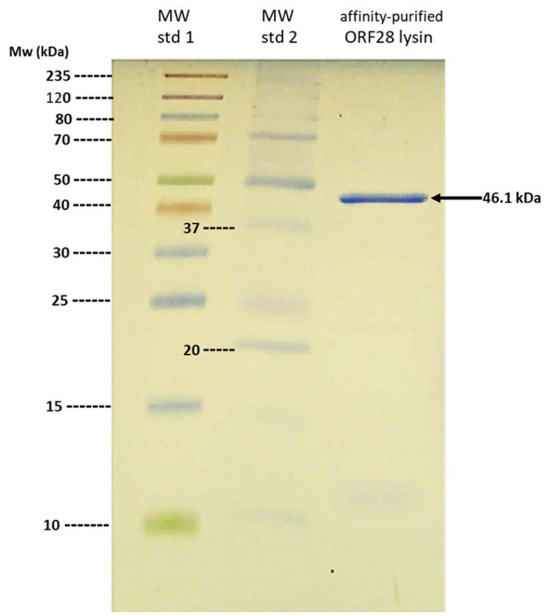


FIGURE 3
SDS-PAGE analysis of purified ORF28 endolysin. Note single 46.1 kDa band from affinity-purified ORF28 endolysin.

HYA/HYD). The p.(Arg52Cys) variant is described as "pathogenic" according to ClinVar database (Accession: RCV000015426.29) in relation to mannose-binding lectin (MBL) deficiency (MIM#614372). Subsequent immunological examination showed a reduced level of MBL to 375 ng/mL (normal value more than 2,880).

By October/November of 2021, the prostatitis worsened, with more intense symptoms in terms of urgencies, nycturia and pelvic pain, and culture of the ejaculate again confirmed *E. faecalis* (Figure 1, Micro 14, 15). For this, the patient began applying probiotics consisting of lactobacilli and the above-mentioned bacteriophage cocktail ($10^9$ pfu/mL), which he continued for the next 2 weeks, 20 mL rectally twice a day, however the symptoms continued to worsen. The patient was then again put on ampicillin-sulbactam (750 mg twice a day orally) and the condition began to improve, however after 4 days, the patient experienced a severe allergic reaction consisting of whole body itching and exanthema. This necessitated the discontinuation of the ampicillin/sulbactam treatment and its replacement by fosfomycin 3 g once a day orally. Again, a similar severe allergic reaction (whole body itching and exanthema) ensued, and the fosfomycin also had to be discontinued. At this point the patient applied the phage endolysin preparation, which had been stored refrigerated since its arrival (prior laboratory studies demonstrated that the endolysin was extremely stable, and could retain its activity for several years, if kept refrigerated) (40). The preparation was diluted 1:5 in saline, and two doses of 5 mL were applied rectally 12 h apart. After the administration of the two doses, the symptoms subsided, but the patient did not have additional doses available, so he could not continue the endolysin therapy.

In July 2022 there was a significant recurrence, and culture of the ejaculate again confirmed *E. faecalis* (Figure 1, Micro16). The total NIH-CPSI score at this point was 24 (a pain score of 7, a urinary symptom score of 8, and a quality-of-life impact score of 9). Additional endolysin received from the Stevens laboratory was immediately applied in the same dose (1 mL of endolysin diluted 1:5 in saline and applied 5 mL rectally every 12 h for 7 days) with significant clinical improvement and bacteriological eradication of enterococcus. The total NIH-CPSI score at this point was 6 (a pain score of 0, a urinary symptom score of 3, and a quality-of-life impact score of 3). After completing the treatment in August of 2022, the culture of the ejaculate was sterile, and the expressed prostatic secretion contained only coagulase-negative staphylococci (Figure 1, Micro 17). The patient reported no untoward reactions during or after the endolysin treatment with any of the administrations. From this period (August 9, 2022) until the last check-up (February 9, 2023), the patient did not have a recurrence of bacterial prostatitis. His CPS score remained at 6 points (a pain score of 0, a urinary symptom score of 3, and a quality-of-life impact score of 3). His most recent bacteriological analysis, in November of 2022 was negative (Figure 1, Micro 18) and MRI of the prostate from December 2022 showed consolidation of inflammatory changes of the prostate by 30% compared to an examination conducted in September 2020. The patient is still taking quercetin 400 mg once a day and Prostacan (*Serenoa rapens* and *Urtica dioica* extract) once a day.

## Discussion

Chronic prostatitis is a chronic recurrent disease with a very complex etiology and pathogenesis, which significantly reduces the quality of life of patients. Predisposing factors are usually hidden immunological deficits, which are manifested by increased susceptibility of the urogenital tract to bacterial invasion (46–48). Numerous recent studies have reported that *E. faecalis* is either the first (49–53) or second (54–57) most frequently isolated organism from CBP cases. *E. faecalis* frequently forms a biofilm in acini or around prostatic calculi, which protects it from the impact of antibiotics (58). Repeated administration of antibiotics is associated with a high risk of the development of resistance, and cumulative toxic effects and allergic reactions. Therefore, the search for alternative approaches is the right way forward.

Here, we present the case of a 39 years-old man with chronic pelvic pain due to CBP. This report points to the torpidity and the chronic relapsing nature of the disease, and the failure of antibiotic therapy. Diagnostically, WES revealed an MBL2 gene polymorphism with genotype A/D instead of wild type AA and subsequent immunologic testing confirmed MBL2 deficiency. Considering this, several alternative treatment options were attempted. From the history of the present case, the following can be stated: bacteriophage cocktails were not effective. Supportive treatment, in the form of phytopharmaceuticals (*Serenoa rapens*, *Urtica dioica* and quercetin extracts), microbiome modification via probiotics, and administration of an autovaccine developed from the patient's own *E. faecalis* strain, all failed to prevent disease reoccurrence and continued infection. We perceive the most significant impact on the course of the disease was due to the application of bacteriophage endolysin rectally, which resulted in the elimination of enterococcus and a long-term asymptomatic period.

Antibiotic therapy is the conventional standard of care for treatment of chronic bacterial prostatitis (4, 59). In the present case, conventional therapy using antibiotics, was ineffective due to the development of resistance to many antibiotics by the *E. faecalis* strain causing the infection, and the intolerably severe allergic reactions caused by the antibiotics that were used. In addition, biofilms that can

form in the infected prostate (60, 61), can be relatively resistant to the antimicrobial effects of antibiotics (62).

Phage therapy has been proposed as a useful alternative to antibiotics. In this regard, several studies have reported the successful use of phage therapy in treating cases of CBP (63–66). A personalized bacteriophage cocktail that was active (*in vitro*) against the patient's own strain of *E. faecalis* was used in treating the patient's infection. In light of the prior reports of successful phage therapy in treating chronic bacterial prostatitis, it is not clear why this was not effective in the present case.

This report demonstrates the utility and efficacy of bacteriophage endolysin therapy in treating recalcitrant infections; particularly those due to drug-resistant bacteria or in cases where effective antibiotic treatment is precluded due to serious adverse reactions. The efficacy of the ORF28 endolysin for controlling this patient's *E. faecalis* infection was presumably due to the ability of the endolysin to lyse and kill the infecting organisms. Previous *in vitro* data demonsterated that the ORF28 endolysin caused rapid and profound lysis of sensitive *E. faecalis* strains (40), and that the patient's *E. faecalis* strain was shown to be sensitive to the endolysin. Thus, it was not unreasonable to anticipate a beneficial effect of the endolysin on the patient's *E. faecalis*-infected prostate, provided that the endolysin could gain access to the infected prostate. Anatomic studies disclose that the rectal venous plexus/hemorrhoidal plexus communicates with the prostatic venous plexus via the vesical venous plexus (67). This could provide an entrée for rectally-applied endolysin to the prostate. In animal studies involving mice and rabbits, rectally-applied bacteriophages could be detected in the circulation within minutes (68). Therefore, the positive outcome that we observed is consistant with both the activity of the phage endolysin, and its potential availability to the site of infection.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/genbank/, GQ452243.

## Ethics statement

The studies involving humans were approved by Institutional Review Board, Temple University. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

## Author contributions

RS: conceptualized the project, isolated and characterized the bacteriophage from which the endolysin was obtained, and provided the genetic information for cloning the endolysin gene. HZ: cloned the endolysin gene and purified the endolysin. RS and HZ: characterized the endolysin. PS: provided infectious disease expertise and expert opinion authorizing the compassionate use of the endolysin. MK: isolated and characterized the bacteriophages used for phage therapy. RP and MR: oversaw the whole exosome sequencing and genomic data analysis. SŠ: oversaw patient management and all clinical aspects of the study. RS and SŠ: wrote the manuscript. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2023.1238147/full#supplementary-material

## References

1. Krieger JN. Classification, epidemiology and implications of chronic prostatitis in North America, Europe and Asia. *Minerva Urol Nefrol.* (2004) 56:99–107.

2. Krieger JN, Lee SWH, Jeon J, Cheah PY, Long ML, Riley DE. Epidemiology of prostatitis. *Int J Antimicrob Agents.* (2008) 31:S85–90. doi: 10.1016/j.ijantimicag.2007.08.028

3. Nickel JC, Moon T. Chronic bacterial prostatitis: an evolving clinical enigma. *Urology*. (2005) 66:2–8. doi: 10.1016/j.urology.2004.12.028

4. Schaeffer AJ. Chronic prostatitis and the chronic pelvic pain syndrome. *N Engl J Med.* (2006) 355:1690–8. doi: 10.1056/NEJMcp060423

5. Bownen D, Dielubanza E, Schaeffer AJ. Chronic bacterial prostatitis and chronic pelvic pain syndrome. *BMJ Clin Evid.* (2015) 8:1802.

6. Meares EM, Stamey TA. Bacteriologic localization patterns in bacterial prostatitis and urethritis. *Invest Urol.* (1968) 5:492–518.

7. Drach GW. Problems in diagnosis of bacterial prostatitis: Gram-negative, Gram-positive and mixed infections. *J Urol*. (1974) 111:630–6. doi: 10.1016/S0022-5347(17)60033-8

8. Rees J, Abrahams M, Doble A, Cooper A. Diagnosis and treatment of chronic bacterial prostatitis and chronic prostatitis/chronic pelvic pain syndrome: a consensus guideline. *BJU Int*. (2015) 116:509–25. doi: 10.1111/bju.13101

9. Roberts RO, Lieber MM, Rhodes T, Girman CJ, Bostwick DG, Jacobsen SJ. Prevalence of a physician-assigned diagnosis of prostatitis: the Olmsted county study of urinary symptoms and health status among men. *Urology*. (1998) 51:579–84. doi: 10.1016/s0090-4295(98)00034-x

10. Krieger JN, Ross SO, Riley DE. Chronic prostatitis: epidemiology and role of infection. *Urology*. (2002) 60:8–12. doi: 10.1016/s0090-4295(02)02294-x

11. Collins MM, Stafford RS, O'Leary MP, Barry MJ. How common is prostatitis? A national survey of physician visits. *J Urol*. (1998) 159:1224–8. doi: 10.1016/S0022-5347(01)63564-X

12. Krieger JN, Riley DE, Roberts MC, Berger RE. Prokaryotic DNA sequences in patients with chronic idiopathic prostatitis. *J Clin Microbiol*. (1996) 34:3120–8. doi: 10.1128/jcm.34.12.3120-3128.1996

13. Nickel JC, Xiang J. Clinical significance of nontraditional bacterial uropathogens in the management of chronic prostatitis. *J Urol*. (2008) 179:1391–5. doi: 10.1016/j.juro.2007.11.081

14. Cox CE, Childs SJ. Treatment of chronic bacterial prostatitis with temafloxacin. *Am J Med*. (1991) 91:134S–9S. doi: 10.1016/0002-9343(91)90326-s

15. Facklam RR, Carvalho MGS, Teixeira LM. History, taxonomy, biochemical characteristics and antibiotic susceptibility testing of enterococci In: MS Gilmore, editor. *The enterococci-pathogenesis, molecular biology, and antibiotic resistance, 2002*. Washington, DC: ASM Press (2002). 1–54.

16. Lebreton F, Willems RJL, Gilmore MS. Enterococcus diversity, origins in nature, and gut colonization In: MS Gilmore, DB Clewell, Y Ike and N Shankar, editors. *Enterococci: from commensals to leading causes of drug resistant infection*. Boston: Massachusetts Eye and Ear Infirmary (2014). 5–64.

17. Sedgley CM, Lennan SL, Appelbe OK. Survival of *Enterococcus faecalis* in root canals *ex vivo*. *Int Endodon J*. (2005) 38:735–42. doi: 10.1111/j.1365-2591.2005.01009.x

18. Murray BE. The life and times of the enterococcus. *Clin Microbiol Rev*. (1990) 3:46–65. doi: 10.1128/CMR.3.1.46

19. Hollenbeck BL, Rice LB. Intrinsic and acquired resistance mechanisms in enterococcus. *Virulence*. (2012) 3:421–33. doi: 10.4161/viru.21282

20. Arias CA, Murray BE. The rise of enterococcus: beyond vancomycin resistance. *Nat Rev Microbiol*. (2012) 10:266–78. doi: 10.1038/nrmicro2761

21. Murray BE, Mederski-Samaroj B. Transferable beta-lactamase. A new mechanism for *in vitro* penicillin resistance in *Streptococcus faecalis*. *J Clin Invest*. (1983) 72:1168–71. doi: 10.1172/JCI111042

22. Bonten MJM, Willems R, Weinstein RA. Vancomycin-resistant enterococci: why are they here, and where do they come from? *Lancet Infect Dis*. (2001) 1:314–25. doi: 10.1016/S1473-3099(01)00145-1

23. Mederski-Samoraj BD, Murray BE. High-level resistance to gentamicin in clinical isolates of enterococci. *J Infect Dis*. (1983) 147:751–7. doi: 10.1093/infdis/147.4.751

24. Uttley AHC, Collins CH, Naidoo J, George RC. Vancomycin-resistant enterococci. *Lancet*. (1988) 68:57–8. doi: 10.1016/s0140-6736(88)91037-9

25. Atkinson BA, Lorian V. Antimicrobial agent susceptibility patterns of bacteria in hospital from 1971 to 1982. *J Clin Microbiol*. (1984) 20:791–6. doi: 10.1128/jcm.20.4.791-796.1984

26. Acar JF, Buu-Hoi AY. Resistance patterns of important Gram-positive pathogens. *J Antimicrob Chemother*. (1988) 21:41–7. doi: 10.1093/jac/21.suppl_c.41

27. Silva Munoz-Price L, Lolans K, Quinn JP. Emergence of resistance to daptomycin during treatment of vancomycin-resistant *Enterococcus faecalis* infection. *Clin Infect Dis*. (2005) 41:565–6. doi: 10.1086/432121

28. Singh KV, Weinstock GM, Murray BE. *Enterococcus faecalis* ABC homologue (Lsa) is required for the resistance of this species to clindamycin and quinupristin-dalfopristin. *Antimicrob Agents Chemother*. (2002) 46:1845–50. doi: 10.1128/AAC.46.6.1845-1850.2002

29. Prystowsky J, Siddiqui F, Chosay J, Shinabarger DL, Millichap J, Peterson LR, et al. Resistance to linezolid: characterization of mutations in rRNA and comparison of their occurrences in vancomycin-resistant enterococci. *Antimicrob Agents Chemother*. (2001) 45:2154–6. doi: 10.1128/AAC.45.7.2154-2156.2001

30. Krause RM. Studies on bacteriophages of hemolytic streptococci. Factors influencing the interaction of phage and susceptible host cell. *J Exp Med*. (1957) 106:365–84. doi: 10.1084/jem.106.3.365

31. Fischetti VA. Bacteriophage lytic enzymes: novel anti-infectives. *Trends Microbiol*. (2005) 13:491–6. doi: 10.1016/j.tim.2005.08.007

32. Nelson D, Loomis L, Fischetti VA. Prevention and elimination of upper respiratory colonization of mice by group a streptococci by using a bacteriophage lytic enzyme. *Proc Natl Acad Sci U S A*. (2001) 98:4107–12. doi: 10.1073/pnas.061038398

33. Borysowski J, Weber-Dabrowska B, Gorski A. Bacteriophage endolysins as a novel class of antibacterial agents. *Exp Biol Med*. (2006) 231:366–77. doi: 10.1177/153537020623100402

34. Nelson DC, Schmelcher M, Rodriguez-Rubio L, Klumpp J, Pritchard DG, Dong S, et al. Endolysins as antimicrobials. *Adv Virus Res*. (2012) 83:301–65. doi: 10.1016/B978-0-12-394438-2.00007-4

35. Pastagia M, Schuch R, Fischetti VA, Huang DB. Lysins: the arrival of pathogen-directed anti-infectives. *J Med Microbiol*. (2013) 62:1506–16. doi: 10.1099/jmm.0.061028-0

36. Love MJ, Bhandari D, Dobson RCJ, Billington C. Potential for bacteriophage endolysins to supplement or replace antibiotics in food production and clinical care. *Antibiotics*. (2018) 7:17. doi: 10.3390/antibiotics7010017

37. Fowler VG Jr, Das AF, Lippka-Diamond J, Schuch R, Pomerantz R, Jauregui-Peredo L, et al. Exebacase for patients with *Staphylococcus aureus* bloodstream infection and endocarditis. *J Clin Invest*. (2020) 130:3751–60. doi: 10.1172/JCI136577

38. Stevens RH, Porras OD, Delisle AL. Bacteriophages induced from lysogenic root canal isolates of *Enterococcus faecalis*. *Oral Microbiol Immunol*. (2009) 24:278–84. doi: 10.1111/j.1399-302X.2009.00506.x

39. Stevens RH, Ektefaie MR, Fouts DE. The annotated complete DNA sequence of *Enterococcus faecalis* bacteriophage ϕEf11 and its comparison with all available phage and predicted prophage genomes. *FEMS Microbiol Lett*. (2011) 2011:9–26. doi: 10.1111/j.1574-6968.2010.02203.x

40. Zhang H, Buttaro BA, Fouts DE, Sanjari S, Evans BS, Stevens RH. Bacteriophage ϕEf11 ORF28 endolysin, a multifunctional enzyme with properties distinct from all other identified *Enterococcus faecalis* phage endolysins. *Appl Environ Microbiol*. (2019) 85:e00555. doi: 10.1128/AEM.00555-19

41. Zhang H, Stevens RH. Intrinsic resistance of *Enterococcus faecalis* strains to ϕEf11 phage endolysin is associated with the presence of ϕEf11 prophage. *Archs Virol*. (2021) 166:249–58. doi: 10.1007/s00705-020-04861-7

42. Rydzanicz M, Zwoliński P, Gasperowicz P, Pollak A, Kostrzewa G, Walczak A, et al. A recurrent *de novo* variant supports KCNC2 involvement in the pathogenesis of developmental and epileptic encephalopathy. *Am J Med Genet A*. (2021) 185:3384–9. doi: 10.1002/ajmg.a.62455

43. Litwin MS, McNaughton-Collins M, Fowler FJ Jr, Nickel JC, Calhoun EA, Pontari MA, et al. The National Institutes of Health chronic prostatitis symptom index: development and validation of a new outcome measure. *J Urol*. (1999) 162:369–75. doi: 10.1016/S0022-5347(05)68562-X

44. Zhang H, Fouts DE, DePew J, Stevens RH. Genetic modifications to temperate *Enterococcus faecalis* phage ϕEf11 that abolish the establishment of lysogeny and sensitivity to repressor, and increase host range and productivity of lytic infection. *Microbiology*. (2013) 159:1023–35. doi: 10.1099/mic.0.067116-0

45. Tinoco JM, Buttaro B, Zhang H, Liss N, Sassone L, Stevens R. Effect of a genetically engineered bacteriophage on *Enterococcus faecalis* biofilms. *Archs Oral Biol*. (2016) 71:80–6. doi: 10.1016/j.archoralbio.2016.07.001

46. Shoskes DA, Keslar KS, Gotwald P, Berglund R, Vij S. Neuroinflammatory gene expression in chronic prostatitis/chronic pelvic pain syndrome patients: insights into etiology and phenotype biology. *Trans Androl Urology*. (2021) 10:3340–7. doi: 10.21037/tau-21-387

47. Peterslund NA, Koch C, Jensenius JC, Thiel S. Association between deficiency of mannose-binding lectin and severe infections after chemotherapy. *Lancet*. (2001) 358:637–8. doi: 10.1016/S0140-6736(01)05785-3

48. Bouwman LH, Roos A, Terpstra OT, De Kniff P, van Hoek B, Verspaget HW, et al. Mannose binding lectin gene polymorphisms confer a major risk for severe infections after liver transplantation. *Gastroenterology*. (2005) 129:408–14. doi: 10.1016/j.gastro.2005.06.049

49. Bundrick W, Heron SP, Ray P, Schiff WM, Tennenberg AM, Wiesinger BA, et al. Levofloxacin versus ciprofloxacin in the treatment of chronic bacterial prostatitis: a randomized double-blind multicenter study. *Urology*. (2003) 62:537–41. doi: 10.1016/S0090-4295(03)00565-X

50. Magri V, Trinchieri A, Pozzi G, Restelli A, Garlaschi MC, Torresani E, et al. Efficacy of repeated cycles of combination therapy for the eradication of infecting organisms in chronic bacterial prostatitis. *Int J Antimicrob Agents*. (2007) 29:549–56. doi: 10.1016/j.ijantimicag.2006.09.027

51. Cai T, Mazzoli S, Bechi A, Addonisio P, Mondaini N, Pagliai RC, et al. *Serenoa repens* associated with *Urtica dioica* (ProstaMEV®) and curcumin and quercitin (FlogMEV®) extracts are able to improve the efficacy of prulifloxacin in bacterial prostatitis patients: results from a prospective randomized study. *Int J Antimicrob Agents*. (2009) 33:549–53. doi: 10.1016/j.ijantimicag.2008.11.012

52. Cai T, Mazzoli S, Meacci F, Boddi V, Mondaini N, Malossini G, et al. Epidemiological features and resistance pattern of uropathogens isolated from chronic bacterial prostatitis. *J Microbiol*. (2011) 49:448–54. doi: 10.1007/s12275-011-0391-z

53. Magri V, Marras E, Perletti G. Chronic bacterial prostatitis: enterococcal disease? *Clin Infect Dis*. (2011) 53:1306–7. doi: 10.1093/cid/cir709

54. Heras-Canas V, Gutiérrez-Soto B, Serrano-Garcia ML, Vázquez-Alonso F, Navarro-Mari JM, Gutiérrez-Fernández J. Chronic bacterial prostatitis. Clinical and microbiological study of 332 cases. *Med Clin*. (2016) 147:144–7. doi: 10.1016/j.medcli.2016.05.018

55. Choi YS, Kim KS, Choi SW, Seol K, Bae WJ, Cho HJ, et al. Microbiological etiology of bacterial prostatitis in general hospital and primary care clinic in Korea. *Prostate Int*. (2013) 1:133–8. doi: 10.12954/PI.13023

56. Stamatiou K, Magri V, Perletti G, Papadouli V, Recleiti N, Mamali V, et al. Chronic prostatic infection: microbiological findings in two Mediterranean populations. *Arch Ital Urol Androl*. (2019) 91:177–81. doi: 10.4081/aiua.2019.3.177

57. Trinchieri A, Abelrahman KM, Bhatti KH, Bello JO, Das K, Gatsev O, et al. Spectrum of causative pathogens and resistance rates to antibacterial agents in bacterial prostatitis. *Diagnostics*. (2021) 11:1333. doi: 10.3390/diagnostics11081333

58. Mazzoli S. Biofilms in chronic bacterial prostatitis (NIH-II) and in prostatic calcifications. *FEMS Immunol Med Microbiol*. (2010) 59:337–44. doi: 10.1111/j.1574-695X.2010.00659.x

59. Wagenlehner FME, Weidner W, Sörgel F, Naber KG. The role of antibiotics in chronic bacterial prostatitis. *Int J Antimicrob Agents*. (2005) 26:1–7. doi: 10.1016/j.ijantimicag.2005.04.013

60. Nickel JC, Costerton JW. Bacterial localization in antibiotic-refractory chronic bacterial prostatitis. *Prostate*. (1993) 23:107–14. doi: 10.1002/pros.2990230204

61. Arakawa S, Matsui T, Gohji K, Okada H, Kamidono S. Prostatitis-the Japanese viewpoint. *Int J Antimicrob Agents*. (1999) 11:201–3. doi: 10.1016/S0924-8579(99)00015-1

62. Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev*. (2002) 15:167–93. doi: 10.1128/CMR.15.2.167-193.2002

63. Letkiewicz S, Miedzybrodzki R, Fortuna W, Weber-Dabrowska B, Górski A. Eradication of *Enterococcus faecalis* by phage therapy in chronic bacterial prostatitis-case report. *Folia Microbiol*. (2009) 54:457–61. doi: 10.1007/s12223-009-0064-z

64. Johri AV, Johri P, Hoyle N, Pipia L, Nadareishvilli L, Nizharadze D. Case report: chronic bacterial prostatitis treated with phage therapy after multiple failed antibiotic treatments. *Front Pharmacol*. (2021) 12:e692614. doi: 10.3389/fphar.2021.692614

65. Letkiewicz S, Miedzybrodzki R, Klak M, Jonczyk E, Weber-Dabrowska B, Gorski A. The perspectives of the application of phage therapy in chronic bacterial prostatitis. *FEMS Immunol Med Microbiol*. (2010) 60:99–112. doi: 10.1111/j.1574-695X.2010.00723.x

66. Górski A, Jończyk-Matysiak E, Łusiak-Szilachowska M, Międzybrodzki R, Weber Dąbrowska B, Borysowski J, et al. Phage therapy in prostatitis: recent prospects. *Front Microbiol*. (2018) 9:1434. doi: 10.3389/fmicb.2018.01434

67. Shafik A. A concept of the anatomy of the anal sphincter mechanism and the physiology of defecation. *Dis Colon Rectum*. (1987) 30:970–82. doi: 10.1007/BF02554289

68. Hoffmann M. Tierversuche zur shleimhautpassage und resorptionsviramie von T3-phagen nach oraler, trachealer und rektaler gabe. *Z Bakteriol Med Hyg Bakteriol Virusfursch Parasitol*. (1965) 198:371–90.

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# The long and sinuous road to phage-based therapy of *Clostridioides difficile* infections

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With the antibiotic crisis and the rise in antimicrobial resistance worldwide, new therapeutic alternatives are urgently needed. Phage therapy represents one of the most promising alternatives but for some pathogens, such as *Clostridioides difficile*, important challenges are being faced. The perspective of phage therapy to treat *C. difficile* infections is complicated by the fact that no strictly lytic phages have been identified so far, and current temperate phages generally have a narrow host range. *C. difficile* also harbors multiple antiphage mechanisms, and the bacterial genome is often a host of one or multiple prophages that can interfere with lytic phage infection. Nevertheless, due to recent advances in phage host receptor recognition and improvements in genetic tools to manipulate phage genomes, it is now conceivable to genetically engineer *C. difficile* phages to make them suitable for phage therapy. Other phage-based alternatives such as phage endolysins and phage tail-like bacteriocins (avidocins) are also being investigated but these approaches also have their own limitations and challenges. Last but not least, *C. difficile* produces spores that are resistant to phage attacks and all current antibiotics, and this complicates therapeutic interventions. This mini-review gives a brief historical overview of phage work that has been carried out in *C. difficile*, presents recent advances in the field, and addresses the most important challenges that are being faced, with potential solutions.

KEYWORDS

***Clostridioides (Clostridium) difficile* infection, bacteriophage (phage), phage therapy, endolysin, diffocin, avidocin, phage engineering**

## Introduction

*Clostridioides difficile* is one of the top priority pathogens according to the US CDC (1). This Gram-positive, strictly anaerobic spore-forming bacillus is the main cause of antibiotic-associated diarrhea. In the early 2000s, major outbreaks occurred in North America and Europe (2). One group of strains, designated as ribotype 027 (R027), has been associated with increased disease severity, poorer clinical outcome, and more frequent relapses (3–5). Current treatments rely on antibiotics, which further disrupt the protective gut microbiota. Spores are resistant to all antibiotics, and once antibiotherapy is stopped, residual spores within the gut or spores ingested from contaminated environments can germinate due to the permissive microbiota. Consequently, many patients experience one or more relapses leading to recurrent *C. difficile* infections (rCDI) (6). The best intervention to treat rCDI is the fecal microbiota transplant (FMT) that swiftly restores the gut microbiota diversity, which is associated with colonization resistance (7). However, this approach presents several limitations including the risk for potential transfer of unwanted microbes and the lack of knowledge on the long-term impact of FMT on health (8). Therefore, other therapeutic strategies are urgently needed. Phage therapy is the administration of bacteriophages (or

phages) that specifically kill target bacteria and is a promising alternative or complement to antibiotherapy in the fight against multidrug-resistant pathogens (9–12). The main advantage of therapeutic phages is their great specificity toward target bacteria, thus sparing other beneficial bacteria. A targeted approach like phage therapy could be a very powerful solution in the case of rCDI. Over the last two decades, the potential of phages or phage derivatives to treat or prevent CDI has been explored. This mini-review summarizes the current advances in phage-based approaches to fight against *C. difficile*. The most urgent challenges that must be addressed and mitigating strategies are also discussed.

## Whole phage treatment

Several phages infecting *C. difficile* have been isolated, but only 33 of them have been sequenced and characterized more deeply. It is important to stress that all *C. difficile* phages described to date have a temperate lifestyle, i.e., these phages lead to lysogeny (13, 14). Upon incorporation of a prophage, a bacterial host becomes automatically resistant to further lytic reinfection by the same or a related phage. It is, therefore, generally discouraged to use temperate phages for therapy, although genetic engineering has the potential to transform temperate phages into an important source of therapeutic agents (15, 16). Moreover, phage resistance due to lysogeny can be mitigated or even eliminated by the temperate phage antibiotic synergy (tPAS) phenomenon, which consists of combining temperate phages with sub-inhibitory concentrations of antibiotics to activate the SOS response and prevent phage entry into the lysogenic cycle (17). tPAS has not been tested in *C. difficile*, but the efficacy of several unmodified temperate phages was assessed under different laboratory settings and in preclinical animal models. Table 1 summarizes these studies and those in which phage-derived antimicrobials were tested.

## Phage cocktails are more efficient than single phage treatments

The lytic potential of different *C. difficile* phages has been assessed in several *in vitro* assays. The main findings are that phages kill vegetative cells efficiently, reducing bacterial counts by several logs (19, 21, 23, 25). However, one common observation with single phage treatments has been culture rebound due to lysogeny. This was well described with phage phiCD27 used in batch fermentations and *in vitro* gut models (19, 20). A well-known method to limit the rise of phage resistance is to use phage cocktails (9). The administration of single or multiple phage combinations was compared *in vitro* (21, 23). Cocktails comprising 3–4 different phages better prevented the culture regrowth compared with cocktails comprising a single phage. Interestingly, some cocktails were shown to prevent *C. difficile* biofilm formation (22). Biofilms are complex ecosystems generally comprising multiple bacterial species embedded into a matrix composed of extracellular polymeric substances (EPS), such as polysaccharides, DNA, amyloids, lipids, and proteins (39). Some phages possess depolymerase activity at the tip of their tail that can degrade EPS and biofilms (40). Although *C. difficile* phages with depolymerase activity have never been described, some phages were shown to penetrate and destabilize already-formed biofilms. However, complete eradiction of *C. difficile* from an already established biofilm was not observed (22). It is worth mentioning that the results varied depending on the targeted *C. difficile* strain, suggesting that optimization of the cocktails would be necessary on a strain-specific basis. The behavior of phages in the presence of human colonic cells in culture was also investigated, and a higher lytic activity of phage phiCDHS1 was observed in the presence of cultured HT-29 cells. This was explained by the high-phage adsorption to the cell line on which *C. difficile* also adheres, promoting phage–bacteria interactions (24).

## Temperate phages are generally unable to completely cure CDI in animal models

The hamster model of CDI has been used to assess the efficacy of temperate phages. The first study was reported by Ramesh et al. (18). The authors found that simultaneous administration of ϕCD140 along with *C. difficile* spores greatly increased the survival of infected hamsters, as opposed to untreated animals that died within 72 h (18). While this first study was globally successful, one of the treated hamsters died due to the rise of phage resistance, which had been attributed to phage receptor mutation or lysogeny. *In vivo* lysogeny was later confirmed in hamsters during treatment with phage phiCD119. The proportion of lysogenic *C. difficile* clones was shown to increase over time, and by day 4 of the experiment, only lysogens could be isolated (41). These results revealed the incapacity of single-temperate phages to treat CDI in hamsters. To circumvent the problem of lysogeny, two- to four-phage cocktails were tested (22). While the luminal bacterial loads were reduced by at least 4 logs, the four-phage cocktail, administered every 8 h, prolonged hamster survival by 3 days compared with untreated animals. Ultimately, all animals died of CDI. Due to hamsters being highly sensitive to *C. difficile* and its toxins, the relevance of this model to the human condition has been questioned (42). Therefore, alternative CDI models, such as the wax moth larvae *Galleria mellonella,* have been recently developed. An optimized four-phage cocktail was tested against infection with a ribotype 014/020 *C. difficile* strain in *G. mellonella* larvae. Prophylactic single-dose cocktail administration ($10^6$ PFU) prior to bacterial inoculation with $10^5$ CFU of vegetative cells led to complete protection and survival of all insects 60 h post-infection, though ∼2-log bacterial counts were still detected at the end of the experiment. When phages were administered simultaneously with bacteria, survival dropped to 72%, whereas treatment with phages 2 h post-infection led to 30% survival after 60 h, and all larvae died at the end of the experiment. Multiple phage doses as well as vancomycin prophylaxis before infection and prior to phage treatment improved the outcome. These results show that the timing of phage inoculation is crucial for the efficacy of phage therapy, and that prophylactic regimens are more effective than remedial regimens, as observed *in vitro* (22, 26). The *G. mellonella* model is easier to manipulate than hamsters or mice and can be useful to test different hypotheses. Whether the observations made with this model are readily transferable to a more complex ecosystem like the mammalian gut remains to be demonstrated.

TABLE 1 Relevant studies investigating the use of phage-based therapeutics against *C. difficile*.

| Treatment/model | General outcome, limitations/challenges | Reference |
|---|---|---|
| **Whole phages** | | |
| CD140;<br>Hamster Model | Phage treatment led to greater hamster survival.<br>Phage resistance arose in one animal, phage treatment did not protect from subsequent *C. difficile* challenge. | (18) |
| phiCD27;<br>*in vitro* batch fermentation assays | Significant reduction in viable *C. difficile* counts, especially in a prophylactic regimen, higher MOI improved treatment, phiCD27 was specific to *C. difficile* strain.<br>Lysogeny was a probable cause of *C. difficile* growth rebound (5/5 *C. difficile* clones isolated at an MOI of 7 were lysogens). | (19) |
| phiCD27;<br>Human *in vitro* gut model | Prophylactic treatment with phage cleared *C. difficile* vegetative cells and decreased toxin production, but spore production was higher.<br>Lysogens were isolated from a replicate where phage treatment failed, and higher spore formation was observed during phage treatment. | (20) |
| phiCDHM1, phiCDHM2, phiCDHM5, phiCDHM6; *in vitro* assays; Hamster model | *In vitro assays:*<br>Dual-phage cocktails reduced lysogeny while three- or four-phage cocktails better prevented lysogeny.<br>*In vivo assays:*<br>Treatment increased infected hamster longevity and reduced *C. difficile* colonization and spore formation but did not protect them from death. Cocktails did not lead to phage resistance.<br>Single- or dual-phage therapy could lead to lysogeny and phage resistance. | (21) |
| phiCDHM1, phiCDHM2, phiCDHM5, phiCDHM6; *in vitro assays; G. mellonella* model | *In vitro assays:*<br>Phages penetrated biofilm *in vitro* and killed biofilm resident bacteria.<br>*In vivo assays:*<br>Prophylactic application resulted in the survival of larvae, phage treatment reduced *C. difficile* colonization. Phage remedial treatment did not prevent the death of larvae. | (22) |
| phiCDHM1, phiCDHM2, phiCDHM5, phiCDHM6 phage cocktails; *in vitro* batch fermentation model | *In vitro assays:*<br>Administration of the cocktail-cleared *C. difficile* from culture during remedial and prophylactic regimen, no *C. difficile* regrowth. Phage treatment did not affect other bacterial species. | (23) |
| phiCDHS1; *in vitro* colonic epithelial cell model | Phage treatment was more effective in the presence of HT-29 cells, reduced *C. difficile* adherence, and phage adsorption was observed on HT-29 cells. Did not consider the mucus layer normally present in the colon. | (24) |
| Wild-type phiCD24-2, engineered phiCD24-2 (carrying CRISPR-Cas3 components); *in vitro assays*; Mouse model with single phage therapy | *In vitro assays:*<br>Reduced or no lysogeny was observed for modified phages, crPhage killed higher counts of vegetative cells and delayed culture rebound of culture.<br>*In vivo assays*:<br>Engineered phage showed a higher reduction in vegetative cells in feces and intestinal bacterial load than WT phage, reduction of lysogeny in engineered phages.<br>Lysogeny still occurred in a phage deleted of key lysogeny genes and CRISPR components. | (25) |
| Cocktail of phiCDHM1, phiCDHM2, phiCDHM5 and phiCDHM6; *Galleria mellonella* model | Prophylactic phage application improved larvae survival, reduced bacterial colonization, lowered toxin levels, and remedial regimen delayed larvae death.<br>Phage remedial treatment only delayed larvae death. | (26) |
| **Diffocins/avidocins** | | |
| Diffocin 4 and diffocin 16; *in vitro assays* | Could be recombinantly expressed in *B. subtilis*. Narrow host spectrum. | (27) |
| Av-CD291.2 (modified R-type bacteriocin); *in vitro assays;* Mouse model of CDI | *In vitro assays:*<br>Av-CD291.2 had a broader activity on multiple ribotype 027 strains than WT diffocins 4.<br>*In vivo:*<br>Prophylactic administration of Av-CD291.2 inhibited *C. difficile* colonization, and Av-CD291.2 did not affect the mouse gut microbiota.<br>Large-scale production and stability in the gut environment will be challenging. | (28) |
| Diffocins derived from RT078 *C. difficile* isolate (HMC114) and Av-CD291 | *In vitro:*<br>HMC114 can kill 21/25 ribotype 027 isolates tested while Av-CD291 killed all ribotype 027 isolates tested. Diffocins could kill strains that produced them. | (29) |
| **Phage endolysins** | | |
| phiCD27 endolysin (CD27L); *in vitro* assays | PhiCD27 endolysin can be used to lyse *C. difficile* cells in culture. Endolysin was specific to *C. difficile* and had a broader host range than its parent phage.<br>Endolysin activity was weaker compared with endolysins produced by other phages that infect other species. | (30) |
| Endolysin catalytic domain (CD27L1-179); *in vitro* assays | Removal of the CBD domain increased the activity of endolysin<br>Removal of the CBD slightly decreased specificity, which caused the lysis of other bacterial species. | (31) |

*(Continued)*

TABLE 1 (Continued)

| Treatment/model | General outcome, limitations/challenges | Reference |
|---|---|---|
| Recombinantly expressed catalytic domain of endolysin PlyCD (PlyCD1-174); *in vitro* assays; *ex vivo* assays | *In vitro assays:*<br>Greater (>4-logs) activity and broader spectrum compared with the full-length PlyCD.<br>Endolysin–vancomycin synergy was observed.<br>*Ex vivo assays:*<br>Recombinant endolysin was able to kill vegetative cells in the colon of mice with approximately 2 log reduction after 1 h of incubation. | (32) |
| CD11 and CDG endolysins; *In vitro* assays | Both endolysins were highly active against *C. difficile*. | (33) |
| Recombinant protein composed of the phiCD2 endolysin catalytic domain (EAD) and human alpha-defensin functional domain ($HD_5$); *in vitro* assays; Mouse model of CDI | *In vitro assays:*<br>Recombinant endolysin killed several *C. difficile* ribotypes, reduced TcdB cytotoxicity, and had lower MICs (0.78 μg/ml) than metronidazole and vancomycin.<br>*In vivo:*<br>Treatment reduced CDI symptoms and reduced *C. difficile* and fecal toxin load in mice. Treated mice recovered while 40% of untreated mice died. | (34) |
| phiMMP01 cell wall hydrolase; *in vitro* assays | Removing the CBD and keeping only the EAD increased lytic activity and expand the activity spectrum.<br>Inhibition of spore outgrowth. Active at various pH and temperatures. | (35) |
| Endolysin CD16/50L; *in vitro* assays | Removing the CBD increased activity and expanded the host spectrum, CBD remains trapped with cellular debris.<br>Endolysin can have off-target hydrolyzation on other clostridial relatives. | (36) |
| Endolysin Ecd09610; and its domain variants; *in vitro* assays | The two C-terminal domains hold the lytic activity and showed the best clearing of the culture. The domain variants were thermoresistant up to 100°C and can be easily produced at high concentrations (contrarily to full-size lysin). Stable after lyophilization.<br>Weak lytic activity was found in some related bacteria. | (37) |
| *Lys6356* and its EAD; *in vitro* assays | Endolysins can be used after spore treatment with germinants and inhibits spore outgrowth. The use of taurocholic acid and glycine did not affect Lys6356 activity.<br>Calcium which is present in the gut and is massively released during spore germination inhibited endolysin activity *in vitro*. | (38) |

## Genetically engineered phages

The first case of a genetically engineered *C. difficile* phage involved the deletion of the genes coding for the *cI* repressor and the integrase in phage phiCD24-2 (43), therefore creating the first strictly lytic *C. difficile* phage (25). The authors also produced a crPhage carrying a mini-CRISPR array targeting the toxin locus, as well as a phage combining both modifications. The crPhages had significantly increased killing power and showed reduced lysogeny while the strictly lytic phages did not lead to lysogeny *in vitro*. The strictly lytic phage was not better than the wild-type phage at reducing bacterial counts *in vitro*, contrary to the *in vivo* condition where it performed better. Unexpectedly, regrowth of *C. difficile* was observed in the mouse gut with the recombinant phages. Clones recovered from fecal samples were lysogens of the modified phages. This unexpected result could not be clearly explained, but it was hypothesized that endogenous prophages could potentially have complemented the lost functions in the recombinant phages (25).

## Phage tail-like particles are potent antimicrobials

Phage tail-like particles are high molecular weight bacteriocins produced by several bacteria as a mechanism to compete with closely related species. Several strains of *C. difficile* produce these particles, which have been called diffocins (29, 44–47). Diffocins are genetically and structurally related to contractile phage tails but without a capsid. Hence, they do not carry genetic material and cannot replicate. Once adsorbed to a susceptible host via a specific receptor, contraction of the tail sheath leads to perforation of the cell membrane by the inner tail tube, causing leakage of the cell content and death (Figure 1) (48). Heterologous expression of functional recombinant diffocins has been successful in *Bacillus subtilis* (27). However, akin to their phage homologs, the host range of diffocins is generally narrow. To circumvent this, a genetically engineered diffocin was created by replacing its receptor-binding protein (RBP) with another from phi027, a prophage conserved in the genome of the R20291 and other R027 epidemic strains (28). This hybrid diffocin, called Avidocin-CD291 (or Av-CD291), was redirected toward strain R20291 and was able to kill all ribotype 027 strains tested *in vitro*, in addition to one or more isolates of ribotype 001, 015, and 087 strains (28). A turning point was reached when diffocins were shown to recognize their bacterial host via binding to the surface layer protein A (SlpA) (49). The authors demonstrated that diffocins' RBPs specifically bind to certain SlpA isoforms (or SLCTs, for surface layer cassette types). Interestingly, they also showed the interchangeability of the diffocin RBPs, and genetically engineered avidocins could be redirected toward different *C. difficile* strains based on their SLCT status (49). Most importantly, the efficacy of Av-CD291 was tested in a mouse model of *C. difficile* spore transmission that mimics the natural human-to-human transmission. Prophylactic administration of purified Av-CD291 in the drinking water completely prevented the colonization of mice, and no *C. difficile* could be detected in fecal samples, as opposed to the placebo group (28). There was no significant change in the composition of the microbiota, suggesting that avidocins

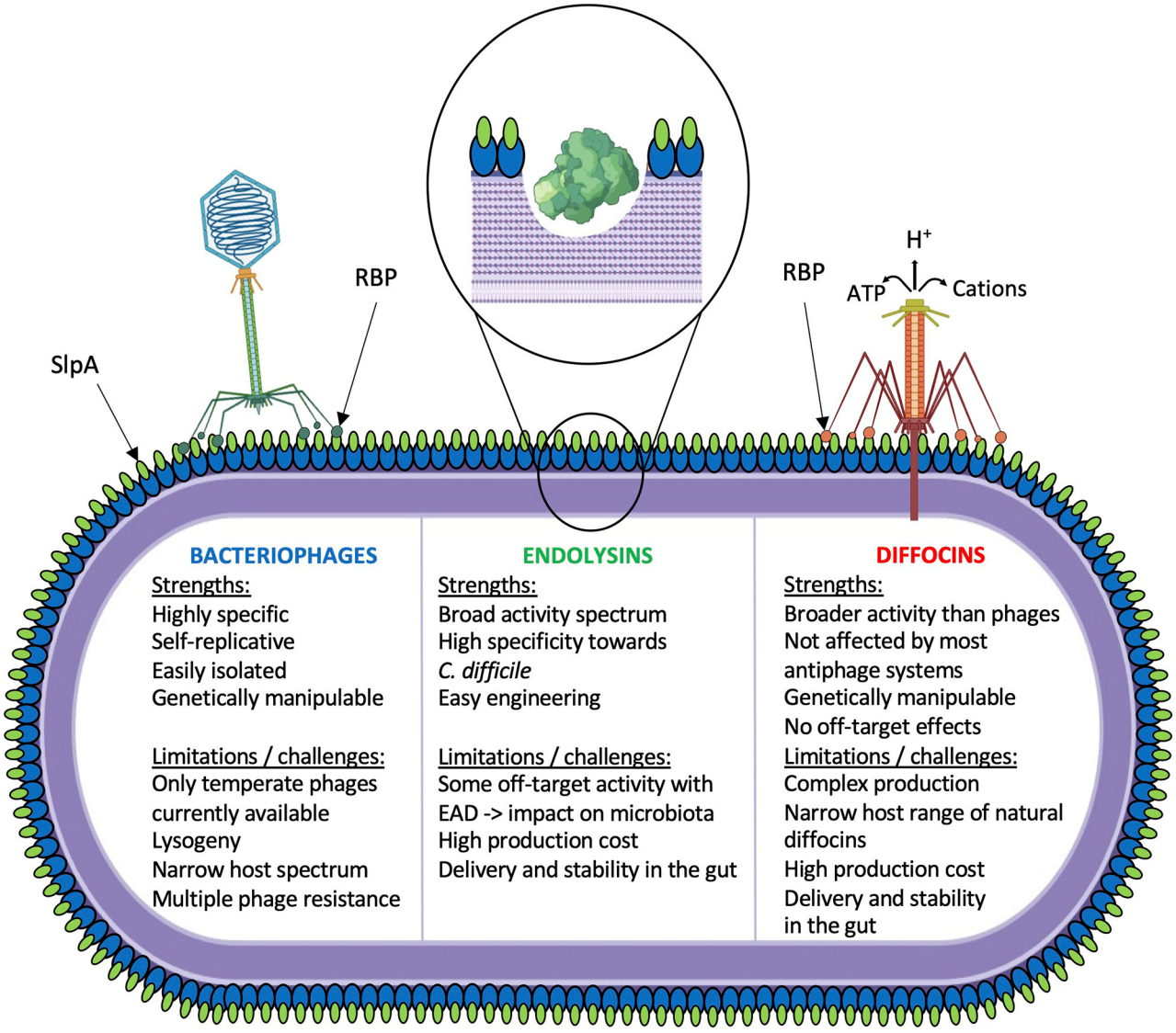


FIGURE 1
Schematic overview of current phage-based investigations. Strengths and limitations/challenges for each strategy are indicated.

are very specific to *C. difficile* and do not disturb the microbiota (28, 29). This study also revealed that Av-CD291 administration in mice did not disturb colonization resistance to *C. difficile* or vancomycin-resistant *Enterococcus faecium* (28).

## Cell wall-degrading enzymes as antimicrobials

Phage endolysins are also promising alternatives to whole phages (Figure 1). Tailed phages must break up the cell wall to escape their host at the end of the lytic cycle. The canonical holin–endolysin pathway involves a small protein, the holin, that accumulates into the cytoplasmic membrane until a programmed time of the lytic cycle at which point it forms pores into the cell membrane. This allows the endolysin to escape the cytoplasm and reach the peptidoglycan layer that it hydrolyzes from within until lysis (50). Gram-positive phage endolysins are generally composed of an enzymatically active domain (EAD) and a cell wall-binding domain (CBD). Most *C. difficile* phage endolysins are N-acetylmuramoyl-L-alanine amidases (35, 51). When purified endolysin is added extracellularly to a bacterial suspension *in vitro*, rapid lysis occurs in a matter of 10–20 min. A few *C. difficile* phage endolysins have been cloned and characterized (Table 1), CD27L being the first to be described (30). Of note, one common observation that has been reported with all *C. difficile* phage endolysins is that the catalytic domain of the enzyme alone (EAD) is sufficient for full activity. In fact, the removal of the CBD was reported to increase the lytic activity of *C. difficile* (30–32, 36, 38). It was also shown that the CBD from the CD16/50L endolysin was responsible for trapping the endolysin within cellular debris after lysis. It was hypothesized that the CBD domain prevents

the endolysin from being released freely into the environment upon cell lysis, therefore preserving uninfected bacteria that can serve as hosts for subsequent phage infection (36). Full-length endolysins were shown to be very specific toward *C. difficile*, as little or no activity was noted on other commensal bacteria, including related Clostridia. However, most truncated endolysins comprising only the EAD displayed a slightly broader host range, killing all *C. difficile* isolates tested, in addition to a few other species, in particular *Clostridium sordellii, Clostridium bifermentans, Bacillus subtilis,* and *Listeria monocytogenes* (30–32, 36, 38).

An interesting feature of gram-positive endolysins is their modular architecture that allows interchanging EAD and CBD. The LHD is a lysin–human defensin fusion protein that results from the fusion of the CBD from phage phiC2 endolysin and the functional domain from the human α-defensin 5 ($HD_5$) (34). LHD was very active on several *C. difficile* strains of different ribotypes, and the minimum inhibitory concentration (0.78 μg/ml) was >4 times lower than that of metronidazole and vancomycin. Interestingly, LHD inhibited the glycosylation activity and toxicity of TcdB, as shown with $HD_5$ (52). Furthermore, treatment of mice infected with the R20291 epidemic strain twice a day for 7 consecutive days with LHD rescued all treated mice from death, as opposed to 60% survival for control mice. Toxin levels and the number of spores were also reduced in the treated group. Interestingly, pre-treatment of bacteria with vancomycin increased the lytic activity of the endolysin $PlyCD_{1-174}$, suggesting a synergistic effect as described with phages (53). In addition, $PlyCD_{1-174}$ endolysin was shown to be active *ex vivo* in a complex mouse fecal environment (32).

## Discussion

Aside from the common challenges that phage therapy faces in general, such as safety, efficacy, the lack of data from clinical trials, resistance, regulatory hurdles, and patentability, several limitations are particularly relevant to CDI. The most urgent problems to address are as follows: (i) the lack of strictly lytic phages, (ii) the narrow host range of current phages, and (iii) the problem of phage resistance. Alternatives to whole phages also have their limitations, such as (vi) the difficulty to produce avidocins or endolysins on a large scale, (vii) the stability of avidocins and endolysins in the intestinal environment, and (viii) the off-target activity of genetically engineered endolysins. However, there are potential solutions to these limitations.

The lack of strictly lytic phages is the most critical problem with *C. difficile* phages, and further screening of environmental samples is not the solution. The most reasonable strategy is the genetic engineering of temperate phages. Such phages have been successfully created and tested in other phage–host models (54, 55), and a human patient infected by *Mycobacterium abscessus* has been treated with one of them (16). The first report of a genetically engineered *C. difficile* phage has proven the feasibility of this approach, although further research is required to better characterize the behavior of genetically engineered phages *in vivo*. It will be particularly important to investigate how therapeutic phages interact with the highly prevalent and diverse endogenous prophages in *C. difficile* genomes (56).

Another important limitation of current *C. difficile* phages is their narrow host range, implying that multiple phages will need to be combined into cocktails to cover the most clinically relevant strains of *C. difficile* (21). Due to recent advances in our understanding of host recognition by *C. difficile* phages, the surface layer protein A (SlpA) seems to be a general receptor used by many phages and diffocins (28, 49, 57–59). It is, therefore, reasonable to foresee the selection of phages based on their RBP to target *C. difficile* strains expressing specific SlpA isoforms. Although RBP can be identified in phage genomes using bioinformatics tools, it will be important to determine if additional phage proteins participate in host recognition or if other receptors exist, as suggested in one study (58).

Resistance is always a critical concern when undertaking phage therapy (12, 60). Several mechanisms of phage resistance exist (61), and an important one is the mutation of the phage receptor. Work on diffocins led to the isolation of two spontaneous *C. difficile* mutants that had an SNP causing severe truncation of the SlpA protein, leading to full resistance to diffocins (49). If strictly lytic phages are developed for CDI treatment, phage resistance through mutation of SlpA might occur as well. However, *in vitro* and *in vivo* data showed that loss of slpA comes with a huge fitness cost. Indeed, *slpA* mutants produce less toxins, sporulate less, are more sensitive to antimicrobial peptides, and are avirulent (49, 62). Therefore, phage resistance through receptor mutation would be compensated by loss of virulence, facilitating patient recovery.

Additional factors that can affect the success of a phage infection will also need to be considered. For instance, active antiphage mechanisms have been described in *C. difficile*, including restriction-modification systems (63) and a type I CRISPR-Cas system (64). A superinfection exclusion mechanism mediated by the phase-variable cell wall protein CwpV has also been described, although its impact on phage resistance *in vivo* needs to be investigated (65). The recent identification of functional anti-CRISPR systems in several *C. difficile* phage genomes (66) suggests that CRISPR-mediated interference could potentially be short-circuited by selecting anti-CRISPR-containing phages or by incorporating anti-CRISPR genes into genetically modified phages. Importantly, incorporation of CRISPR cassettes into cargo phage genomes to target the host cell [e.g., toxin or other virulence genes (25)] will require considering the fact that some naturally occurring lysogens might express anti-CRISPR systems and therefore negatively interfere with the engineered phages.

Avidocins are very appealing alternatives to whole phages, but their large-scale production will be challenging because they are a complex assemblage of multiple components. On the other hand, endolysins are much simpler to produce and have a broader host range, which is a clear advantage. However, off-target killing might be a problem, as some of the other clostridial species that can be lysed by certain recombinant endolysins are beneficial species, such as *C. scindens* which has been shown to protect against CDI through primary bile acid conversion (67).

Sporulation of *C. difficile* is also one important hurdle in the treatment of CDI. Spores are naturally produced in the gut during infection and become resistant to most antimicrobials. Hence, combination therapy that includes spore germinants could promote germination, therefore ensuring maximum killing of the infected strain. This strategy has, however, been shown to interfere

with endolysin activity, as the massive release of calcium during germination is shown to inhibit the activity of LysCD6356, at least *in vitro* (38). The importance of this observation to the *in vivo* condition requires further investigation. Nevertheless, even if residual spores remain after phage-based treatment, the risk of relapse should be lower than with conventional antibiotics because the gut microbiota will be spared in the process.

In conclusion, we are still a few steps from a viable phage-based product to fight CDI, but recent progress in our understanding of phage–host interactions and the development of more efficient molecular tools to genetically engineer phages will certainly lead to exciting advances in the next few years.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## References

1. CDC. *COVID-19: U.S. Impact on Antimicrobial Resistance, Special Report 2022*. Atlanta, GA: U.S. Department of Health and Human Services, CDC (2022). Available online at: https://www.cdc.gov/drugresistance/covid19.html

2. He M, Miyajima F, Roberts P, Ellison L, Pickard DJ, Martin MJ, et al. Emergence and global spread of epidemic healthcare-associated *Clostridium difficile*. *Nat Genet.* (2013) 45:109–13. doi: 10.1038/ng.2478

3. Chakra CNA, Pépin J, Sirard S, Valiquette L. Risk factors for recurrence, complications and mortality in *Clostridium difficile* infection: a systematic review. *PLoS One.* (2014) 9:e98400. doi: 10.1371/journal.pone.0098400

4. Almutairi MS, Gonzales-Luna AJ, Alnezary FS, Fallatah SB, Alam MJ, Begum K, et al. Comparative clinical outcomes evaluation of hospitalized patients infected with *Clostridioides difficile* ribotype 106 vs. other toxigenic strains. *Anaerobe.* (2021) 72:102440. doi: 10.1016/j.anaerobe.2021.102440

5. Ofori E, Ramai D, Dhawan M, Mustafa F, Gasperino J, Reddy M. Community-acquired *Clostridium difficile*: epidemiology, ribotype, risk factors, hospital and intensive care unit outcomes, and current and emerging therapies. *J Hosp Infect.* (2018) 99:436–42. doi: 10.1016/j.jhin.2018.01.015

6. Eyre DW, Walker AS, Wyllie D, Dingle KE, Griffiths D, Finney J, et al. Predictors of first recurrence of Clostridium difficile infection: implications for initial management. *Clin Infect Dis.* (2012) 55:S77–87. doi: 10.1093/cid/cis356

7. Yadegar A, Pakpoor S, Ibrahim FF, Nabavi-Rad A, Cook L, Walter J, et al. Beneficial effects of fecal microbiota transplantation in recurrent *Clostridioides difficile* infection. *Cell Host Microbe.* (2023) 31:695–711. doi: 10.1016/j.chom.2023.03.019

8. DeFilipp Z, Bloom PP, Soto MT, Mansour MK, Sater MRA, Huntley MH, et al. Drug-resistant E. coli bacteremia transmitted by fecal microbiota transplant. *N Engl J Med.* (2019) 381:2043–50. doi: 10.1056/NEJMoa1910437

9. Hatfull GF, Dedrick RM, Schooley RT. Phage therapy for antibiotic-resistant bacterial infections. *Annu Rev Med.* (2021) 73:1–15. doi: 10.1146/annurev-med-080219-122208

10. Moelling K, Broecker F, Willy C. A wake-up call: we need phage therapy now. *Viruses*. (2018) 10:688. doi: 10.3390/v10120688

11. Jones JD, Trippett C, Suleman M, Clokie MRJ, Clark JR. The future of clinical phage therapy in the United Kingdom. *Viruses.* (2023) 15:721. doi: 10.3390/v15030721

12. Schmidt C. Phage therapy's latest makeover. *Nat Biotechnol.* (2019) 37:581–6. doi: 10.1038/s41587-019-0133-z

13. Heuler J, Fortier LC, Sun X. *Clostridioides difficile* phage biology and application. *Fems Microbiol Rev.* (2021) 45:fuab012. doi: 10.1093/femsre/fuab012

14. Nale JY, Thanki AM, Rashid SJ, Shan J, Vinner GK, Dowah ASA, et al. Diversity, dynamics and therapeutic application of *Clostridioides difficile* bacteriophages. *Viruses.* (2022) 14:2772. doi: 10.3390/v14122772

15. Monteiro R, Pires DP, Costa AR, Azeredo J. Phage therapy: going temperate? *Trends Microbiol.* (2018) 27:368–78. doi: 10.1016/j.tim.2018.10.008

16. Dedrick RM, Guerrero-Bustamante CA, Garlena RA, Russell DA, Ford K, Harris K, et al. Engineered bacteriophages for treatment of a patient with a disseminated drug-resistant *Mycobacterium abscessus*. *Nat Med.* (2019) 25:730–3. doi: 10.1038/s41591-019-0437-z

17. Al-Anany AM, Fatima R, Hynes AP. Temperate phage-antibiotic synergy eradicates bacteria through depletion of lysogens. *Cell Rep.* (2021) 35:109172. doi: 10.1016/j.celrep.2021.109172

18. Ramesh V, Fralick JA, Rolfe RD. Prevention of *Clostridium difficile* -induced ileocecitis with Bacteriophage. *Anaerobe.* (1999) 5:69–78. doi: 10.1006/anae.1999.0192

19. Meader E, Mayer MJ, Gasson MJ, Steverding D, Carding SR, Narbad A. Bacteriophage treatment significantly reduces viable *Clostridium difficile* and prevents toxin production in an *in vitro* model system. *Anaerobe.* (2010) 16:549–54. doi: 10.1016/j.anaerobe.2010.08.006

20. Meader E, Mayer MJ, Steverding D, Carding SR, Narbad A. Evaluation of bacteriophage therapy to control *Clostridium difficile* and toxin production in an in vitro human colon model system. *Anaerobe.* (2013) 22:25–30. doi: 10.1016/j.anaerobe.2013.05.001

21. Nale JY, Spencer J, Hargreaves KR, Buckley AM, Trzepiński P, Douce GR, et al. Bacteriophage combinations significantly reduce *Clostridium difficile* growth *in vitro* and proliferation *in vivo*. *Antimicrob Agents Ch.* (2016) 60:968–81. doi: 10.1128/AAC.01774-15

22. Nale JY, Chutia M, Carr P, Hickenbotham PT, Clokie MRJ. 'Get in early'; biofilm and wax moth (*Galleria mellonella*) models reveal new insights into the therapeutic potential of *Clostridium difficile* bacteriophages. *Front Microbiol.* (2016) 7:1383. doi: 10.3389/fmicb.2016.01383

23. Nale JY, Redgwell TA, Millard A, Clokie MRJ. Efficacy of an optimised bacteriophage cocktail to clear *Clostridium difficile* in a batch fermentation model. *Antibiotics.* (2018) 7:13. doi: 10.3390/antibiotics7010013

24. Shan J, Ramachandran A, Thanki AM, Vukusic FBI, Barylski J, Clokie MRJ. Bacteriophages are more virulent to bacteria with human cells than they are in bacterial culture; insights from HT-29 cells. *Sci Rep-UK.* (2018) 8:5091. doi: 10.1038/s41598-018-23418-y

25. Selle K, Fletcher JR, Tuson H, Schmitt DS, McMillan L, Vridhambal GS, et al. *In vivo* targeting of *Clostridioides difficile* using phage-delivered CRISPR-Cas3 antimicrobials. *MBio.* (2020) 11:e00019–20. doi: 10.1128/mBio.00019-20

26. Nale JY, Chutia M, Cheng JKJ, Clokie MRJ. Refining the *Galleria mellonella* model by using stress marker genes to assess *Clostridioides difficile* infection and recuperation during phage therapy. *Microorg.* (2020) 8:1306. doi: 10.3390/microorganisms8091306

27. Gebhart D, Williams SR, Bishop-Lilly KA, Govoni GR, Willner KM, Butani A, et al. Novel high-molecular-weight, R-type bacteriocins of *Clostridium difficile*. *J Bacteriol.* (2012) 194:6240–7. doi: 10.1128/JB.01272-12

28. Gebhart D, Lok S, Clare S, Tomas M, Stares M, Scholl D, et al. A modified R-type bacteriocin specifically targeting *Clostridium difficile* prevents colonization of mice without affecting gut microbiota diversity. *MBio.* (2015) 6:e02368–14. doi: 10.1128/mBio.02368-14

29. Sangster W, Hegarty JP, Stewart DB. Phage tail-like particles kill *Clostridium difficile* and represent an alternative to conventional antibiotics. *Surgery.* (2015) 157:96–103. doi: 10.1016/j.surg.2014.06.015

30. Mayer MJ, Narbad A, Gasson MJ. Molecular characterization of a *Clostridium difficile* bacteriophage and its cloned biologically active endolysin. *J Bacteriol.* (2008) 190:6734–40. doi: 10.1128/JB.00686-08

31. Mayer MJ, Garefalaki V, Spoerl R, Narbad A, Meijers R. Structure-based modification of a *Clostridium difficile*-targeting endolysin affects activity and host range. *J Bacteriol.* (2011) 193:5477–86. doi: 10.1128/JB.00439-11

32. Wang Q, Euler CW, Delaune A, Fischetti VA. Using a novel lysin to help control *Clostridium difficile* infections. *Antimicrob Agents Chemother.* (2015) 59:7447–57. doi: 10.1128/AAC.01357-15

33. Mehta KK, Paskaleva EE, Wu X, Grover N, Mundra RV, Chen K, et al. Newly identified bacteriolytic enzymes that target a wide range of clinical isolates of *Clostridium difficile*. *Biotechnol Bioeng.* (2016) 113:2568–76. doi: 10.1002/bit.26029

34. Peng Z, Wang S, Gide M, Zhu D, Patabendige HMLW Li C, et al. A novel bacteriophage lysin-human defensin fusion protein is effective in treatment of *Clostridioides difficile* infection in mice. *Front Microbiol.* (2019) 9:3234. doi: 10.3389/fmicb.2018.03234

35. Mondal SI, Akter A, Draper LA, Ross RP, Hill C. Characterization of an endolysin targeting *Clostridioides difficile* that affects spore outgrowth. *Int J Mol Sci.* (2021) 22:5690. doi: 10.3390/ijms22115690

36. Phothichaisri W, Chankhamhaengdecha S, Janvilisri T, Nuadthaisong J, Phetruen T, Fagan RP, et al. Potential role of the host-derived cell-wall binding domain of endolysin CD16/50L as a molecular anchor in preservation of uninfected *Clostridioides difficile* for new rounds of phage infection. *Microbiol Spectr*. (2022) 10:e0236121. doi: 10.1128/spectrum.02361-21

37. Sekiya H, Yamaji H, Yoshida A, Matsunami R, Kamitori S, Tamai E. Biochemical characterizations of the putative endolysin Ecd09610 catalytic domain from *Clostridioides difficile*. *Antibiotics.* (2022) 11:1131. doi: 10.3390/antibiotics11081131

38. Alyahya K, Baillie L. Assessing the feasibility of employing a combination of a bacteriophage-derived endolysin and spore germinants to treat relapsing *Clostridioides difficile* infection. *Microorganisms.* (2023) 11:1651. doi: 10.3390/microorganisms11071651

39. Flemming HC, Hullebusch ED. van, Neu TR, Nielsen PH, Seviour T, Stoodley P, et al. The biofilm matrix: multitasking in a shared space. *Nat Rev Microbiol.* (2023) 21:70–86. doi: 10.1038/s41579-022-00791-0

40. Knecht LE, Veljkovic M, Fieseler L. Diversity and function of phage encoded depolymerases. *Front Microbiol.* (2020) 10:2949. doi: 10.3389/fmicb.2019.02949

41. Revathi G, Fralick JA, Rolfe RD. *In vivo* lysogenization of a *Clostridium difficile* bacteriophage ϕCD119. *Anaerobe.* (2011) 17:125–9. doi: 10.1016/j.anaerobe.2011.05.012

42. Chen X, Katchar K, Goldsmith JD, Nanthakumar N, Cheknis A, Gerding DN, et al. A mouse model of *Clostridium difficile*-associated disease. *Gastroenterology.* (2008) 135:1984–92. doi: 10.1053/j.gastro.2008.09.002

43. Sekulovic O, Garneau JR, Néron A, Fortier LC. Characterization of temperate phages infecting *Clostridium difficile* isolates of human and animal origins. *Appl Environ Microb.* (2014) 80:2555–63. doi: 10.1128/AEM.00237-14

44. Nagy E, Földes J. Electron microscopic investigation of lysogeny of *Clostridium difficile* strains isolated from antibiotic-associated diarrhea cases and from healthy carriers. *APMIS.* (1991) 99:321–6. doi: 10.1111/j.1699-0463.1991.tb05156.x

45. Fortier LC, Moineau S. Morphological and genetic diversity of temperate phages in *Clostridium difficile*. *Appl Environ Microb.* (2007) 73:7358–66. doi: 10.1128/AEM.00582-07

46. Hargreaves KR, Colvin HV, Patel KV, Clokie JJP, Clokie MRJ. Genetically diverse *Clostridium difficile* strains harboring abundant prophages in an estuarine environment. *Appl Environ Microbiol.* (2013) 79:6236–43. doi: 10.1128/AEM.01849-13

47. Nale JY, Shan J, Hickenbotham PT, Fawley WN, Wilcox MH, Clokie MRJ. Diverse temperate bacteriophage carriage in *Clostridium difficile* 027 strains. *PLoS ONE.* (2012) 7:e37263. doi: 10.1371/journal.pone.0037263

48. Patz S, Becker Y, Richert-Pöggeler KR, Berger B, Ruppel S, Huson DH, et al. Phage tail-like particles are versatile bacterial nanomachines—a mini-review. *J Adv Res.* (2019) 19:75–84. doi: 10.1016/j.jare.2019.04.003

49. Kirk JA, Gebhart D, Buckley AM, Lok S, Scholl D, Douce GR, et al. New class of precision antimicrobials redefines role of *Clostridium difficile* S-layer in virulence and viability. *Sci Transl Med.* (2017) 9:eaah6813. doi: 10.1126/scitranslmed.aah6813

50. Cahill J, Young R. Phage lysis: multiple genes for multiple barriers. *Adv virus Res.* (2018) 103:33–70. doi: 10.1016/bs.aivir.2018.09.003

51. Mondal SI, Draper LA, Ross RP, Hill C. Bacteriophage endolysins as a potential weapon to combat *Clostridioides difficile* infection. *Gut Microbes*. (2020) 12:1813533. doi: 10.1080/19490976.2020.1813533

52. Giesemann T, Guttenberg G, Aktories K. Human α-defensins inhibit *Clostridium difficile* toxin B. *Gastroenterology.* (2008) 134:2049–58. doi: 10.1053/j.gastro.2008.03.008

53. Nieuwenhuyse BV, Linden DV. der, Chatzis O, Lood C, Wagemans J, Lavigne R, et al. Bacteriophage-antibiotic combination therapy against extensively drug-resistant Pseudomonas aeruginosa infection to allow liver transplantation in a toddler. *Nat Commun.* (2022) 13:5725. doi: 10.1038/s41467-022-33294-w

54. Park JY, Moon BY, Park JW, Thornton JA, Park YH, Seo KS. Genetic engineering of a temperate phage-based delivery system for CRISPR/Cas9 antimicrobials against *Staphylococcus aureus*. *Sci Rep.* (2017) 7:44929. doi: 10.1038/srep44929

55. Lynch KH, Seed KD, Stothard P, Dennis JJ. Inactivation of *Burkholderia cepacia* complex phage KS9 gp41 identifies the phage repressor and generates lytic virions. *J Virol.* (2010) 84:1276–88. doi: 10.1128/JVI.01843-09

56. Labrie SJ, Moineau S. Abortive infection mechanisms and prophage sequences significantly influence the genetic makeup of emerging lytic lactococcal phages. *J Bacteriol.* (2006) 189:1482–7. doi: 10.1128/JB.01111-06

57. Royer ALM, Umansky AA, Allen MM, Garneau JR, Ospina-Bedoya M, Kirk JA, et al. *Clostridioides difficile* S-layer protein A (SlpA) serves as a general phage receptor. *Microbiol Spectr.* (2023) 11:e03894–22. doi: 10.1128/spectrum.03894-22

58. Phetruen T, Chanarat S, Janvilisri T, Phanchana M, Charoensutthivarakul S, Phothichaisri W, et al. Receptor binding protein of prophage reversibly recognizes the low-molecular weight subunit of the surface-layer protein SlpA in *Clostridioides difficile*. *Front Microbiol.* (2022) 13:998215. doi: 10.3389/fmicb.2022.998215

59. Whittle MJ, Bilverstone TW, Esveld RJ. van, Lücke AC, Lister MM, Kuehne SA, et al. A novel bacteriophage with broad host range against *Clostridioides difficile* ribotype 078 supports SlpA as the likely phage receptor. *Microbiol Spectr.* (2022) 10:e02295–21. doi: 10.1128/spectrum.02295-21

60. Nick JA, Dedrick RM, Gray AL, Vladar EK, Smith BE, Freeman KG, et al. Host and pathogen response to bacteriophage engineered against *Mycobacterium abscessus* lung infection. *Cell.* (2022) 185:1860–74.e12. doi: 10.1016/j.cell.2022.04.024

61. Rostøl JT, Marraffini L. (Ph)ighting phages: how bacteria resist their parasites. *Cell Host Microbe.* (2019) 25:184–94. doi: 10.1016/j.chom.2019.01.009

62. Ormsby MJ, Vaz F, Kirk JA, Barwinska-Sendra A, Hallam JC, Lanzoni-Mangutchi P, et al. An intact S-layer is advantageous to *Clostridioides difficile* within the host. *PLoS Pathog.* (2023) 19:e1011015. doi: 10.1371/journal.ppat.1011015

63. Purdy D, O'Keeffe TAT, Elmore M, Herbert M, McLeod A, Bokori-Brown M, et al. Conjugative transfer of clostridial shuttle vectors from *Escherichia coli* to *Clostridium difficile* through circumvention of the restriction barrier. *Mol Microbiol.* (2002) 46:439–52. doi: 10.1046/j.1365-2958.2002.03134.x

64. Boudry P, Semenova E, Monot M, Datsenko KA, Lopatina A, Sekulovic O, et al. Function of the CRISPR-Cas system of the human pathogen *Clostridium difficile*. *MBio.* (2015) 6:e01112–15. doi: 10.1128/mBio.01112-15

65. Sekulovic O, Bedoya MO, Fivian-Hughes AS, Fairweather NF, Fortier L. The *Clostridium difficile* cell wall protein CwpV confers phase-variable phage resistance. *Mol Microbiol.* (2015) 98:329–42. doi: 10.1111/mmi.13121

66. Muzyukina P, Shkaruta A, Guzman NM, Andreani J, Borges AL, Bondy-Denomy J, et al. Identification of an Anti-CRISPR protein that inhibits the CRISPR-Cas Type I-B system in clostridioides difficile. *BioRxiv [Preprint].* (2023). doi: 10.1101/2023.05.22.541795

67. Buffie CG, Bucci V, Stein RR, McKenney PT, Ling L, Gobourne A, et al. Precision microbiome reconstitution restores bile acid mediated resistance to *Clostridium difficile*. *Nature.* (2015) 517:205–8. doi: 10.1038/nature13828

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# Regulations of phage therapy across the world

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Phage therapy, a century-long treatment targeting bacterial infection, was widely abandoned after the clinical availability of antibiotics in the mid-20th century. However, the crisis of antimicrobial resistance today led to its revival in many countries. While many articles dive into its clinical application now, little research is presenting phage therapy from a regulatory perspective. Here, we focus on the regulations of phage therapy by dividing sections into Eastern Europe where it was never abandoned and Western Europe, Australia, the United States, India, and China where it only re-attracted researchers' attention in recent decades. New insights about its regulations in China are provided as little English literature has specifically discussed this previously. Ultimately, by introducing the regulations in phage therapy for human health across representative countries, we hope to provide ideas of how countries may borrow each other's adapting legislation in phage therapy to best overcome the current regulatory hurdles.

KEYWORDS

**antimicrobial resistance, phage therapy regulation, clinical trial, investigational new drug, personalized phage therapy**

## Introduction

The formal discovery of phage can be traced back to 1915 when Frederick Twort and Felix d'Herelle independently characterized its ability to cause "transmissible bacterial lyses" (Chanishvili, 2012). D'Herelle named it "bacteriophage" and started the first phage therapy in 1919 in Paris (Chanishvili, 2012). Following some successful cases, phage therapy was sensational for doctors and spread around the world (Chanishvili, 2012). However, it was widely abandoned since the commercial availability of penicillin in the mid-20th century, only remaining in Poland and the former Soviet Union (Lin et al., 2017; Fauconnier, 2019). Antibiotics, in contrast, had quickly taken over the market by virtue of their properties of standardized manufacture, administration, and their broad-spectrum antimicrobial ability.

Nevertheless, the extensive use of antibiotics brought increasing emergence and spread of antimicrobial resistance (AMR). What made the situation worse is the decreasing discovery rate of new antibiotics when the previous ones were rendered ineffective. The antibiotics crisis led to the renaissance of phage therapy in where it was once fully replaced by antibiotics. Increasing studies on the safety and efficacy of phage therapy in animals and humans were reported. Besides phage therapy that has been the standard of care in parts of the former Soviet Union for over 80 years, there are dozens of therapeutic phage pipelines entering phase 1–3 trials with investigational new drug (IND) approval in the West. However, in many countries experiencing the renaissance of phage therapy, there is still a lot of room for improvement regarding corresponding regulations and guidelines that may greatly facilitate the process. In this article, we will separately discuss the current and potential future regulations of phage therapy for

human health in representative countries as an important reference for legislators, pharmaceutical companies, and researchers.

## Current progress in clinical phage therapy research

While there have been many investigator-initiated trials (IITs) of phage therapy since its discovery, more and more industry-sponsored trials (ISTs) that symbolize private investors' commercial interest are emerging, representing investors' growing confidence in this rapidly developing field.

### Investigator-initiated trials

From 1922 to 2022, phage therapy in more than 6,300 patients covered clinical departments of pneumology, urology, orthopedics, dermatology, otolaryngology, ophthalmology, gastroenterology, cardiology, and critical care medicine; involved treatments of infections of *Enterobacter*, *Acinetobacter*, *Pseudomonas*, *Staphylococcus*, *Enterococcus*, *Salmonella*, *Shigella*, *mycobacteria*, *Vibrio*, *Burkholderia*, *Serratia*, *Neisseria gonorrhoeae*, and other common clinical bacteria; and was implemented across the world in Europe, America, Asia, and Africa [summarized from Diallo and Dublanchet (2023)]. Phage therapy may bring promising clinical improvements and bacterial eradication results, while causing less or similar adverse events that are generally mild and resolved after the treatment than the control groups (Uyttebroek et al., 2022). In contrast to numerous case studies reported, randomized controlled clinical trials (RCTs) that are more in line with modern medical criteria are very limited, and many are primarily concerned with phage therapy's safety. As shown in Table 1 with nine investigator-initiated RCTs published from 2005 to 2021. All these trials confirmed the safety of phage therapy relatively to the control groups. Also, several studies tested the efficacy of phage therapy compared to standard treatments (Wright et al., 2009; Sarker et al., 2016; Jault et al., 2019). However, the efficacy of phage therapy compared to the control groups demonstrated varying results, sometimes being superior but sometimes being inferior (Table 1). Though, these trials derived many suggestions for future studies, such as the possibility of raising phage dosage of administration to ensure effective working concentration and achieve better bacterial killing effect.

Account for any potential phage loss to achieve the optimal multiplicity of infection, allowing rapid phage replication to occur. In summary, these studies demonstrated the undoubtful potential of phage therapy but admitted that more systematic studies according to modern medical standards are needed.

### Industry-sponsored trials

Alongside the IITs that indicate the good performance of phage therapy, biotech and pharmaceutical companies are racing to develop new drugs based on formulated phage cocktails, phage endolysins, or even a phage collection. Table 2 highlights the current or planned ISTs of phage therapy reported to ClinicalTrials.gov since 2017. Several candidate pipelines have entered phase 3 clinical trials, and we expect to see commercial phage products available within 2–5 years. Worth mentioning, the United States Food and Drug Administration (FDA) revolutionarily approved INDs for Adaptive Phage Therapeutics' personalized PhageBank therapy. Currently, the company has three pipelines of PhageBank therapy in phase 1–3 trials. Overall, most pipelines acquired their INDs in the United States, and other countries are relatively lagging in this stage.

## Regulations of phage therapy across the world

### Regulations in Poland, Georgia, and Russia

Although many countries only recently rediscovered their interest in phage therapy since the emergence of AMR crisis, it has always been widely used in Eastern Europe. In particular, phage therapy has been a part of the health care practice in Georgia, Poland, and Russia since its initial discovery (Międzybrodzki et al., 2018). Though, corresponding legislations as well as research and medical standards went through tremendous changes in recent decades to fit modern regulations more appropriately. In Poland, regulations in phage therapy became much more completed and systemized by falling under stricter administration and establishing ethical guidelines after Poland joined the EU in 2004 and established the Phage Therapy Unit (PTU) in 2005. Phage therapy in Poland was considered an experimental treatment based on several Polish legislations, particularly the Medical and Dental Professions Act of December 5, 1996 in addition to the Constitution of Poland and the ethical code of the Polish Association, and EU legislations on its member states (Żaczek et al., 2022). Phage therapy in Poland is also under Directive 2001/20/EC of the European Parliament and of the Council and Directive 2005/28/EC that regulate clinical trials and Good Clinical Practice (Hartmann and Hartmann-Vareilles, 2006). In Poland, the compassionate use (use of unapproved drugs to benefit patients) of phage therapy is together governed by the Helsinki Declaration and Guideline on Compassionate Use of Medicinal Products along with the Polish legislation (Żaczek et al., 2022). Worth noticing though, Directive 2001/20/EC was repealed in 2022 and replaced by European Medicines Agency (2023). While a transition period exists, it must be waited to see how this new legislation will impact clinical trials in phage therapy later. Based on the above regulations, phage therapy in Poland is now under systematic control, but it is only administered to patients in the PTU (Żaczek et al., 2022).

In contrast, phage therapy in Georgia and Russia is in an entirely different scenario. In Georgia and Russia, phage cocktails like "Intestiphage" and "Pyophage" can be directly purchased without a prescription (Abedon et al., 2011). However, their policies differ in many ways, especially regarding personalized phage therapy. In Georgia, pre-prepared phage and personalized phage medicines are considered pharmaceuticals. The pre-prepared phage medicines are under legislation for market authorization, while personalized phage medicines are allowed to be manufactured in pharmacies specially licensed by the Georgian Ministry of Healthcare through magistral preparation (Fauconnier, 2019). Phage products are similarly classified as pharmaceuticals in Russia, with manufacture and storage procedures listed in pharmacopeia GPM.1.8.1.0002.15 (Międzybrodzki et al., 2018; Pharmacopoeia.ru, n.d.). However,

TABLE 1 The investigator-initiated RCTs of phage therapy.*

| Name | Phase | Enrollment | Adm route | Target | Indication | Outcome | Developer | Ref. |
|---|---|---|---|---|---|---|---|---|
| WPP-201 | 1 | 42 | Topical | *P. aerogenosa, S. aureus,* and *E. coli* | Chronic venous leg ulcers | Safety demonstrated | Southwest Regional Wound Care Center, United States | Rhoads et al. (2009) |
| Pyophage | 2/3 | 113 | IU | Multiple common bacteria | Urinary tract infection | Favorable safety profile, non-inferior to standard-of-care antibiotic treatment. | Balgrist University Hospital, Georgia | Leitner et al. (2021) |
| NS | 1/2 | 24 | Oral | *E. coli* | Chronic otitis | Safety demonstrated; clinical improvements; and bacteria reduction in the test group only. | Royal National Throat, Nose, and Ear Hospital, United Kingdom | Wright et al. (2009) |
| Microgen Coli Proteus and T4-like phage cocktail | NA | 120 | Oral | *E. coli* | Acute bacterial diarrhea | Safety demonstrated; no substantial intestinal phage replication; the test group showed no amelioration over control group. | Dhaka Hospital of the International, Bangladesh | Sarker et al. (2016) |
| PP1131 | 1/2 | 27 | Topical | *P. aeruginosa* | Burn wound | Safety demonstrated; the control group reduced bacterial burden at faster pace | Burn centers in France and Belgium | Jault et al. (2019) |
| NS | NA | 15 | Oral | NS | Healthy | Safety demonstrated | Nestle Research Center, Switzerland | Bruttin and Brüssow (2005) |
| Microgen Coli Proteus | NA | 15 | Oral | *E. coli and Proteus* | Healthy | Safety demonstrated | Bangladesh | McCallin et al. (2013) |
| Microgen Coli Proteus | 1 | NS | Oral | *E. coli* | Healthy | Safety demonstrated | Bangladesh | Sarker et al. (2017) |
| PreforPro | NA | 43 | Oral | NS | Mild to moderate gastro-intestinal distress but no diagnosed | Safety demonstrated | Colorado State University, United States | Gindin et al. (2019) |

*NS, Non-specified; NA, Not applicable; Adm. route, Route of administration; Ref., References; IU, Intraurethral/intravesical.

personalized phage therapy is forbidden, and only NPO Mikrogen is authorized by the State Register of the Ministry of Health of the Russian Federation to manufacture market medicinal phage cocktails (Vlassov et al., 2020). Furthermore, commercial phage products from Russia and Georgia are not recognized by the western medicine regulatory agencies, so exports of such products are difficult.

Hence, phage therapy is encountering developmental obstacles in these once-leading countries. Both countries, moreover, lack of double-blinded clinical trials, and potentially more regulations in this golden standard may improve their research progress in phage therapy (Abedon et al., 2011; Międzybrodzki et al., 2018).

## Regulations in the United Kingdom, France, and Belgium

In contrast to the long-term use of phage therapy in Eastern Europe, its application in Western Europe such as the United Kingdom, France, and Belgium is relatively scattered, but recent news demonstrated major advancements in its regulations in these countries. Since 2011, phage therapy was classified as a medicinal product by European Medicines Agency, but there were disputes about whether it should be classified as biological medicinal product according to Commission Directive 2001/83/EC or advanced therapy medicinal product based on Commission Directive 2003/63/EC (Naureen et al., 2020). However, because of its increasing importance, European Pharmacopoeia Commission sought to better specify phage therapy's regulations by enacting a new general chapter named *Phage therapy active substances and medicinal products for human and veterinary use (5.31)* in 2021 and tasked this process to the newly established Bacteriophages Working Party (BACT WP). While the draft is being made, BACT WP is open to the public for advice until June 2023 (Council of Europe, 2023). This sets the precedence of modern mainstream medicine regulatory agency incorporating phage therapy in pharmacopoeia for human and veterinary use.

While there is no licensed phage therapy in the United Kingdom, the Medicine and Healthcare products Regulatory Agency regulates different aspects of phage therapy, classifying natural phage as biological medicine and overseeing the compassionate use of phage therapy (Jones et al., 2023). Under the regulation, domestically

TABLE 2 The ISTs of phage therapy since 2017 acquired from Clinicaltrials.gov.

| Name | Phase | Adm route | Target | Indication | Starting year | Developer | Country | NCT. Id |
|---|---|---|---|---|---|---|---|---|
| Preforpro | 3 | Oral | NS | Vaginal infection | May 1, 2023 (est) | Deerland Enzymes | United Kingdom | NCT05590195 |
| Pyobacteriophage | 3 | IH | Multiple common bacteria | Acute tonsillitis | October 2, 2020 | Tashkent Pediatric Medical Institute | Uzbekistan | NCT04682964 |
| LBP-EC01 | 2/3 | IU & IV | *E. coli* | Urinary tract infection | July 13, 2022 | Locus Bioscience | United States | NCT05488340 |
| AP-PA02 | 2 | IH | *P. aeruginosa* | Chronic pulmonary infection | January 10, 2023 | Armata Pharmaceuticals, Inc. | United States | NCT05616221 |
| PhageBank | 2 | NS | *S. aureus* | Diabetic foot osteomyelitis | November 24, 2021 | Adaptive Phage Therapeutics, Inc. | United States | NCT05177107 |
| PhageBank | 2 | IO | Multiple common bacteria | Chronic prosthetic join infection on hip/knee | March 27, 2023 | Adaptive Phage Therapeutics, Inc. | United States | NCT05269134 |
| PP1493 & PP1815 | 2 | IA | *S. aureus* | Prosthetic joint infection | June 15, 2022 | Pherecydes Pharma | France | NCT05369104 |
| YPT-01 | 1/2 | IH | *P. aeruginosa* | Cystic fibrosis infection | March 29, 2021 | Yale University | United States | NCT04684641 |
| BACTELIDE | 1/2 | Topical/IH | *S. aureus, P. aeruginosa,* or *K. pneumoniae* | Pressure ulcer infection | January, 2022 (est.) | Phagelux Inc. | United States | NCT04815798 |
| PhageBank | 1/2 | IO & IV | Multiple common bacteria | Chronic prosthetic join infection on hip/knee | May, 2022 (est.) | Adaptive Phage Therapeutics, Inc. | United States | NCT05269121 |
| BX005-A | 1/2 | Topical | *S. aureus* | Atopic dermatitis | May 2022 (est.) | BiomX | United States | NCT05240300 |
| ShigActive | 1/2 | Oral | *Shigellosis* | Experimental Shigella challenge | February 23, 2023 | Intralytix | United States | NCT05182749 |
| EcoActive | 1/2 | Oral | *E. coli* | Crohn's Diseases | May 1, 2019 | Intralytix | United States | NCT03808103 |
| VRELysin | 1/2 | Oral | *Enterococcus* | Intestinal infection | April 1, 2023 (est.) | Intralytix | United States | NCT05715619 |
| NS | 1/2 | Topical | *S. aureus* | Diebetic foot ulcers infection | June 1, 2022 (est.) | Center Hospitalier Universitaire de Nîmes | France | NCT02664740 |
| TP-102 | 1/2 | Topical | *P. aeruginosa, S. aureus, A. baumanni* | Diabetic foot ulcer infection | March 22, 2021 | Technophage | Israel | NCT04803708 |
| WRAIR-PAM-CF1 | 1/2 | IV | *P. aeruginosa* | Cystic fibrosis infection | October 3, 2022 | National Institute of Allergy and Infectious Diseases | United States | NCT05453578 |
| AP-PA02 | 1/2 | IH | *P. aeruginosa* | Chronic lung infection | December 22, 2020 | Armata Pharmaceuticals, Inc. | United States | NCT04596319 |
| AP-SA02 | 1/2 | IV | *S. aureus* | Bacteremia | April 26, 2022 | Armata Pharmaceuticals, Inc. | United States | NCT05184764 |
| BX004-A | 1/2 | IH | *P. aeruginosa* | Chronic pulmonary infection | June 21, 2022 | BiomX, Inc. | United States | NCT05010577 |
| PGX-0100 | 1 | IH | *S. aureus, P. aeruginosa,* or *K. pneumoniae* | Burn infection | January 2022 (est.) | Phagelux Inc. | Australia | NCT04323475 |
| BX002-A | 1 | Oral | NA | NA—Healthy individual | October 28, 2020 | BiomX, Inc. | United States | NCT04737876 |
| SNIPR001 | 1 | Oral | NA | NA—Healthy individual | March 24, 2022 | SNIPR Biome Aps. | United Kingdom | NCT05277350 |
| PrePhage | 1 | Nasal | NS | Necrotizing Enterocolitis in Preterm infant | April 1, 2023 (est.) | Rigshospitalet Hospital | Denmark | NCT05272579 |

*Clinical trials that are canceled, discontinued, or have entered a new phase are not included. Information is up to date since the last check on June 25, 2023. NA, Not applicable; NS, Non-specified; Adm. route, Route of administration; NCT Id., Clinical Trial register ID; IV, Intravenous; IH, Inhalation; IO, Intraoperative; IU, Intraurethral/intravesical; and IA, Intra-articular injection.

produced phage must follow GMP, and for clinical trials, only phages produced according to GMP may be used, while GMP is not required for imported phage for unlicensed use. Following more research, many proposals now advocate for more public funding for phage research and licensure coordinated by National Health Service (Jones et al., 2023). In response, the United Kingdom Parliament recently published an inquiry to draw evidence from different fields and experts to discuss the future of phage therapy and its regulations (UK Parliament, 2023). By the submission deadline, the inquiry received one oral transcript and 34 written evidence from various fields (Lin et al., 2023). Learning from the suggestions from the research institutes, healthcare providers, and capital interests within and beyond the United Kingdom, the parliament can hopefully gain a better understanding of the current situation of phage therapy and legislates accordingly to facilitate its applications.

Alongside following the EU guidelines as its member state, France also regulates phage therapy through the National Agency for the Safety of Medicines and Health Products (ANSM). Besides overseeing the compassionate use of phage therapy, the ANSM also formed temporary specialized scientific committees in 2016 and 2019 to investigate the potential of phage therapy in France (Procaccia, 2021). After research, the ANSM now treats phage therapy seriously and considers forming organized production and legal framework for it, pushing for the enactment of national guidance and approval platform for phage therapy in 2019 (Procaccia, 2021).

In contrast to the preliminary ideas in regulating phage therapy in the United Kingdom and France, Belgium features an established, innovative, and distinctive regulatory framework. Belgian Federal Agency for Medicines held the task to establish systematic regulations for phage therapy after Belgian Chamber of Representatives discussed the advantages and plights of phage therapy's regulations in 2016 (Sacher, 2018). Following the discussion between different groups in Belgian, the government started to regulate phage therapy based on magistral preparation (Pirnay et al., 2018). In short, magistral preparation allows a pharmacist to produce medicinal products based on a physician's prescription for each patient in pharmaceutical standards (Pirnay et al., 2018). Official pharmacopoeia such as European Pharmacopoeia and Belgian Pharmacopoeia guide the requirement for active ingredients in such a preparation (Fauconnier, 2018). The Minister of Public Health provides additional guidelines when information from those pharmacopoeia are insufficient, and a Belgian Approved Laboratory, which is an accredited quality control agent by the government, certifies whether non-authorized ingredients can be incorporated in the preparation (Fauconnier, 2018). Compared to many other countries in the EU, magistral preparation in Belgian allows tailored therapy to individual patients whereas the compassionate treatment can only be applied under emergency with exceptional nature.

## Regulations in Australia

Another country where research and health professionals are embracing phage therapy in recent years is Australia. Connected by Phage Australia, a national alliance aiming to systemize phage therapy, researchers and clinicians feature expansive network across the country based in centers like hospitals and research institutes. While the stakeholders are working toward professionalizing the therapy, it is not readily available to the public yet (Lin et al., 2019). However, currently three pathways, special access scheme (SAS), clinical trial notification (CTN), and clinical trial exemption (CTX), allow phage therapy to be administered after a referral process from family doctor or specialist to infectious disease specialist to Phage Australia (Lin et al., 2019; Bacteriophage.news, 2022). SAS serves like compassionate practice of using treatments that are not on Australian Register of Therapeutic Goods. CTN and CTX, on the other hand, are clinical trial pathways that differ in some ways. In CTN, the Human Research Ethics Committee and research sponsor are responsible for reviewing and approving the research protocol followed by clinical trial, and the Australian Therapeutic Good Administration (TGA) is notified. In contrast, CTX is the pathway in which TGA reviews the protocol and often involves novel treatments. What determines the procession of CTN or CTX is whether the Human Research Ethics Committee has the expertise to assess the safety of the treatment. If it has, then it follows CTX, or else it follows CTN. Furthermore, Phage Australia uniquely adopts the Standardized Treatment and Monitoring Protocol for Adults and Pediatric Patients (STAMP) protocol to evaluate the clinical process for phage therapy (Phage Australia, n.d.). This has immense implications as by studying and standardizing the process, different phages may be used, greatly facilitating personalized phage therapy. The STAMP process is paired up with SAS (Phage Australia, n.d.). With a national phage alliance, various pathways for phage therapy, and an innovative approach in focusing on the holistic process rather than a specific phage, Australia demonstrates its promising future in phage therapy.

## Regulations in the United States

In the United States, Office of Vaccines Research and Review in the Center for Biologics Evaluation and Research of FDA classified phage therapy as biological product and regulate it accordingly, and its manufacture must follow standards like GMP, preclinical research, and clinical trial (Furfaro et al., 2018). While there is no FDA-approved phage therapy yet, the United States has the most phage-related ISTs, some being at phase 3 clinical trial (Table 1). The first clinical trial assessing genetically modified phage was also approved in the United States (NCT05488340). Applications of INDs for phage therapy are similarly subjected to the review of FDA just as other drugs, and clinical trials may be started if FDA does not hold on the application after 30 days (Suh et al., 2022). Also, like the compassionate use of phage therapy elsewhere, FDA allows the extended access to the INDs of phage therapy under exceptional situations for patients when they cannot enroll in clinical trials (U.S. Food and Drug Administration, 2021). The Institutional Review Board and FDA must be notified about the extended use of phage therapy (Suh et al., 2022). Regarding personalized phage therapy, FDA made revolutionary progress by approving the IND of Adaptive Phage Therapeutics' phage bank therapy, featuring the only phage bank therapy with IND approval in the world (Adaptive Phage Therapeutics, 2021).

## Regulations in India

While phage therapy in India just emerged in recent years, phage research can be traced far back when British scientists discovered unknown that killed cholera bacteria in 1896. The unknown is highly suspected to be phage as one of the first phage

discoveries (Ranjith et al., 2019). In 1940, the government established the Central Drug Laboratory, Kasauli, to work on biological products including phage based on Drugs and Cosmetics Act (1940) (Ranjith et al., 2019). Phage therapy, however, was not much promoted until 2017 when Vitalis Phage Therapy was founded by Pranav Johri, who treated his multi-drug resistant infection using phage therapy in Eliava Phage Therapy Center in Georgia, and now cooperates with the center to provide phage therapy for others (Sacher, 2019). Today, because no clear regulations is made regarding phage therapy, it in India is offered as compassionate Phage Therapy (cPT) on the compassionate base and is regulated by the Declaration of Helsinki and coordinated by the Central Drugs Standard Control Organization that enables cPT in India as it allows the importation of unregulated drugs like phages for compassionate treatment (Ranjith et al., 2019; Johri, 2023). Realizing the potential of phage therapy, the Indian government is trying to promote phage therapy as the Indian Council of Medical Research (ICMR) has been gathering phage researchers and stakeholders to discuss relevant details, so more specific regulations and research centers can be expected in India (Johri, 2023).

## Regulations in China

It was as early as 1958 when the Shanghai Jiao Tong University School of Medicine successfully treated a patient with *P. aeruginosa* burn infection using phage therapy (Liang et al., 2023). However, the ethics approval system was not yet established. It was not until 2019 when Shanghai Institute of Phage conducted the first IIT of personalized phage therapy at Shanghai Public Health Clinical Center and Zhongshan Hospital Fudan University under the ethics approval framework (Bao et al., 2020).

Related studies have also been conducted in Shenzhen later (Tan et al., 2021). Following the progress in Shanghai and several other hospitals in Shenzhen, Xi'an also launched IITs of phage therapy recently. However, no company has acquired IND to conduct IST in China. According to Chinese regulations, commercial phage therapy applications can go through two pathways. Firstly, phage products with fixed ingredients should be regulated as innovative biological products (Lu et al., 2023). Their INDs need to be submitted to the Center for Drug Evaluation (CDE) under National Medical Product Administration (NMPA), which are then subjected to IST regulated by Chinese Good Clinical Practice and Measures for the Administration of Drug Registration (Lu et al., 2023). Secondly, personalized phage therapies need to go through IIT under Management Measures for Clinical Research (Huang, 2022). Based on successful results, researchers from a medical institution may apply for restrictive medical technology in Provincial Health Commission, and upon approval, the personalized phage therapy can be conducted at certain institutions (Huang, 2022; Yang, 2023). Although phage-related legislations are yet to be created, legislators are increasingly aware of this rapidly developing field. For instance, On January 31, 2023, the Development and Reform Commission of Shenzhen Municipality issued three documents consecutively to emphasize the support that will be placed in nine core technologies that are facing bottlenecks, one being phage therapy (Development and Reform Commission of Shenzhen Municipality, 2023).

## Future prospects

In summary of the countries discussed above, it can be seen that compassionate use is a major pathway that patients rely on to access phage therapy overall, especially in the countries that started to embrace the therapy in recent decades. The representative countries in which the compassionate use being a major access to phage therapy include the United Kingdom, France, Belgium, Australia, India, China, and the United States despite that the terms that these countries use in referring to compassionate use may be different. It can be argued that even Poland, in which phage therapy has been a long tradition, practices the compassionate use of phage therapy after it adopted more related regulations as it currently defines phage therapy as experimental treatment and regulates it like compassionate treatment in many ways. In addition, phage therapy can also be accessed if clinical trial is available in many of these countries. However, given the considerable demand and various types of research involving clinical trials done, phage therapy demonstrates its enormous potential that made these countries to come up with more strategies in recent years to extend it beyond the scope of compassionate use. Among the new policies, Belgium features the most significant and innovative regulation by establishing a thorough regulatory framework for phage therapy, the unique magistral preparation in Western Europe, enabling the public access to phage therapy. The other countries are also working toward public access. Belgium's counterparts, the United Kingdom and France, are on the way to innovate their current regulations for phage therapy by posing inquiries to specialists and forming expert committees. More broadly, the European Pharmacopoeia Commission announced to add a new general chapter specifically regulating phage therapy, and this will be the party's primary goal from 2023 to 2025 (Council of Europe, 2023). India is on the similar track as the ICMR is also gathering professional perspectives and the local company has partnered with the experienced Eliava Institute. Besides, Australia formed a national alliance to promote the STAMP protocol that are similar to magistral phage in some ways, like focusing on the process rather than a particular product. Over the sea, the United States unprecedentedly granted INDs for personalized phage bank treatment to Adaptive Phage Therapeutics. In 2021, FDA held a series of conferences among phage experts to discuss the prospects and regulations of phage therapy (U.S. Food and Drug Administration, 2021). Recognizing the importance of phage therapy through ongoing compassionate treatments, the conference encouraged more well-controlled clinical trials to support the official licensure of phage therapy.

In this process of forming novel regulations for phage therapy, China seems to fall behind as no major turning point has occurred yet despite the government's emphasis on supporting phage therapy at some regional levels. To further promote phage therapy, the governments and researchers should borrow regulatory experiences from other treatments. In general, the concerns related to the biological characteristics of phage can be addressed by learning from the regulations for treatments with similar characteristics, such as viral vector vaccines, oncolytic viruses, and neutralizing antibodies. Also, regulations in fixed-ingredient phage cocktails can be referred to other treatments that are similarly subjected to IST. For instance, cellular immunotherapy like Neoantigen-Targeting T Cell suspension for intravenous infusion uses a similar procedure as a phage cocktail to first get IND from CDE and conduct IST upon NMPA approval.

Moreover, many personalized therapies including cellular therapy and fecal microbiota transplantation approved by the Provincial Health Commission or National Medical Product Administration allow personalized phage therapy to take an example of. Such experiences are models for improvement in regulations of phage therapy in China.

In summary, just as how studying phage greatly promoted the advancement in various fields including virology, genetics, molecular biology, and synthetic biology, progress in phage therapy can assist pharmaceutical regulatory authorities to update and optimize related policies, especially regarding personalized therapies that have enormous future potential. Diving into these discussions in this article, we believe that the revolutionary landmarks in phage therapy represent its renaissance in the mainstream of the modern medical field.

## Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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## Conflict of interest

QY was an intern at CreatiPhage Biotechnology. NW was a cofounder of CreatiPhage Biotechnology.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## References

Abedon, S. T., Kuhl, S. J., Blasdel, B. G., and Kutter, E. M. (2011). Phage treatment of human infections. *Bacteriophage* 1, 66–85. doi: 10.4161/bact.1.2.15845

Adaptive Phage Therapeutics (2021). Adaptive phage therapeutics announces FDA clearance of IND application for APT's phage bank for the treatment of prosthetic joint infections—Adaptive phage therapeutics. Available at: https://aphage.com/adaptive-phage-therapeutics-announces-fda-clearance-of-ind-application-for-phagebank-for-the-treatment-of-prosthetic-joint-infections/

Bacteriophage.news (2022). STAMP protocol approved for phage therapy in Australia. Available at: https://www.bacteriophage.news/stamp-protocol-phage-therapy-in-australia/

Bao, J., Wu, N., Zeng, Y., Chen, L., Li, L., Yang, L., et al. (2020). Non-active antibiotic and bacteriophage synergism to successfully treat recurrent urinary tract infection caused by extensively drug-resistant *Klebsiella pneumoniae*. *Emerg. Microb. Infect.* 9, 771–774. doi: 10.1080/22221751.2020.1747950

Bruttin, A., and Brüssow, H. (2005). Human volunteers receiving *Escherichia coli* phage T4 orally: a safety test of phage therapy. *Antimicrob. Agents Chemother.* 49, 2874–2878. doi: 10.1128/AAC.49.7.2874-2878.2005

Chanishvili, N. (2012). Phage therapy—history from Twort and d'Herelle through soviet experience to current approaches. *Adv.Virus Res.* 83, 3–40. doi: 10.1016/B978-0-12-394438-2.00001-3

Council of Europe (2023). Public consultation on new general chapter on phage therapy active substances and medicinal products for human and veterinary use in Pharmeuropa 35.2—European Directorate for the Quality of Medicines & HealthCare. Available at: https://www.edqm.eu/en/home/-/asset_publisher/wQkauHDDLDSk/content/public-consultation-on-new-general-chapter-on-phage-therapy-active-substances-and-medicinal-products-for-human-and-veterinary-use-in-pharmeuropa-35.2

Development and Reform Commission of Shenzhen Municipality (2023). Development and Reform Commission of Shenzhen Municipality issued a notice on three policy measures, one being "Several Measures to Promote the High-quality Development of biomedical Industrial Clusters in Shenzhen" [深圳市发展和改革委员会关于印发《深圳市促进生物医药产业集群高质量发展的若干措施》等三个政策措施的通知—2023年第3期(总第1273期)]. Available at: http://www.sz.gov.cn/zfgb/2023/gb1273/content/post_10403843.html

Diallo, K., and Dublanchet, A. (2023). A century of clinical use of phages: a literature review. *Antibiotics* 12:751. doi: 10.3390/antibiotics12040751

European Medicines Agency (2023). *Human regulatory, Clinical Trials Regulation*. Available at: https://www.ema.europa.eu/en/human-regulatory/research-development/clinical-trials/clinical-trials-regulation

Fauconnier, A. (2018). "Guidelines for bacteriophage product certification" in *Bacteriophage Therapy*. eds. J. Azeredo and S. Sillankorva, vol. *1693* (New York: Springer), 253–268.

Fauconnier, A. (2019). Phage therapy regulation: from night to Dawn. *Viruses* 11:352. doi: 10.3390/v11040352

Furfaro, L. L., Payne, M. S., and Chang, B. J. (2018). Bacteriophage therapy: clinical trials and regulatory hurdles. *Front. Cell. Infect. Microbiol.* 8:376. doi: 10.3389/fcimb.2018.00376

Gindin, M., Febvre, H. P., Rao, S., Wallace, T. C., and Weir, T. L. (2019). Bacteriophage for gastrointestinal health (PHAGE) study: evaluating the safety and tolerability of supplemental bacteriophage consumption. *J. Am. Coll. Nutr.* 38, 68–75. doi: 10.1080/07315724.2018.1483783

Hartmann, M., and Hartmann-Vareilles, F. (2006). The clinical trials directive: how is it affecting Europe's noncommercial research. *PLoS Clin.Trials* 1:e13. doi: 10.1371/journal.pctr.0010013

Huang, C. (2022). Scientific interpretation on "management of medical technology clinical application (In Chinese). *Soft Science of Health*, 36, 68–72. doi: 10.3969/j.issn.1003-2800.2022.05.014

Jault, P., Leclerc, T., Jennes, S., Pirnay, J. P., Que, Y.-A., Resch, G., et al. (2019). Efficacy and tolerability of a cocktail of bacteriophages to treat burn wounds infected by *Pseudomonas aeruginosa* (PhagoBurn): a randomised, controlled, double-blind phase 1/2 trial. *Lancet Infect. Dis.* 19, 35–45. doi: 10.1016/S1473-3099(18)30482-1

Johri, P. (2023). Antimicrobial resistance and phage therapy in India. The Microbiologist. Available at: https://www.the-microbiologist.com/features/antimicrobial-resistance-and-phage-therapy-in-india/1386.article

Jones, J. D., Trippett, C., Suleman, M., Clokie, M. R. J., and Clark, J. R. (2023). The future of clinical phage therapy in the United Kingdom. *Viruses* 15:721. doi: 10.3390/v15030721

Leitner, L., Ujmajuridze, A., Chanishvili, N., Goderdzishvili, M., Chkonia, I., Rigvava, S., et al (2021). Intravesical bacteriophages for treating urinary tract infections in patients undergoing transurethral resection of the prostate: A randomised, placebo-controlled, double-blind clinical trial. *Lancet Infect. Dis.* 21, 427–436. doi: 10.1016/S1473-3099(20)30330-3

Liang, S., Qi, Y., Yu, H., Sun, W., Raza, S. H. A., Alkhorayef, N., et al. (2023). Bacteriophage therapy as an application for bacterial infection in China. *Antibiotics* 12:417. doi: 10.3390/antibiotics12020417

Lin, R., Ameneh, K., and Anton, P. (2023). The antimicrobial potential of bacteriophages—UK Parliament Inquiry 2023. Available at: https://www.phageaustralia.org/blog/antimicrobial-potential-of-phages

Lin, R. C., Fabijan, A. P., Attwood, L., and Iredell, J. (2019). State of the regulatory affair: Regulation of phage therapy in Australia. Available at: https://phage.directory/capsid/phage-therapy-regulation-australia

Lin, D. M., Koskella, B., and Lin, H. C. (2017). Phage therapy: an alternative to antibiotics in the age of multi-drug resistance. *World J. Gastrointest. Pharmacol. Ther.* 8, 162–173. doi: 10.4292/wjgpt.v8.i3.162

Liu, Q., Lu, M., Hu, H., Chen, L., and Yao, W. (2022). Research on the current status of clinical trial supervision for tumor neoantigen vaccine in China (In Chinese) *Chin. Pharm.* 33, 2827–2830. doi: 10.6039/j.issn.1001-0408.2022.23.02

Lu, J., Xu, L., Wei, W., and He, W. (2023). Advanced therapy medicinal products in China: regulation and development. *Med. Commun.* 4:e251. doi: 10.1002/mco2.251

McCallin, S., Alam Sarker, S., Barretto, C., Sultana, S., Berger, B., Huq, S., et al. (2013). Safety analysis of a Russian phage cocktail: from MetaGenomic analysis to oral application in healthy human subjects. *Virology* 443, 187–196. doi: 10.1016/j.virol.2013.05.022

Międzybrodzki, R., Hoyle, N., Zhvaniya, F., Łusiak-Szelachowska, M., Weber-Dąbrowska, B., Łobocka, M., et al. (2018). "Current updates from the long-standing phage research centers in Georgia, Poland, and Russia" in *Bacteriophages*. eds. D. R. Harper, S. T. Abedon, B. H. Burrowes and M. L. McConville (Springer, Cham), 1–31. doi: 10.1007/978-3-319-40598-8_31-1

Naureen, Z., Malacarne, D., Anpilogov, K., Dautaj, A., Camilleri, G., Cecchin, S., et al. (2020). Comparison between American and European legislation in the therapeutical and alimentary bacteriophage usage. *Acta Bio Med. Atenei Parmensis* 91:e2020023. doi: 10.23750/abm.v91i13-S.10815

Phage Australia (n.d.). 4. Our approach. Available at: https://www.phageaustralia.org/approach (Accessed August 9, 2023).

Pharmacopoeia.ru (n.d.). GPM. 1.8.1.0002.15 Immunobiological medicinal products—Pharmacopoeia.ru. Available at: https://pharmacopoeia.ru/en/ofs-1-8-1-0002-15-immunobiologicheskie-lekarstvennye-preparaty/ (Accessed June 13, 2023).

Pirnay, J.-P., Verbeken, G., Ceyssens, P.-J., Huys, I., De Vos, D., Ameloot, C., et al. (2018). The Magistral phage. *Viruses* 10:64. doi: 10.3390/v10020064

Procaccia, C. (2021). Phage therapy: the medicine of yesterday and tomorrow (OPECST)—Policy Briefs & Reports—EPTA network. Available at: https://eptanetwork.org/database/policy-briefs-reports/2127-phage-therapy-the-medicine-of-yesterday-and-tomorrow-opecst

Ranjith, G., Pujar, A. A., Dubey, A. K., and Venkatesh, P. (2019). Antimicrobial resistance and phage therapy in the Indian context. *Curr. Sci.* 117, 562–564. doi: 10.18520/cs/v117/i4/562-564

Rhoads, D. D., Wolcott, R. D., Kuskowski, M. A., Wolcott, B. M., Ward, L. S., and Sulakvelidze, A. (2009). Bacteriophage therapy of venous leg ulcers in humans: results of a phase I safety trial. *J. Wound Care* 18, 237–243. doi: 10.12968/jowc.2009.18.6.42801

Sacher, J. (2018). Belgium's new brand of phage therapy. Available at: https://phage.directory/capsid/belgium-therapy

Sacher, J. (2019). Improving access to phage therapy in India. Available at: https://phage.directory/capsid/phage-therapy-access-india

Sarker, S. A., Berger, B., Deng, Y., Kieser, S., Foata, F., Moine, D., et al. (2017). Oral application of E scherichia coli bacteriophage: safety tests in healthy and diarrheal children from B angladesh. *Environ. Microbiol.* 19, 237–250. doi: 10.1111/1462-2920.13574

Sarker, S. A., Sultana, S., Reuteler, G., Moine, D., Descombes, P., Charton, F., et al. (2016). Oral phage therapy of acute bacterial diarrhea with two Coliphage preparations: a randomized trial in children from Bangladesh. *EBioMedicine* 4, 124–137. doi: 10.1016/j.ebiom.2015.12.023

Suh, G. A., Lodise, T. P., Tamma, P. D., Knisely, J. M., Aslam, A. J., Barton, S., et al. (2022). Considerations for the use of phage therapy in clinical practice. *Antimicrob. Agents Chemother.* 66:e0207121. doi: 10.1128/aac.02071-21

Tan, X., Chen, H., Zhang, M., Zhao, Y., Jiang, Y., Liu, X., et al. (2021). Clinical experience of personalized phage therapy against Carbapenem-resistant *Acinetobacter baumannii* lung infection in a patient with chronic obstructive pulmonary disease. *Front. Cell. Infect. Microbiol.* 11:631585. doi: 10.3389/fcimb.2021.631585

U.S. Food and Drug Administration (2021). Science and regulation of bacteriophage therapy. Available at: https://www.fda.gov/media/159399/download

UK Parliament (2023). The antimicrobial potential of bacteriophages—committees—UK Parliament. Available at: https://committees.parliament.uk/work/7045/the-antimicrobial-potential-of-bacteriophages/publications/

Uyttebroek, S., Chen, B., Onsea, J., Ruythooren, F., Debaveye, Y., Devolder, D., et al. (2022). Safety and efficacy of phage therapy in difficult-to-treat infections: a systematic review. *Lancet Infect. Dis.* 22, e208–e220. doi: 10.1016/S1473-3099(21)00612-5

Vlassov, V. V., Tikunova, N. V., and Morozova, V. V. (2020). Bacteriophages as therapeutic preparations: what restricts their application in medicine. *Biochem. Mosc.* 85, 1350–1361. doi: 10.1134/S0006297920110061

Wright, A., Hawkins, C. H., Änggård, E. E., and Harper, D. R. (2009). A controlled clinical trial of a therapeutic bacteriophage preparation in chronic otitis due to antibiotic-resistant *Pseudomonas aeruginosa*; a preliminary report of efficacy. *Clin. Otolaryngol.* 34, 349–357. doi: 10.1111/j.1749-4486.2009.01973.x

Yang, A. (2023). CreatiPhage—The Pioneer in China. Phage Directory. Available at: https://phage.directory/capsid/creatiphage-china

Żaczek, M., Górski, A., Weber-Dąbrowska, B., Letkiewicz, S., Fortuna, W., Rogóż, P., et al. (2022). A thorough synthesis of phage therapy unit activity in Poland—its history, milestones and international recognition. *Viruses* 14:1170. doi: 10.3390/v14061170

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# Phage therapy: a revolutionary shift in the management of bacterial infections, pioneering new horizons in clinical practice, and reimagining the arsenal against microbial pathogens

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The recent approval of experimental phage therapies by the FDA and other regulatory bodies with expanded access in cases in the United States and other nations caught the attention of the media and the general public, generating enthusiasm for phage therapy. It started to alter the situation so that more medical professionals are willing to use phage therapies with conventional antibiotics. However, more study is required to fully comprehend phage therapy's potential advantages and restrictions, which is still a relatively new field in medicine. It shows promise, nevertheless, as a secure and prosperous substitute for antibiotics when treating bacterial illnesses in animals and humans. Because of their uniqueness, phage disinfection is excellent for ready-to-eat (RTE) foods like milk, vegetables, and meat products. The traditional farm-to-fork method can be used throughout the food chain to employ bacteriophages to prevent food infections at all production stages. Phage therapy improves clinical outcomes in animal models and lowers bacterial burdens in numerous preclinical investigations. The potential of phage resistance and the need to make sure that enough phages are delivered to the infection site are obstacles to employing phages *in vivo*. However, according to preclinical studies, phages appear to be a promising alternative to antibiotics for treating bacterial infections *in vivo*. Phage therapy used with compassion (a profound understanding of and empathy for another's suffering) has recently grown with many case reports of supposedly treated patients and clinical trials. This review summarizes the knowledge on the uses of phages in various fields, such as the food industry, preclinical research, and clinical settings. It also includes a list of FDA-approved bacteriophage-based products, commercial phage products, and a global list of companies that use phages for therapeutic purposes.

KEYWORDS

**bacteriophage, veterinary medicine, preclinical AND clinical trials, immune response, treatment challenges**

## Introduction

If the crisis of antimicrobial resistance is not addressed, it is anticipated that by 2050, the societal and financial costs will total US$100 trillion, resulting in 70,000 annual deaths (1). This number, although controversial, still emphasizes the severe problem we face regarding therapeutic alternatives for multidrug-resistant (MDR) bacterial infections (2, 3). Significant health risks are posed by pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA) and multi-resistant *Pseudomonas aeruginosa* (MDR-PA). The World Health Organization (WHO) recently published a list of MDR-priority pathogens and called for more research into antimicrobial resistance (4). Equally concerning is the requirement for rapid development of new antibiotics to replace older ones that are losing their efficacy. As a result, many researchers and clinicians are looking at bacteriophage as the most potential substitute for or adjunct to antibiotics to treat bacterial infections in the face of rising antibacterial resistance. Bacteriophages are viruses that uniquely and specifically target and eliminate bacteria. They work cooperatively in microbiological ecosystems in the human body and the environment and do not harm mammalian cells. As natural biological regulators, bacteriophages integrate into the One Health Strategy for animals, humans, and the environment (5). The first phages to be identified were phages against *Escherichia coli*, *Shigella dysenteriae*, and *Vibrio cholerae* (6, 7). Although there are several reports on phage treatment, they are uncontrolled or anecdotal and must adhere to the standards of contemporary evidence-based medicine (8). After the discovery of antibiotics, phages ceased being used as antibacterial agents in Western countries. Given our present in-depth understanding of the biology of bacteriophages, which is crucial in supporting developments in molecular biology, the odds of success the second time around are significantly greater. However, evidence from clinical and animal model studies strongly suggests that phage treatment is secure and may be advantageous (9–11).

This review offers recommendations for clinicians considering experimental phage treatment based on a thorough literature evaluation due to knowledge gaps. The study also intends to give an evidence-based assessment of the situations in which this experimental therapy may be considered and to acquaint clinicians with phage therapy's preclinical and clinical usage.

## Bacteriophages vs antibiotics

### Enzybiotics

According to Veiga-Crespo et al. (12), the term "enzybiotics" refers to phages, viruses that target and lyse bacteria and may potentially aid in the treatment of bacterial infections. These can be phage-encoded lytic enzymes, such as lysins, and extracellular polymeric substance (EPS) depolymerase. Even though many EPS depolymerases and certain lysins are connected to virion particles (13), most lysins are endolysins, which are "from-within cell-wall degrading enzymes." Enzybiotics, however, are added from outside after purification. Gram-positive bacteria's peptidoglycan is not shielded by an outer membrane, making it susceptible to phage lysins. In order to cross the outer membrane barrier in Gram-negative bacteria, phage lysins often must be altered. Fusion proteins that combine natural lysin with an antibacterial peptide might accomplish this. According to the study by Yang et al. (14), one of these constructs, PlyA, showed good efficacy against *A. baumannii* and *P. aeruginosa* and growing cultures but not against cells in the stationary phase unless combined with agents that permeabilize the outer membrane. Due to a lack of awareness and comprehension, engineered bacteriophages could pose a problem in terms of public acceptance and regulatory approvals of engineered bacteriophages could delay their usage (15). In contrast to antibiotics, phages have genomes and can proliferate while parasitizing their host; hence, engineering them could cause additional problems and necessitate the addition of legal clauses addressing the social issues associated with genetic modifications. Additionally, when lysins are used via intravenous administration, neutralizing antibodies are produced, reducing their antibacterial efficacy after multiple administrations (16). However, few endolysin immunogenicity studies have shown that immune serum does slow but is not sufficient to block the antibacterial ability of endolysin (17–19). Since they have a short plasma half-life, are immunogenic and potentially toxic, cause an inflammatory reaction to bacterial debris, and are ineffective in lysing intracellular bacteria, their use as an antibacterial agent in human treatment raises concerns (20).

## Delivery system for phage and Endolysin

Despite therapeutic potential of phages and endolysins, these alternative agents must overcome various practical challenges posed by the host system, such as limited bioavailability, loss of action, non-targeted delivery, rapid clearance by the reticuloendothelial system, and antibody-mediated inactivation (21).

Numerous methods for encapsulating phage and endolysin have been discovered recently (21). These delivery methods treat acute and chronic infections in animal models by altering host immunological response to therapeutic entities and increasing pharmacokinetic parameters (22). Many phage encapsulation studies have investigated the possibilities of different drug delivery systems, primarily natural polymers, synthetic polymers, liposomes, and electrospun fibers. GIT infections have been thoroughly researched as a potential polymeric phage encapsulation therapy target. These polymers shield phages from harsh acidic environments that may otherwise result in phage inactivation or loss of phage titer. In addition to safeguarding phages from harsh environments, these polymeric encapsulation materials also give permeability to the mucosal membrane, where bacterium pathogens may live, and get protected from digestive enzymes and bile fluids (23). According to Gondil et al., phage delivery systems are very effective when an infection is still in its early stages and can be effectively eliminated with just one dose of a phage formulation (24). However, late administration of phage formulation necessitates concomitant antibiotic administration or multiple doses of phage formulation to prevent the spread of infection. Different delivery systems of bacteriophage and endolysin are mentioned in Table 1.

## Bacteriophage and its applications

Bacteriophages have been used and reported worldwide in various valuable applications. Figure 1: graphically represents the use of phage in clinical, preclinical, agriculture, fishery, and food

TABLE 1 Showing phage delivery systems.

| Delivery system | Types | Protective Properties |
|---|---|---|
| Stabilized dry phage preparation (powders) | 1. Lyophilized Phages<br>2. Spray drying | The biotherapeutic material is still active after lyophilization, enabling long-term storage. The particle sizes produced range from nanometers to micrometers.<br>Particle sizes between 1 and 5 mm are typically produced via spray drying. Such nano- or microparticle manufacturing enables the creation of phage powders that are simple to administer for treating respiratory infections because distribution via inhalers enables effective nebulization (25–28). |
| Encapsulation | 1. Liposomes<br>2. Transferosome<br>3. Hydrogels<br>4. Electrospinning | Liposomes shield the cargo from enzymatic attack, hydrolysis (low pH), and inactivation by immune system components. They can penetrate bacterial biofilms. It also helps the retention of phages at the site of infection. Oral liposome formulations are the most effective method for treating gastrointestinal illnesses (21, 29, 30).<br>With better skin penetration and higher soft tissue protection than a free-phage cocktail, transferosomes—liposomes incorporating detergent are effective delivery systems (31).<br>Phage hydrogel encapsulation demonstrated high antibacterial activity in an alginate encapsulation and was successfully shielded from the acidic stomach pH. A phage cocktail in $CaCO_3$ demonstrated higher antibacterial activity (32).<br>It is possible to produce a wide variety of materials. A fiber-encapsulated phage can be quickly deposited into other substances (33). |
| Immobilization with fibers | Bandages and dressings | The topical delivery of phages in the form of bandages and dressings for wounds and packing materials with acidic pH and antibacterial enzymes is made possible by surface immobilization of the phages (34). |

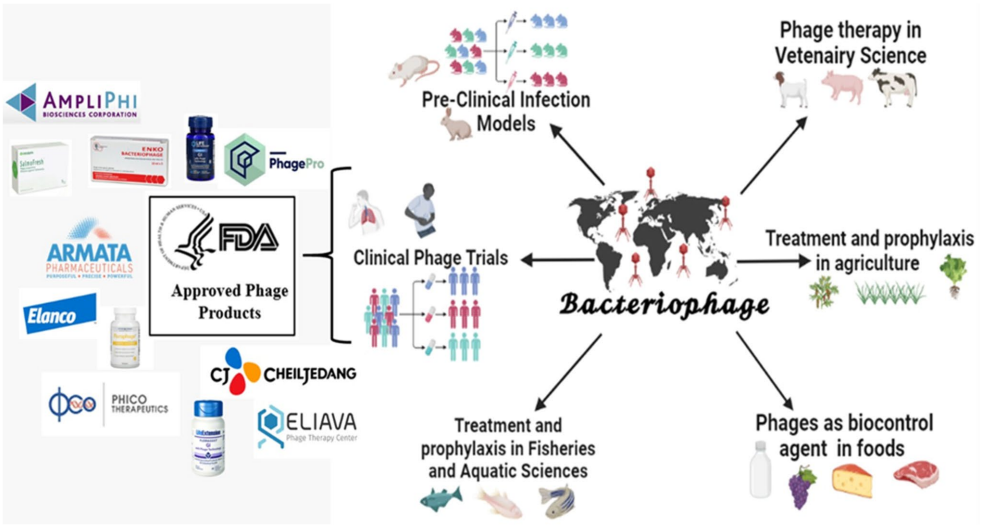


FIGURE 1
Applications of bacteriophages and its FDA-approved products (created by the help of bio-render tool).

industries. In addition, the therapeutic efficacy of the phage has been proven using preclinical and clinical settings. Based on these results, many pharmaceutical companies are still running clinical trials using bacteriophage products. However, the FDA has approved various phage products competing with the existing antimicrobials.

## Bacteriophages in preclinical studies

Recent years have seen a resurgence in interest in phage treatment, which uses bacteriophages to treat bacterial illnesses. This is because antibiotic-resistant bacteria are becoming more common. However, before going on to clinical trials in people, preclinical phage application involves evaluating the tolerance and efficacy of phage therapy in animal models.

Preclinical trials of phage therapy in animals typically involve infecting the animals with a specific bacterial pathogen and then administering phages to see if they can reduce bacterial loads and improve clinical outcomes. In addition, the animals are monitored for signs of toxicity or adverse reactions to the phages. Phage therapy improves clinical outcomes in animal models and lowers bacterial burdens in numerous preclinical studies. Nevertheless, there are challenges to using phages *in vivo*, such as the risk of phage resistance and the requirement to ensure that enough phages are delivered to the infection site.

## Pharmacokinetics and pharmacodynamics of bacteriophages

Lytic phages, unlike conventional antibiotics, are unique biological agents capable of replicating within susceptible bacteria. They present complex pharmacokinetic (PK) profiles that encompass various facets, including absorption, distribution, metabolism, and elimination. The bedrock of phage dosage determination lies in the quantity of administered phage particles. Often, the choice of assays for quantifying phages in dosing solutions and biological samples, like blood and urine, is overlooked (35).

The primary method for quantifying phages involves counting visible plaques on agar plates containing susceptible bacteria. However, this approach may not encompass the entire phage population, as it only considers those capable of causing extensive lysis and resulting in visible plaques. Additionally, the concept of "efficiency of plating," expressed as the ratio of plaque-forming units (pfu) of phages on the target bacterial strain relative to a reference strain, is integral to phage quantification. Using a bacterial host strain with lower plating efficiency can lead to underestimations, necessitating adjustments for an accurate count of infective phages against the target strain.

In the clinical arena, quantitative PCR traditionally monitors the kinetics of viral load for human viruses like HIV, CMV, Hepatitis B & C, and SARS-Cov-2, aiding in disease assessment and evaluating the efficacy of antiviral therapies. Recently, quantitative PCR-based assays have been applied to monitor phage PK in patients with severe bacterial infections undergoing intravenous adjunctive phage therapy. However, these assays face limitations in distinguishing infective phages from non-infective ones or phage DNA/RNA fragments. Therefore, further research is imperative to establish correlations between PCR-based assays and therapeutic outcomes. In conclusion, the pressing need for sensitive and validated methods of quantifying phages remains a paramount concern in the field of phage PK/PD research.

Phage therapy, unlike antibiotics relying on the widely accepted minimum inhibitory concentration (MIC) for evaluating pharmacodynamics (PD), grapples with standardizing antibacterial activity assessment. Traditional approaches encompass agar-based spot tests and efficiency of plating assays, while fluid environments like broth employ planktonic killing assays to assess phage effectiveness. These different testing methods yield divergent results in terms of the host range for the same phage; direct spot tests exhibit the broadest coverage, followed by efficiency of plating and broth killing assays. This variability arises from distinct killing kinetics and the emergence of phage resistance under different testing conditions. Evaluating the strengths and limitations of each method comprehensively is crucial for enhancing phage efficacy assessment (35, 36).

Recognizing that the dynamics of phage resistance observed in controlled laboratory settings may not necessarily mirror what transpires in animals or humans is essential. Initiatives like Clinical Phage Microbiology aim to provide guidance for clinical decision-making, underscoring the significance of incorporating knowledge related to phage pharmacokinetics and pharmacodynamics (PK/PD) to optimize therapy. Developing a robust phage PK/PD framework requires the standardization of PD parameters and the establishment of dependable measurement methods to inform treatment protocols. This endeavor holds immense promise for advancing the field of phage therapy and its application in clinical practice.

However, there are only a few studies that cover the pharmacology of bacteriophage therapy, and even fewer that focus on the pharmacokinetics of phage therapy. Pharmacology focuses on how drugs interact with the body; it is further divided into pharmacokinetics, which evaluates how the body impacts drugs, and pharmacodynamics, which examines how drugs impact the body. In addition, the dosage quantity of a given phage cocktail was crucial for achieving enhanced pharmacokinetics; the high dosage. As it is crucial to produce the pharmacodynamic effects of the treatment, pharmacokinetics explains how well a drug can accumulate in the locality of the targeted tissues and is summarized in absorption, distribution, metabolism, and excretion (36).

According to Abedon et al. (35), drug dilution causes drug densities to decrease during both absorption and distribution, which may result in an increase in drug density in the targeted bodily organ. Different medication delivery methods are used depending on the pharmacokinetics. A variety of criteria are taken into consideration when choosing a drug's delivery method, including the target tissue, the drug's sensitivity to body enzymes, the patient's convenience, and immunity. The formulation of the phage is yet another crucial factor in establishing efficient pharmacokinetics. The stability of phages is improved by using various formulation techniques. When treating various infections in both animal models and humans, it is important to take into account the different pharmacokinetics concepts that are necessary for the phage to penetrate the target bacteria, the achievement of an adequate phage concentration in the target's locality, and an ample antibacterial response against the target.

Since 2010, all relevant research results regarding phages *in vivo* in preclinical studies have been compiled (Table 2). The published reports are systematically presented under the headings *viz.* infection syndrome, the animal used in the study, route of phage administration, microbial organism, clinical outcome, and findings, and reported adverse events, if any. Overall, preclinical studies on phages as an alternative to antibiotics for treating bacterial infections show promise *in vivo*. However, more investigation is required to completely comprehend the safety and effectiveness of phage therapy and identify the best dosage and delivery techniques for various bacterial infections and animal species (Table 3).

## Bacteriophage therapy in clinical infections

While phage therapy used with compassion (a profound awareness and empathy for another's suffering) has recently increased with numerous case reports claimed to have cured patients, clinical trials intended to demonstrate its efficacy per current regulatory requirements have officially failed. Given the issue of rising antibiotic resistance, there needs to be an important decision on the role of phage therapy in contemporary medicine. The dramatic surge in case reports of treated patients reflects the growing interest in phage therapy. Additionally, various journals (Front Microbiol., Front Pharmacol., Viruses, Antibiotics, Pathogens, Microorganisms, etc.) have recently published special issues and study subjects on phage therapy (121).

TABLE 2 Potential advantages of bacteriophage treatment over antibiotic treatment.

| Characteristics features | Phage treatment | Antibiotics treatment | References |
|---|---|---|---|
| Specificity | Highly specific | Broad range of action | (37) |
| Effect on Normal flora | Minimal effect on normal flora with no dysbiosis and chances of developing secondary infections | Possess a broad spectrum of activities likely to affect microbial balance in patients and that are likely to generate severe secondary infections. | (38, 39) |
| Toxicity | Almost non-toxic | varying degrees of toxicity that range from mild to severe | (39–41) |
| Biofilm Penetration | Ability to penetrate effectively | cannot penetrate unless applied in large doses | (42–48) |
| Possibility of resistance | Reduced potential to induce bacterial resistance | High possibility of resistance | (39, 49–51) |
| Replication at the site of infection | Replicate at the infection site, making them accessible where they are most needed. | They do not always concentrate at the site of infection; instead, they are metabolized and excreted from the body. | (40, 50–52) |
| Adaptation to bacterial mutation | Can rapidly adapt to bacterial mutation | Unable to adjust to bacterial mutation | (40, 50) |
| Cross-resistance | Lack of cross-resistance to phages | Resistance mechanisms can also impact the effectiveness of various classes of antibiotics to a specific family of antibiotics. | (38, 39, 41, 50, 53, 54) |
| Dosage | Sometimes require multiple doses | Repeat doses are necessary | (40, 51) |
| Environment impact | Low impact on the environment | High environment impact | (40, 49, 55) |
| Effect on inflammatory responses | Possible effect on the inflammatory response | No effect on the inflammatory response | (56, 57) |
| Cycle of development | New phages (against phage-resistant bacteria) can rapidly be developed and be accomplished in days or weeks. | The long and expensive development cycle may take several years. | (55, 58) |

Since their discovery, phages have been widely used in Eastern Europe and the former Soviet Union; as a result, their medical systems now incorporate the therapeutic use of phages. However, a thorough scientific analysis of this potential therapy has recently been conducted (41, 122). Abedon offered a list of essential requirements that should be carefully considered and reported in phage therapy (123). The effectiveness of clinical research depends on the proper characterization and selection of the phages, the participants (humans), and the target microorganisms. Though necessary, additional information like formulations, dosages, and potencies are only helpful when used with clearly defined and well-planned goals. The quality of future studies would increase with more thorough reporting, enabling the replication and expansion of earlier investigations. Choosing suitable disease targets for phage therapy is another factor to consider. Phage mixtures provide a wide range of activity when contemplating monotherapy or combination therapy techniques and lessen the possibility of resistance development. Additionally, it significantly increases the difficulty of determining how each phage in a cocktail would affect inflammation, the possibility of gene transfer, and the emergence of phage resistance (124).

In several world regions, bacteriophage therapy has been utilized for many years to treat bacterial infections. As reviewed by Uyttebroek et al. (125), Kutter et al. (41) Abedo et al. (35) and described in Marza et al. (126), encouraging results have been documented. Although bacteriophage therapy has been used for many years, there are few clinical studies in this field, raising many doubts about its effectiveness against infectious diseases. However, the growing demand for novel antimicrobial therapies drives the development of bacteriophage therapeutics for diverse diseases. These require the completion of extensive clinical trials per United States FDA or European EMA requirements (127). The phage's bactericidal activity, and concerns about potential toxic shock must also be addressed.

## Musculoskeletal infections

Fish et al. (128) presented a continuous case series examining the effectiveness of treating infected and poorly vascularized toe ulcers with the exposed bone when the recommended antibiotic therapy with topically applied *S. aureus*-specific phages failed. After providing standard wound care, the phage solution was topically given to the ulcerations weekly. All infections responded to the phage therapies, and infected bone debridement resulted in an average healing time of 7 weeks for the ulcers. In order to retain hallux function and treat an ulcer with extremely poor vascularity, 18 weeks of therapy were required.

The case report by Fish et al. (129) successfully treated distal phalangeal osteomyelitis in a 63-year-old diabetic woman with long-term bacteriophage follow-up. In a different study, Fish et al. (130) reported using the commercial formulation of the extensively researched anti-*Staphylococcal* bacteriophage Sb-1 from Eliava Bio Preparations to cure diabetic toe ulcers successfully. In difficult-to-close toe ulcers containing contaminated/infected bone, topical administration of a single *S. aureus*-targeting bacteriophage proved successful.

According to a study by Ferry et al. (131) local injections of a bacteriophage mixture during debridement, antibiotics, and implant retention (DAIR) procedures to treat recurrent *S. aureus* chronic joint prosthesis infection (PJI) were effective, safe, and associated with clinical success.

TABLE 3 A list of recent studies that are pertinent to the use of phages *in vivo* in preclinical trials.

| Infection Syndrome | Animal (Species/Strain) | Route of administration | Target bacteria | Clinical outcome | Outcome assessed | Adverse events | Article (references) and Country |
|---|---|---|---|---|---|---|---|
| Urinary tract infections | Mice (C57BL/6NCrl) | Intraperitoneal | *Cronobacter turicensis* | The kidney's bacterial load was reduced by 70% after receiving phage ($10^{11}$) PFU/mL without impacting the kidney's antioxidant status. | Bacterial load | None | Tothova et al. (59) Slovakia |
| | Mice (Kunming) | Intraperitoneal | *Salmonella enteritidis* | Single phage ($10^{10}$) PFU/mL treatment given an hour after a bacterial challenge prevented 40% of the mice from developing a fatal illness. | Mortality | None | Tang et al. (60) China |
| Bacteremia | Mice (BALB/c) | Intravenous | *S. enterica serovar* Paratyphi B | Phage administered two weeks after infection was utterly effective in sterilizing the animals; it also showed that phage-resistant bacteria could be excellent vaccines with reduced virulence. | Mortality and bacterial load | None | Capparelli et al. (51) Italy |
| | Mice (BALB/c) | Intraperitoneal | *Klebsiella pneumoniae* | The frequency of bacterial mutations was decreased by employing a phage cocktail comprising three lytic phages (GH-K1, GH-K2, and GH-K3) with various but overlapping host strains. Furthermore, phage cocktail treatment saved more animals than single phage treatment. | Mortality | None | Gu et al. (61) China |
| | Mice (ICR) | | *Pseudomonas aeruginosa* | Using various MOIs, a single phage was injected into immunocompetent and neutropenic mice (1, 10, and 100). All MOIs tested resulted in 80–100% rescue of normal mice. However, the phage did not offer protection to the infected neutropenic mice. | Mortality | None | Tiwari et al. (62) Republic of Korea |
| | Mice (ICR) | Intraperitoneal | *Staphylococcus aureus* | Mice exhibiting considerable phage replication throughout time, particularly in the liver and spleen, were administered intraperitoneal phage S13' six hours after infection. This treatment prevented the development of lung-derived septicaemia and lowered the severity of the infection. | Mortality | None | Takemura Uchiyama et al. (63) Japan |
| | Mice (C57BL/6) | Intraperitoneal vs. Oral (Intragastric) | *K. pneumoniae* | A single dosage of NK5 less than $2 \times 10^8$ PFU/mL administered intraperitoneally or intragastrically 30 min after *K. pneumoniae* infection prevented animals from dying in a dose-dependent manner. While i.p. injection produced better effects with late phage treatment (30 min), intragastric administration provided more robust protection with early phage administration (6-24 h). | Mortality | None | Hung et al. (64)Taiwan |
| | Rat (Sprague Dawley rat pups) | Intraperitoneal or Subcutaneous | *Escherichia coli* | Sepsis and meningitis models were used to assess the therapeutic efficacy of a single dosage of phage ($10^8$ PFU/mL) given 7 or 24 h after infection. In these animals, survival was 100 and 50%, respectively. | Mortality | None | Pouillot et al. (65) France |
| | Mice (BALB/c) | Intraperitoneal | *Enterococcus faecalis* | A single intraperitoneal phage injection ($4 \times 10^5$ PFU/) given an hour after the bacterial challenge was enough to prevent all mice from bacteraemia and cause faster bacterial clearance in the blood of protected mice than in unprotected mice. However, phage should be controlled and used appropriately to avoid imbalance in the gut microbiota. | Mortality | None | Cheng et al. (66) China |
| | Mice (BALB/c) | Intraperitoneal | *Acinetobacter baumannii* | The study assessed the safety, effectiveness, and delivery strategies of the Abp1 (phage) in treating local and systemic *A. baumannii* infections. When phage was administered to animals right after bacterial infection, they had a 100% survival rate. Abp1 effectiveness is equal to polymyxin B (10 mg/kg). | Mortality | None | Yin et al. (67), China |
| | Mice (BALB/c) | Intravenous | *E, coli* | When given within 60 min of bacterial infection, bacteriophage had a 100% curative effect and saved all affected mice's lives. | Mortality | None | Schneider et al. (68) Hungary |
| | Rat (Wistar) | Intramuscular | *P. aeruginosa* | In cases of endocarditis, a single dosage of phage therapy eliminated 7 log colony-forming units (CFU)/g of fibrin clots in 6 h. Phage-resistant mutants formed again after 24 h, which was prevented by administering ciprofloxacin with the phage. Single-dose phage therapy successfully treated 64% of rats *in vivo* and destroyed 2.5 log CFU/g vegetation in 6 h. | Mortality | None | Oechslin et al. (69) France |
| | Mice (BALB/c) | Intraperitoneal | *A. baumannii* | Endocarditis: A single dosage of phage therapy eliminated 7 log colony forming units (CFU)/g of fibrin clots in 6 h; mutant strains resistant to the phage proliferated once more after 24 h but were eradicated by administering ciprofloxacin in combination. Single-dose phage therapy successfully treated 64% of rats *in vivo* and destroyed 2.5 log CFU/g vegetation in 6 h. | Mortality | None | Patel Shesh R et al. (70) India |
| | Mice (BALB/c) | Intraperitoneal | *K. pneumoniae* | A single dose of the phage cocktail with $10^5$ PFU/mouse protected the mice from fatal consequences at any stage of septicaemia. However, a higher phage dose of $10^{12}$ PFU/mouse was lethal in the early hours of septicaemia, while this high dose is non-fatal in the later stages. | Mortality | None | Singh et al. (71) India |
| Gastrointestinal tract Infections | Mice (BALB/c) | Oral | *E. coli* | In a dose-dependent way, mixing the cocktail with water to consume for 24 h significantly decreased ileal *E. coli* concentrations and only slightly decreased fecal *E. coli* concentrations. | Bacterial load | None | Maura et al. (72) France |

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TABLE 3 (Continued)

| Infection Syndrome | Animal (Species/Strain) | Route of administration | Target bacteria | Clinical outcome | Outcome assessed | Adverse events | Article (references) and Country |
|---|---|---|---|---|---|---|---|
| | Rabbit (Outbred New Zealand White rabbits) | Oral | *Vibrio cholerae* | In contrast to the treatment given before a bacterial challenge, administering the phage cocktail orally after one considerably reduced the number of bacteria excreted. | Bacterial load | None | Jaiswal et al. (73) India |
| | Mouse (Swiss Albino) | Oral | *Vibrio cholerae* | Mice treated with a phage cocktail reduced the number of colonies per gram by 3 logs. However, mice treated with ciprofloxacin reduced viable counts to 5 logs/g of tissue homogenates. While the oral rehydration solution failed to reduce the number of viable bacteria, disease progression was much slower. | Mortality and Bacterial load | None | Jaiswal et al. (74)India |
| | Mice (BALB/c) | Oral and Intraperitoneal | *V. parahaemolyticus* | Mice that received phage treatment an hour after receiving i.p. bacteria (MOI of 0.1, 1, and 10) were shielded against illness and mortality. | Mortality | None | Jun et al. (75) South Korea |
| | Hamster (Syrian golden) | Oral | *Clostridium difficile* | Compared to untreated animals, phage treatment delayed the onset of symptoms by 33 h and reduced *C. difficile* colonization 36 h after infection. | Mortality | None | Nale et al. (76) United Kingdom |
| | Mice (BALB/c) | Drinking water | *E. coli* | The levels of *E. coli* were dramatically reduced throughout the gut after a single dosage of a combination of the three bacteriophages, with far less disruption of the microbiota diversity than antibiotics. | Bacterial load | None | Galtier al (77). France |
| | Mice (BALB/c) | Oral | *Salmonella enteritidis* | After ten days of treatment, oral administration of phage ($10^9$ PFU/mL) protected mice from salmonellosis and prevented weight loss. | Mortality and Bacterial load | None | Nikkhahi et al. (78) Iran |
| | Mice (BALB/c) | Oral | *E. coli* | Over the ten-day study, mice were protected from enteropathogenic *E. coli* by a single dose of phage ($2 \times 10^9$ PFU/mL). | Mortality | None | Vahedi et al. (79) Iran |
| | Mice (Swiss albino) | Intraperitoneal and Oral (Drinking water) | *S. typhimurium* | In Swiss albino mice, *S. typhimurium* acute infection and chronic carrier were established. However, when mice received phage ($10^5$ PFU/mL) intraperitoneally at 24-h intervals, the severity of the acute disease was lessened, and they were back to normal after nine days. While a high count ($10^{12}$ PFU/mL) phage cocktail given orally within seven days of feeding completely cured the carrier condition. | Bacterial load | None | Yadav et al. (80) India |
| | Mice (BALB/c) | Oral or enteral | *Salmonella enterica* | Animals infected with *S. enteritidis* were treated with a single dose of phage SE20 ($2 \times 10^8$ PFU/mL), which caused the animals to develop hepatomegaly and splenomegaly but not gastrointestinal problems. | Bacterial load | None | Dallal et al. (81) Iran |
| | Mice (ICR) | Intraperitoneal | *V. vulnificus* | All phage-treated mice (MOI = 10) died within 48 h, while the survival rate of phage-treated mice (MOI = 100 and 1,000) was 50 and 70%, respectively, after seven days. Nevertheless, untreated mice died in 12 h. | Mortality and bacterial load | None | Kim et al. (82), South Korea |
| Respiratory tract infections (Upper respiratory tract infections) | Mice (BALB/c) | Intranasal | *S. aureus* | By day seven after treatment, a single phage (MOI 1 or 10) had completely eradicated *S. aureus* from the nares compared to the control group. Mupirocin treatment yielded a comparable result, whereas phage treated with mupirocin exhibited 100% clearance by day 5. | Bacterial load | None | Chhibber et al. (83) India |
| | Sheep (Marine-cross) | Intranasal | *S. aureus* | It was discovered that a phage cocktail ($10^6$ PFU/mL) was used for flushing frontal sinusitis once daily for three days in decolonized nostrils and was safe for short-term use. Treatment with EDTA has a comparable result. Phage and EDTA, however, worked better together. | Bacterial load | None | Drilling et al. (84) Australia |
| | Sheep | Intranasal (Extension canula) | *S. aureus* | Application of phage to the frontal sinuses of sheep was safe for 20 days; the paranasal sinus mucosa did not experience any inflammatory infiltration or tissue damage. | Bacterial load | None | Drilling et al. (85) Australia |
| | Sheep (Merine cross wethers) | Intranasal | *P. aeruginosa* | In sheep frontal sinuses, a 7-day-old biofilm was dramatically decreased by a phage cocktail ($10^8$ to $10^{10}$ PFU/mL) applied twice daily with no safety issues noted. | Bacterial load | None | Fong et al. (2019) (86) Australia |
| Lower respiratory tract infections | Mice (BALB/c) | Intranasal | *P. aeruginosa* | A single dose of phage ($10^8$ PFU/mL) administered to immunocompetent mice 2 h after the onset of infection (curative treatment) enabled a survival rate of over 95%. On the other hand, a 4-day preventive medication (a single dose) led to a 100% survival rate. | Mortality | None | Morello et al. (87) France |
| | Mice (BALB/c) | Intranasal | *P. aeruginosa* | Phage cocktail reduced biofilms in cystic fibrosis bronchial epithelial CFBE41o cells. Tests on infected mice with a lux-tagged strain showed a 4-log reduction in the lungs after 6 h of treatment. | Mortality | None | Alemayehu et al. (88) Ireland |
| | Mice (BALB/c) | Intranasal vs. Intraperitoneal | *Burkholderia cenocepacia* | The analysis of several phage delivery techniques. Compared to mice receiving intraperitoneal injections of phage therapy, those receiving aerosolized phage therapy significantly reduced bacterial load. | Bacterial load | None | Semler et al. (27) Canada |

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TABLE 3 (Continued)

| Infection Syndrome | Animal (Species/Strain) | Route of administration | Target bacteria | Clinical outcome | Outcome assessed | Adverse events | Article (references) and Country |
|---|---|---|---|---|---|---|---|
| | Mice (Swiss-webster) | Intranasal | *K. pneumoniae* | Mice were protected from lethal pneumonia by an intranasal injection of $2\times 10^9$ PFU/mouse two hours after *K. pneumoniae* inoculation. The phage-treated mice had a lower lung bacterial burden than the untreated control group. In addition, reduced weight loss and inflammatory cytokines in their lungs were observed. | Mortality | None | Cao, Fang et al. (89) China |
| | Mice (BALB/c) | Intraperitoneal | *K. pneumoniae* | Even when therapy was initiated three days after the development of pneumonia, liposome-encapsulated phage (LP) was effective in curing infection. On the other hand, non-liposomal phage offered protection when given 24 h after infection. | Mortality | None | Singla et al. (90) India |
| | Mice (BALB/c) | Intravenous | *S. aureus* | Similar survival rates were seen in mice treated after 72 h of infection with a single dosage of clindamycin (8 mg/kg/body weight), $10^8$ PFU/mL phage, or combination therapy (clindamycin plus phage). However, the mice in the phage control group were more active than those in the clindamycin, phage, and combination therapy groups. | Mortality and bacterial load | None | Oduor et al. (91) Kenya |
| | Mice (BALB/c) | Intranasal | *P. aeruginosa* | Phage administered at an MOI of 10 could eliminate the lungs of infection 24–48 h after infection. Phage exposed to UV light had no protective effect, however. | Mortality and bacterial load | None | Waters et al. (92) United Kingdom |
| | Sheep | Intranasal (Extension canula) | *S. aureus* | NOVO12 (a two-phage cocktail) was applied topically to the frontal sinus for 20 days, and this treatment was considered safe because it did not cause any tissue damage or inflammatory reactions. | Mortality | None | Drilling et al. (93) Australia |
| | Mice (Swiss mouse) | Endotracheal route | *P. aeruginosa* | The bacterial load in the lungs was decreased by 5.3 logs compared to the untreated group when phage dry powder formulation (2 mg/mice) at a dosage of $2\times 10^7$ PFU/mg was applied two hours after a bacterial challenge. | Bacterial load | None | Chang et al. (94) Australia |
| | Mice (C57BL/6) | Intraperitoneal | *P. aeruginosa* | When administered 4 h after infection, single phage treatments ($10^9$ PFU/mL) significantly reduced lung infection in mice (>4 logs) and shielded them (>65%) from a lethal disease. At the same time, animals in the untreated groups all died by day three post-infection. | Mortality | None | Jeon et al. (95) Republic of Korea |
| | Mice (BALB/c) | Intravenous | *E. coli* | Antibiotics and bacteriophages had comparable outcomes, but bacteriophages were more effective at reducing bacterial burden. Additionally, bacteriophage therapy did not promote excessive inflammation but tended to reduce it and corrected blood cell count irregularities more quickly than antibiotics. | Mortality and Bacterial load | None | Dufour et al. (96) France |
| | Mice (BALB/c) | Intraperitoneal | *K. pneumoniae* | After a 10-min bacterial challenge, the mice were administered phage ($1.75\times 10^8$ PFU/animals), which ensured 100% survival and no signs of infection. This prevented all of the mice from death. This therapeutic effect was not observed when the phage suspension was administered an hour after the bacterial challenge. In this instance, the survival rate was still 100% after 24 h, but the mice's general health significantly declined, and 48 h after infection, their survival rate decreased to 12.5% (1/8 mice). No rescue effect was observed when a phage was given three hours after a bacterial challenge. | Mortality and bacterial load | None | Horváth et al. (97), Hungary |
| | Mice (BALB/c) | Intraperitoneal and oral | *K. pneumoniae* | The phage Kp_Pokalde_002 was administered intraperitoneally (IP) and orally to the infected mice at an MOI of 1.0 ($\sim 1\times 10^7$ PFU/mouse). Phage treatment was used to treat sick mice by oral and intravenous methods. As a result, the bacterial burden in the blood and lungs decreased significantly (3-7 log10 CFU/mL) in the treatment group. | Mortality and bacterial load | None | Dhungana et al. (98) Nepal |
| | Rats (Wistar) | Respiratory and intravenous | *S. aureus* | The animals received aerophages, intravenous (IV) phages, IV linezolid, IV and aerophages, and a combination of IV linezolid and aerophages. Aerophage therapy considerably increased survival (by 50%). In addition, Aerophages and IV phage therapy significantly improved survival compared to either treatment alone, even though IV phage therapy alone only led to a 50% survival rate. Finally, aerophages were added, but no synergistic impact was seen. | Mortality and bacterial load | None | Prazek et al. (99), Switzerland |
| | Mice (Wild-type C57BL/6J) | Intraperitoneal | *K. pneumoniae* | Phages were injected intraperitoneally (IP) at various times (1, 8 and 24 h after bacterial infection) and at various MOIs (an MOI of 1 or 10). The treatment duration, not the phage dose, significantly impacted survival. A control group of mice that received saline treatment developed a severe illness quickly in all of them. However, mice treated with viable phages survived those effectively receiving saline. | Mortality and bacterial load | None | Hesse et al. (100), United States |
| Skin and soft tissue infections | Mice (BALB/c) | Intraperitoneal | *K. pneumoniae* | When administered immediately after a bacterial challenge, the Kpn5 phage efficiently treated mice with a burn wound infection and could save about 26.66% of the mice even after 18 h of bacterial challenge. In addition, pro-inflammatory cytokines (IL-1beta and TNF-alpha) and anti-inflammatory cytokines (IL-10) were also found in much-reduced concentrations in the serum and lungs of mice treated with phage. | Mortality | None | Kumari et al. (101) India |

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TABLE 3 (Continued)

| Infection Syndrome | Animal (Species/Strain) | Route of administration | Target bacteria | Clinical outcome | Outcome assessed | Adverse events | Article (references) and Country |
|---|---|---|---|---|---|---|---|
| | Mice (BALB/c) | Topical | *K. pneumoniae* | Phage Kpn5 mixed with hydrogel applied topically at an MOI of 200 on the burn wound site could prevent infection in mice compared to multiple silver nitrate and gentamicin treatments. | Mortality | None | Kumari et al. (102) India |
| | Pig (Yorkshire) | Topical | *S. aureus, P. aeruginosa and A. baumannii* | The pig wound infection model used a phage cocktail of six different phages. Results varied depending on the species, but mechanical debridement generally had a positive effect. Compared to the pig model, the rodent model demonstrated more significant improvement. | Mortality | None | Mendes et al. (103) Portugal |
| | Mice (BALB/c) | Topical | *S. aureus* | The efficacy of the phage MR-10, which was locally applied at a dose of $10^8$ PFU/mL (MOI-100), was equivalent to that of the antibiotic linezolid. However, combination therapy using bacteriophage and linezolid was more successful in preventing the infection process than antibiotics or phage used alone. | Bacterial load | None | Chhibber et al. (104) India |
| | Mice (BALB/c) | Sub cutaneous | *Mycobacterium ulcerans* | 33 days after the bacterial challenge, a single subcutaneous injection of mycobacteriophage D29 reduced pathology and prevented the development of ulcers. This protection significantly reduced the number of *M. ulcerans*, increased levels of cytokines (including IFN-), and generated a lymphocytic/macrophage-profiled cellular infiltration. | Bacterial load | None | Trigo et al. (105) Portugal |
| | Mice (BALB/c) | Intraperitoneal | *E. coli* | Bacteriophage (T4) was administered intraperitoneally after being modified by phage display to contain anticancer Tyr-Ile-Gly-Ser-Arg (YIGSR) peptides. In mice receiving YIGSR-displaying phage treatment, tumor growth was slowed. In addition, mice receiving phage treatment had considerably lower wound severities, bacterial loads, and inflammatory markers. | Bacterial load | None | Dabrowska et al. (106) Poland |
| | Rats (Wistar) | Topical | *A. baumannii* | Comparing the phage-exposed group to the antibiotic-treated uncontrolled diabetic rats and the control group, a substantial decrease in infection, epithelialization time, and wound contraction was seen. Furthermore, the rats, including the phage controls, showed no signs of generalized sepsis and seemed remarkably healthy. | Bacterial load | None | Shivaswamy et al. (107) India |
| | Mice (Swiss albino) | Injection | *P. aeruginosa* | The number of bacterial cells was significantly reduced. The number of phages was significantly increased when an 11-phage cocktail was added to subcutaneous bags containing two catheter sections loaded with biofilm. | Bacterial load | None | Basu et al. (108) India |
| | Mice (BALB/c) | Subcutaneous and topical | *A. baumannii* | Treatment with the phage Abp1 ($5.0 \times 10^8$ PFU/mL) had significant therapeutic effects in mice models with local and systemic *A. baumannii* infection. Compared to mice who received either systemically administered phage or no treatment, mice that received locally applied phage had much-reduced wound sizes. | Mortality | None | Yin et al. (67) China |
| | Rat (Wistar) | Intramuscular | *S. aureus* | Rats treated with the transfersome-entrapped phage mixture recovered from the experimental thigh infections in 7 days compared to the 20 days required for untreated animals. Even when given 12 h after infection, the transfersome-entrapped phage cocktail protected all test animals (without fatalities). | Mortality | None | Chhibber et al. (31) India |
| | Mice (BALB/c) | Topical | *K. pneumoniae* | A phage cocktail comprising five and one individual phage was compared for their effects. Despite the successful results of single-phage therapy, the phage cocktail demonstrated superior outcomes and significantly delayed the emergence of resistant mutants. | Bacterial load and wound size | None | Chadha et al. (109) India |
| | Mice (BALB/c) | Intraperitoneal | *K. pneumoniae* | The efficacy of a liposome-encapsulated phage cocktail against a free phage cocktail in treating a mouse burn wound infection was compared. The animals treated with a liposomal entrapped phage cocktail had a significantly lower bacterial load in the blood and major organs. | Mortality and Bacterial load | None | Chadha et al. (110) India |
| | Mice (BALB/c) | Topical | *S. aureus* | A single injection of a 10 MOI bacteriophage proved very successful in treating diabetic mice. Surprisingly, linezolid was ineffective in diabetic mouse models. Concomitant therapy (phages + linezolid) did not show any antibacterial synergy. A single injection of the phage had the same impact on non-diabetic control animals as linezolid had on non-diabetic infected animals. | Bacterial load | None | Albac et al. (111) France |
| | Mice (Swiss) | Topical or superficial | *S. aureus* | Phage therapy enhanced clinical recovery and decreased local bacterial load 7 and 14 days after infection. Unlike antibiotics, phage therapy did not cause the gut microbiota of the treated animals to become less diverse. The mouse microbiota's alpha and beta diversity was reduced by amoxicillin. Additionally, while phage treatment did not affect the microbiota, it disrupted architecture even seven days after treatment ended. | Mortality and bacterial load | None | Huon et al. (112), France |

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TABLE 3 (Continued)

| Infection Syndrome | Animal (Species/Strain) | Route of administration | Target bacteria | Clinical outcome | Outcome assessed | Adverse events | Article (references) and Country |
|---|---|---|---|---|---|---|---|
| | Mice (BALB/c) | Subcutaneous | *S. aureus* | Linezolid and subcutaneous phage injections were administered after the mouse air pouch model was established. The phage MR-5 dramatically decreased bacterial load (extracellular and intracellular), both alone and in combination with linezolid (showing synergy), accelerating the clearance of pouch infection. | Mortality and bacterial load | None | Kaur et al. (113) India |
| | Mice (BALB/c) | Topical | *S. aureus* | A single phage (a combination of three phages, J-Sa36, Sa83, and Sa87) reduced the bacterial burden with an efficacy comparable to or greater than vancomycin treatment. On the other hand, wounds from mice given saline treatment did not heal and grew larger, more infected, ulcerated, and suppurative. | Bacterial load | None | Kifelew et al. (114), Australia |
| Eye and ear infections | Dog | Topical | *P. aeruginosa* | A single phage cocktail ($10^5$ PFU/ear) injected directly into the auditory canal after 48 h significantly decreased clinical scores without exhibiting any evidence of inflammation or additional adverse effects. | Bacterial load | None | Hawkins et al. (115), United Kingdom |
| | Mice (C57BL/6) | Topical | *P. aeruginosa* | Phage KPP12 eye drops administered as a single dose significantly accelerated the healing process while maintaining the structural stability and transparency of the infected cornea. The treatment with KPP12 also reduced neutrophil infiltration and significantly enhanced bacterial clearance in the infected cornea. | Bacterial load | None | Fukuda et al. (116) Japan |
| | Mice (C57BL/6) | Topical | *P. aeruginosa* | In the animal model for keratitis, a cocktail of two phages reduced bacteria. The keratitis caused by *P. aeruginosa* could be entirely prevented by the phages. Additionally, phages may prevent equine keratitis better than the current preventative use of antibiotics. | Bacterial load | None | Furusawa et al. (117) Japan |
| Other infections | Rat (Wistar) | Intramuscular | *E. faecalis* | When used at 37°C, phage survival was not impeded by formulation in poloxamer P407 media. This continued to be evident for a month. After formulation with poloxamer P407, the phage cocktail showed antibacterial activity and eradicated planktonic *E. faecalis* after 1, 2, 8, 14, 21, and 28 days. | Bacterial load | None | Schlezinger et al. (118) Israel |
| | Mice (SD mice) | Intraperitoneal | *A. baumannii* | Phage ($5.0 \times 10^8$ PFU) in PBS was administered intraperitoneally right after infection. Phages were reinjected after 12 h. In the control group, on the first day after infection, the infected mouse began to die, and within a week, all 12 died. In the phage treatment group, the sick mouse died three days later, and eight mice were still alive one week later. | Mortality | None | Jiang et al. (119) China |
| | *Galleria mellonella* | Haemolymph | *A. baumannii* | This study showed that combining the phage (MOI = 1) and meropenem, which increased larval survival from 35 to 77%, resulted in the greatest prolongation of *G. mellonella* larval survival. | Mortality | None | Grygorcewicz et al. (120), Poland |





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Cano et al. (132) reported intravenous injections of a single phage (KpJH462) to a 62-year-old diabetic patient with prosthetic infection due to *K. pneumoniae* complex, resolving local symptoms and signs of infection and restoring function. In addition, a trend toward reduction in biofilm biomass was observed 22 h after exposure to KpJH462.

A case study of a 72-year-old man with a chronic methicillin-resistant *S. aureus* prosthesis infection was published in 1971 by Doub et al. (133) Bacteriophage therapy was stopped following the third intravenous dose due to a rare, reversible transaminitis. Despite this, the patient's severe chronic infection was successfully treated and eradicated.

A persistent methicillin-sensitive *S. aureus* (MSSA) prosthetic knee joint infection in a 61-year-old woman was successfully treated after a second cycle of bacteriophage therapy given during a two-stage replacement procedure, according to Ramirez-Sanchez et al. (134) The study also highlighted the success of bacteriophage therapy with a single lytic phage, the safety and effectiveness of intravenous and intra-articular infusions, and the development of serum neutralization with continued treatment.

The study by Onsea et al. (135) reported the successful use of bacteriophage therapy for patients with severe musculoskeletal infections. There was no recurrence of infection with the causal strains following a single phage therapy with concurrent antibiotics, with follow-up periods ranging from 8 to 16 months. In addition, the phage application protocol has not been associated with harmful side effects.

In a case report, Chan et al. (136) described the therapeutic application of phage OMKO1 to a chronic *P. aeruginosa* infection of an aortic Dacron graft with a related aortocutaneous fistula. Phage OMKO1 and ceftazidime were administered only once, and the infection seemed to clear up with no signs of recurrence.

## Urinary tract infections

Ujmajuridze et al. (137) conducted a prospective cohort study to examine the efficacy of prophylactic bladder instillation in patients undergoing transurethral prostate resection. Nine patients got postoperative phage therapy by bladder instillation after a preoperative examination of asymptomatic individuals for the presence of uropathogens in midstream urine. No adverse events were reported. In six cases, the bacterial burden was also decreased. The outcomes of this two-phase, prospective trial indicated that tailored bacteriophage therapy for treating UTI might be efficient and secure.

A Dutch case study by Kuipers et al. (138) showed how bacteriophage was utilized successfully to treat chronic recurring urinary tract infections in a kidney transplant recipient caused by extended-spectrum lactamase (ESBL)-positive *K. pneumoniae*.

The study by Corbellino et al. (139) on long-term, multisite colonization by an MDR *K. pneumoniae* strain in a patient with a single kidney, cutaneous ureterostomy, and permanent ureteral stent resolved after 3 weeks of treatment with personalized BT given both orally and intrarectally.

In refractory *P. aeruginosa* urinary tract infection accompanied by bilateral ureteric stents and bladder ulcers, Khawaldeh et al. (140) demonstrated the benefit of adjuvant bacteriophage therapy after recurrent failure of antibiotics alone. The dynamics of bacteria and bacteriophages in urine point to a self-perpetuating and self-limiting infection, and no bacteriophage-resistant bacteria formed.

In a clinical trial by Quin et al. (141), phage therapy was utilized to treat a man with multiple urinary tract infections caused by MDR *K. pneumoniae*. However, three phage therapies were unsuccessful due to polyclonal co-infectious cells in his renal pelvis and bladder. Therefore, a percutaneous nephrostomy was done on the patient (PCN) following analysis. In addition to receiving antibiotic therapy, a mixture of bacteriophages chosen for their ability to attack all 21 diverse isolates were simultaneously irrigated via the kidney and bladder. The patient eventually made a full recovery with an improved bladder.

Other studies investigating the use of bacteriophage therapy in individuals with persistent urinary tract infections have shown its efficacy in overall clinical improvement (symptom alleviation and prevention of UTI recurrence) and bacterial eradication (142–144).

## Skin and soft tissue infections

Equal healing rates were observed in the control and test groups of Rhodes D. et al.'s phase I clinical trial on bacteriophage therapy for treating human venous leg ulcers. In the study, 39 patients had their chronic blood vessel leg ulcers treated for 12 weeks with either a bacteriophage product or a placebo. No adverse events or safety issues were recorded (145).

The PhagoBurn Section 1/2 study by Jault P. et al. (5) was another clinical trial that compared standard care (Sulfa-drug silver emulsion cream) with a cocktail of twelve anti-*P. aeruginosa* lytic phages applied via an alginate dressing for a seven-day treatment of burn wound infection in 25 patients. Unfortunately, bacteriophage therapy has shown to be ineffective due to several factors, including variations in the maximum bacterial load between treatment groups and a significantly lower-than-expected bacteriophage dosage due to a concentration decrease during manufacturing and the fall in titres (during storage) were not checked before using them in the clinical trial.

A study by Patel et al. (146) using a customized bacteriophage preparation in managing chronic nonhealing wounds reported 100% microbiological eradication after 3 months with a healing rate of 81.2%. Additionally, the study suggested that specific phage therapy is equally effective whether a patient has diabetes or not, despite healing being slower in the former cluster. Another study by Gupta P et al. (147) examined the impact of topical bacteriophage therapy on chronic nonhealing wounds infected with *E. coli*, *S. aureus*, and *P. aeruginosa*. They found that after 3 to 5 doses, there was a significant improvement in wound healing with no signs of infection, both clinically and microbiologically.

Both studies offered nearly unambiguous proof that topical phage therapy helped patients who had not responded to conventional therapy completely heal their wounds. Furthermore, the isolates from the research population's chronic wounds showed significant resistance to the most widely used antibiotics. Neither study's participants experienced adverse effects, tissue breakdown, or recurrent infections during or after therapy.

In order to determine the impact of bacteriophage on the healing process, Bhartiya et al. (148) conducted a non-randomized, prospective, open-label, blinded, case–control study on infected acute traumatic wounds. The results of this investigation are promising. In patients receiving phage therapy, the average time needed to achieve sterility, complete wound granulation, and primarily intended healing was half that of the control group. The financial analysis also favored

bacteriophage therapy (BT), as only 1/3 of the costs were incurred in the BT group compared to the control group.

Marza et al. (126) reported clinical improvement and bacterial elimination after phage therapy in a patient with an infected burn wound. In contrast, Rose T. et al. (149) discovered that phage therapy had no therapeutic effects on burn wounds. Bacteriophage use, however, had no adverse effects.

## Ophthalmic infections

Infectious keratitis induced by Vancomycin-resistant *S. aureus* was described in the study by Fadlallah et al. (150) Phage treatment was given intravenously and topically in the form of eye drops and nasal spray. There were no concurrent antibiotics given. The infection was effectively treated, as evidenced by stabilizing ocular symptoms and observing negative cultures. The six-month follow-up period had showed no adverse effects and no reinfection.

## Intensive care for patients with sepsis

Many studies have not been done on patients who received phage therapy for severe sepsis. *S. aureus*, *P. aeruginosa*, *E. coli*, *K. pneumoniae*, *Proteus mirabilis*, *Morganella morganii*, and *Enterobacter* spp. were the pathogens that were frequently isolated. Phage treatment was given intravenously, intramuscularly, or both intramuscularly and locally. Of the 109 patients, 85 (78%) received antibiotics simultaneously. Adverse incidents were not reported. Follow-up lasted anywhere between 20 days and 4 months. As a result, 90 (83%) of the 109 patients had improved vital signs, and two wholly eradicated their bacteria. Unfortunately, phage therapy proved ineffective in 20 patients; three died despite the initial improvement, and five died shortly after the treatment began (151–153).

## Gastrointestinal infections

A 68-year-old diabetic patient with necrotizing pancreatitis was described in a case report by Schooley et al. (154) with an MDR *A. baumannii* infection. The patient's clinical course was reversed, the bacteria were cleared up, and they recovered after receiving these bacteriophages intravenously and through percutaneously into the abscess cavities.

## Cardiovascular infections

Three studies detail individuals with aortic graft and left ventricular assist system infections that were difficult to cure and were infected by *P. aeruginosa* or *S. aureus* (136, 155, 156). Two patients received local phage therapy, while one received intravenous phage therapy. Antibiotics were given concurrently to all patients. After receiving a local phage application, one patient reported nausea. There was a clinical improvement in all cases; one patient showed evidence of bacterial eradication. The follow-up period lasted 7-9 months.

## Respiratory infections

Several case studies have been published regarding phage therapy and patients with respiratory infections (157–166). Phage therapy was administered intravenously, orally, and intravenously using a nebulizer. Phage treatment was well tolerated in each case and had no adverse side effects. Furthermore, clinical improvement was observed in most cases, including general improvement and reduced sputum and coughing.

## Other infections

Nine patients with chronic rhinosinusitis caused by *S. aureus* were treated with intranasally given phage therapy in a prospective study by Ooi et al. (167). The treatment was examined for safety and preliminary efficacy. Phage therapy was well tolerated, and the side effects (such as diarrhea, epistaxis, signs of an upper respiratory infection, oropharyngeal discomfort, rhinitis, and a drop in serum bicarbonate) were modest. All nine patients saw improvements in their clinical conditions, and the bacterial load was reduced; however, only two (22%) of the nine patients had their bacteria wholly eradicated. There was a seven-day follow-up period (167).

Despite many positive case studies, there still needs to be robust scientific evidence from well-planned, monitored and regulated clinical trials supporting bacteriophage therapy. However, more recently, a rise in the number of bacteriophage therapy-related articles, books, and reviews and commercial bacteriophage companies focusing on a specific market suggests that the scientific communities and pharmaceutical companies are becoming more eager to integrate bacteriophage therapy into conventional medical practice (127).

Phage therapy may be helpful for bacterial illnesses that are challenging to cure. Additionally, this treatment is generally considered safe because it has a low incidence of side effects and is given via various administration routes. Even though phage therapy appears to be a promising strategy in the fight against untreatable infections and antimicrobial resistance, high-quality studies are desperately needed to advance our understanding of the long-term effects of this treatment. When taken orally and intravenously, getting more knowledge about the pharmacokinetics and pharmacodynamics of the bacteriophage (cocktails) is necessary. Additionally, endotoxin, bacterial, and viral contamination testing for bacteriophage solutions should be done (Table 4).

## Worldwide phage development organizations

Phage therapy has been used for many years in certain countries, primarily in Eastern Europe, although it has yet to gain widespread acceptance in Western nations. This is partly due to the need for more commercial phage production, making it difficult to standardize and regulate phage therapy products.

TABLE 4 Ongoing clinical trials of phage.

| Name of the study | Phase | Registry date | Clinical trial registry number | Trial result | Public data |
|---|---|---|---|---|---|
| A phase 1b/2 trial of the safety and microbiological activity of bacteriophage therapy in Cystic fibrosis subjects colonized with *Pseudomonas aeruginosa* | I/II | 12 July 2022 | NCT05453578 | – | Trial design (168) |
| Cystic Fibrosis bacteriophage study at Yale (CYPHY) | I/II | 24 December 2020 | NCT04684641 | – | Trial design (169) |
| A randomized, placebo-controlled, double-blind clinical trial of therapeutic bacteriophage preparation in chronic antibiotic-resistant wound infections at Banaras Hindu University, Varanasi, India. | I/II | 8 December 2021 | CTRI/2021/12/038527 | – | Trial design (170) |
| Phage Therapy for the treatment of urinary tract infection | I/II | 13 September 2022 | NCT05537519 | – | Trial design (171) |

However, several companies have recently been established to develop and commercialize phage-based products. These companies use various methods to produce phages, including isolating phages from environmental samples, genetically engineering phages, and producing phages using fermentation or other bioprocessing techniques. One of the main challenges in commercial phage production is ensuring consistent quality and purity. This is important to ensure that the phages are safe and effective for medical applications. In addition, companies use various methods to standardize and test their phage products, including use of bioassays to measure phage activity and testing for endotoxins and other contaminants. Several companies worldwide are developing and commercializing phage-based therapies for various bacterial infections. Here are some examples, summarized in Table 5, including the name of the company, its location, and the company website.

## FDA-approved bacteriophage-based products and regulatory challenges

Phage therapy treatments have only been carried out in Western medicine under the laws of the Helsinki Declaration, which was adopted by the 18th World Medical Association General Assembly (Helsinki, Finland, June 1964) as unproven interventions in clinical practice or out of compassion and with the patient's informed consent (175). A new legal framework has just been installed in Belgium that permits phages to be used as an active pharmaceutical ingredient in a magistral preparation as long as specific logical requirements are met. A medicinal preparation is described as "any medicinal product prepared in a pharmacy following a medical prescription for an individual patient," in line with Article 3 of Directive 2001/83 of the European Parliament and Article 6 quarter 3 of the Belgian medicinal law of 25 March 1964 (176, 177). The preparation must be explicitly made by a pharmacist from the various components per current pharmaceutical standards and under a Medical Doctor's (MD) prescription for a selected patient (177). In 2006, the United States Food and Drug Administration (FDA) approved using a phage as an antibacterial (preventive) agent in "the ready-to-eat food," The process of developing and marketing phage therapy medical products (PTMPs) can theoretically be completed while adhering to all regulations, but it is exceedingly expensive and time-consuming. Furthermore, given the ongoing evolutionary dynamics between bacteria and phages, it is possible that the phage products that are eventually launched on the market are already obsolete or will do so shortly due to the lengthy development times concerning the divergent evolution of the targeted bacterial populations (178).

The current approach for producing and marketing pharmaceutical products was primarily designed for static chemical drugs like antibiotics and typically includes the following elements: Producing using good manufacturing practices (GMP), Preclinical research, including *in vitro* and animal pharmacokinetic, pharmacodynamic and toxicological investigations\Phase I to IV clinical trials and centralized marketing authorization (177).

The PhagoBurn study showed that producing PTMPs under the established pathway is very expensive, time-consuming, and only sometimes leads to high-quality and effective products. The licensed GMP research product's titer was reduced drastically (1000-fold) 15 days after manufacturing, and manufacturing and licensing consumed the majority of the time and money allotted to the study (5). Phage therapy is a form of "experimental treatment" legal in Poland. The modified Act of 5 December 1996 on the Medical Profession, issued in the Polish Law Gazette, 2011, No. 277 item 1,634, and Article 37 of the Declaration of Helsinki, serve as the foundation for this framework. Phage treatment is practically feasible in Poland under specific circumstances, such as informed consent, a doctor's application, and a bioethics panel's approval, but only without any other potentially viable and established treatment alternative (177). Under the 'compassionate use' regulation, phage therapy may occasionally be used in various nations. Australia and France are examples (140, 179). Despite the European Medicines Agency (EMA)'s advice, each nation appears to employ a unique approach to this treatment.

The U.S. Food and Drug Administration (FDA) has been attempting to create a legal framework for employing bacteriophages as a therapeutic modality. In the United States, phage therapy has been considered an investigational new drug (IND) and regulated under the FDA's IND program. This program allows for the clinical testing of new drugs and biologics in humans before they are approved for use in the general population. Recently, the FDA has taken steps to establish a more formal regulatory framework for phage therapy. In 2019, the agency released a draft guidance document on developing bacteriophage products for treating bacterial infections. The guidance document provides recommendations for developing and submitting data to support the safety and efficacy of phage products (180).

The FDA's draft guidance recommends that phage products be evaluated in well-controlled clinical trials designed to demonstrate safety and efficacy. The guidance also recommends manufacturing phage products using appropriate quality control measures to ensure

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TABLE 5 Global distribution of companies exploiting phages for therapeutic purposes (7, 172–174).

| Company | Locations | Web site address |
|---|---|---|
| Adaptive Phage Therapeutics | Maryland, United States | https://aphage.com/ |
| AmpliPhi Bioscience Corporation | Virginia, United States | http://www.ampliphibio.com |
| Armata Pharmaceuticals, Inc | California, United States | https://www.armatapharma.com/ |
| Aziya Immuno-preparat | Tashkent, Uzbekistan | https://aziyaimmunopreparat.uz/ |
| BigDNA | Edinburgh, United Kingdom | http://www.bigdna.com/ |
| Biopharm Ltd. | Tbilisi, Georgia | https://biopharm-ge.com/ |
| Biophage Pharma Inc. | Montreal, Canada | http://www.biophage.com/ |
| BiomX | United States | https://www.biomx.com/ |
| CJ CheilJedang Corporation | Seoul, South Korea | https://www.cj.co.kr/ |
| Elanco Food Solutions | Illinois, United States | https://www.elanco.com/ |
| EBI Food safety | Wageningen, the Netherlands | https://www.ebifoodsafety.com/ |
| Ellis Day Skin Sciences | California, United States | https://www.ellisdayskinscience.com/ |
| Eliava Bio Preparations Ltd. | Tbilisi, Georgia | https://pha.ge/ |
| Exponential Biotherapies, Inc. | Virginia, United States | http://www.expobio.com/ |
| Gangagen Biotechnologies Pvt. Ltd. | Bangalore, India | https://gangagen.com/ |
| Gangagen Inc. | California, United States | https://gangagen.com |
| Hexal Genentech | Holzkirchen, Germany | http://www.hexal.de/ |
| Innophage | Porto, Portugal | http://www.innophage.com/ |
| Intralytix | Maryland, United States | http://www.intralytix.com |
| Jafral Ltd. | Ljubljana, Slovenia | https://jafral.com/ |
| JSC Biochimpharm | Tbilisi, Georgia | https://biochimpharm.ge/ |
| Locus Biosciences | North Carolina, United States | https://www.locus-bio.com/ |
| MB Pharma | Prague, Czech Republic | https://www.mbph.cz/?lang=en |
| MicroMir | Moscow, Russia | https://micromir.bio/eng |
| Microgen | Moscow, Russia | https://www.microgen.ru/en/ |
| Novolytics | Coventry, United Kingdom | http://www.novolytics.co.uk/ |
| New Horizons Diagnostics | Maryland, United States | http://www.nhdiag.com/index.htm |
| OmniLytics Inc. | Utah, United States | https://www.omnilytics.com/ |
| Phage International, Inc. | California, United States | https://www.phageinternational.com/ |
| Phage Biotech Ltd. | Rehovot, Israel | http://www.phage-biotech.com/ |
| Phage Therapy Center | Tbilisi, Georgia | https://www.phagetherapycenter.com/ |
| Phico Therapeutics | Cambridgeshire, United Kingdom | https://www.phicotx.co.uk/ |
| Pherecydes Pharma | Ile-de-France, France | https://www.pherecydes-pharma.com/en/ |
| Proclara Biosciences | Massachusetts, United States | http://www.proclarabio.com/ |
| Phagelux | Sandy, Utah, United States | http://www.phageluxagrihealth.com/en/ |
| Phagex | Kyiv, Ukraine | https://bacteriophages.info/en/ |
| Special Phage Services Pty, Ltd. | New South Wales, Australia | http://www.specialphageservices.com.au/ |
| SciPhage Biotechnology | Colombia, United States | https://sciphage.com/ |
| Targanta Therapeutics | Massachusetts, United States | http://www.targanta.com/ |
| Viridax™ Corporation | Florida, United States | http://www.dreamingrock.com/viridax/eviridax/ |

consistency and purity. It can be an essential step in regulating phage therapy. It provides a roadmap for companies developing phage products and clinicians interested in using phage therapy to treat bacterial infections. The guidance also provides a basis for discussion between the FDA and industry stakeholders on developing safe and effective phage products (181). Phage products have also been successfully produced globally in various applications, supporting their safety and efficacy after the approval of the FDA and other regulating bodies. Companies' names, product details, agency approval, and related applications of phages are compiled in Tables 6, 7.

TABLE 6 List of regulatory body-approved phage-based products for food-borne pathogens in food.

| Product and company | Regulatory approbation | Applications | References |
|---|---|---|---|
| ListShield, Intralytix, Inc. (United States) | US FDA (2006) for direct application onto foods | Salami, sausage, shellfish (Ready-to-eat food), and food contact surfaces and environments: | (182) |
| EcoShield, Intralytix, Inc. (United States) | FDA (2011) cleared as "Food Contact Notification". (Safe and suitable antimicrobial) | Red meat parts and trim intended to be ground | (183) |
| SalmoFresh, Intralytix, Inc. (United States) | FDA listed suitable in the production of organic foods | Poultry, fish, and shellfish, freshly processed fruits and vegetables | (184) |
| LISTEX Micreos EBI, Food Safety, (Netherlands) | FDA-Approved | Ready to eat meat, fish, and cheese | (185) |
| Agriphage, Omnilytics, United States | Environmental Protection Agency (EPA 2005) for use in agriculture | In agriculture, fruits, and vegetables | (186) |
| Compyshield™, Intralytix, Inc. (United States) | FDA Approved | Food additive for raw red meat | (187) |
| Shiga Shield™, Intralytix, Inc. (United States) | FDA Approved | *Shigella* removal from meat and vegetables | (188, 189) |
| EnkoPhagum, Brimorose Technology Corporation (United States) | FDA Approved | Removal of *Salmonella, Shigella, E. coli,* and *Staphylococcus* in meat products | (188) |
| SalmoPro™, Phagelux (China) | FDA Approved | As an antibacterial processing aid in food | (190) |
| Secure Shield E1, FINK TEC GmbH (Germany) | FDA Approved | Used in beef products, turkey, and other foods. | (191) |

The information and references provided by the companies mentioned above further explain and support the scientific evidence regarding the potential of phage therapy as an antibiotic substitute and the FDA's recommendations for creating bacteriophage therapy products.

## Limitations and future prospect of implementing phage therapy

While laying out a roadmap of modern phage therapy's difficulties, it becomes clear that our structured and organized environment brings many obstacles. The first is the uncertain nature of bacteriophages, which are not pure biological macromolecular complexes (like therapeutic proteins) but non-living creatures. As a result, it becomes difficult for regulatory agencies to approve medical therapies or therapeutic substances because legislative approval procedures are intricate, expensive, and time-consuming. Patients may also reject such therapies due to erroneous worries and a lack of knowledge because this approach uses "live viruses" for treatment (207).

Some additional problems attributed to early phage therapy failure and their probable solution and required approach are summarized below:

1. *Narrow host range of phages*: Due to the high specificity of phages, many negative results may have been acquired due to the inability to select lytic phages for the targeted bacterial species. Before utilizing phages for therapeutic purposes, ascertaining the etiologic agent's susceptibility to phages (208) and using polyvalent phage cocktails that lyse most etiologic agent strains will be a better approach for favorable results.
2. *Insufficient purity of phage preparation*: Early therapeutic phages were in crude lysates of the host bacteria and contained a variety of contaminants (including endotoxins) that may have neutralized the phage's effects. To create phage preparations with a high purity level, ion-exchange chromatography, high-speed centrifugation, and other advanced purification methods should be utilized (208).
3. *Poor stability and viability of phage preparations*: Commercial phage preparations were supplemented with mercurial, oxidizing agents or heat-treated to ensure bacterial sterility (50). Many of these treatments also may have inactivated the phages, resulting in ineffective phage preparations. Advanced purification techniques can purify phages and ensure they are bacterium-free. The viability and titer of phages should be determined before using them therapeutically.
4. *Lack of understanding of the heterogeneity and mode of action of phages (i.e., lytic vs. lysogenic)*: Some researchers may have used lysogenic phages instead of lytic phages due to their inability to distinguish between them. The temperate phages are not recommended for therapy because they do not "kill" the target bacteria and can cause the transfer of undesired genes. A careful choice should be made when looking for lytic phages. This is essential for preventing potential horizontal gene transfer by lysogenic phages of genes encoding bacterial toxins, antibiotic resistance, etc. (209).
5. *Exaggerated claims of the effectiveness of commercial phage preparation*: One illustration of this would be the Enterophagos, marketed as applicable against herpes infections, urticaria, and

TABLE 7 List of commercial phage products for humans.

| Product and company | Regulatory approval | Route of administration | Application | References |
|---|---|---|---|---|
| PhagUTI, Pherecydes, (France) | Phase I/II | Undefined | Treating *E. coli* Urinary tract infections (UTI) | (192) |
| EcoActive, Intralytix (United States) | FDA-approved IND, Phase 1/2a | Oral | Targeting adherent-invasive *E. coli* | (193) |
| AP-PA02; AP-PA03, Armata (United States) | FDA-approved IND, Phase 1b/2 | Inhalation | Treatment of *P. aeruginosa*-related respiratory tract infections, particularly in Cystic Fibrosis patients. | (158) |
| PGX0100, Phagelux (China) | FDA-approved IND, preclinical | Transdermal | Spray and gel for burn care | (194) |
| Staphylococcal bacteriophage, Microgen (Russia) | Russian Federation national standard certification | Inhalation | Treatment of *Staphylococcal* Intestinal Disorders and Suppurative Inflammation | (195) |
| AP-SA01; AP-SA02, Armata (United States) | FDA-approved IND, Phase 1b/2 | Intravenous | Diabetic foot ulcer treatment and management of resistant and refractory *S. aureus* bacteremia | (114, 158) |
| *Staphylococcal* Bacteriophage, Eliava Bio Preparation (Georgia) | Georgian Approval | Oral or intrarectal | Preventing and treating *Staphylococcal* infections and postoperative wound infections | (196) |
| BX003, BiomX (United States) | Phase I | Oral | The treatment aims at gut-residing *K. pneumoniae* bacterial strains in patients with primary sclerosing cholangitis (PSC) and Inflammatory bowel disease (IBD). | (197) |
| ShigActive™, Intralytix (United States) | FDA-approved IND,2021 | Oral | Prevention of human diseases caused by *Shigella* infection | (198) |
| *Streptococcal* bacteriophage, Microgen (Russia) | Russian Federation national standard certification | Oral, topical, and intrarectal | Treatment diseases caused by *Streptococcus* spp. | (195) |
| Phagyo®spray, Biochimpharm (Georgia) | Georgian Approval | Topical | Treatment and prophylaxis of bacterial purulent–inflammatory infections (multiple microorganisms) | (199) |
| Septaphage®table, Septaphage, Phagyo®, PhageStaph, Biochimpharm (Georgia) | Georgian Approval | Oral | | |
| Travelphag™, Biochimpharm (Georgia) | Georgian Approval | Oral | For bacterial infections, indigestion | |
| *Salmonella* groups A, B, C, D, bacteriophage, Microgen (Russia) | Russian Federation national standard certification | Oral, intrarectal | Treatment and preventative measures for *Salmonella*-related diseases | (195) |
| *E. coli-Proteus* bacteriophage, Microgen (Russia) | Russian Federation national standard certification | Oral, topical, and intrarectal | Treatment and prevention of enteric and inflammatory disorders that are purulent, dysbacteriosis caused by the bacteria *Proteus*, and enterotoxigenic *E. coli* | |
| *Klebsiella* purified polyvalent bacteriophage, Microgen (Russia) | Russian Federation national standard certification | Oral, topical, and intrarectal | specific lysis of *K. pneumoniae*, *K. odorifera*, and *K. rhinosclerosis* | |
| Dysentery polyvalent, bacteriophage, Microgen (Russia) | Russian Federation national standard certification | Oral and intrarectal | specific lysis of the bacterium that causes bacillary dysentery | |
| Complex Pyobacteriophage, Microgen (Russia) | Russian Federation national standard certification | Oral, topical, and intrarectal | Specific lysis of *E. coli*, *K. pneumoniae*, *Streptococcus*, *Enterococcus*, *Proteus*, and *K. pneumoniae*. | |
| Bacteriophage dysenteric polyvalent "MediPhag," Aziya Immunoprepara (Uzbekistan) | Marketed | Oral | A white gelatin capsule with capsules of *Shigella*-fighting bacteriophages that have been dried and frozen. | (200) |
| LYZODOL®, MB Pharma (Czech Republic) | Marketed | Oral | Against respiratory infections caused by *Propionibacterium acnes*, *Lelliottia amnigena*, *S. aureus*, and *K. pneumoniae* | (201) |
| Phagogyn, MicroMir (Russia) | Marketed | Topical | A gel containing 74 phages protects against reproductive system bacterial infections. | (202) |
| Phagodent, MicroMir (Russia) | Marketed | Topical | Contains 72 phage complexes to balance oral microbiota | |
| Phagoderm, MicroMir (Russia) | Marketed | Topical | A 64-phage skin gel that prevents bacterial skin infections. | |
| Otophagus, MicroMir (Russia) | Marketed | Topical | A gel comprising 69 phages protects the ear, nose, and throat against bacterial and suppurative inflammation. | |
| Iskraphage, MicroMir (Russia) | Marketed | Topical | Gel for hygiene and normalization of the skin microbiota. | |
| Pyofag®, Phagex (Ukraine) | Marketed | Oral and topical | Treatment of pathogenic agents in purulent inflammation and intestinal diseases caused by *P. aeruginosa*, *Proteus vulgaris*, *Proteus mirabilis*, *Streptococcus pyogenes*, *S. aureus*, and *E. coli*. | (172) |
| Intestifag® polyvalent bacteriophage, Phagex (Ukraine) | Marketed | Oral and topical | Fights *Shigella*, *Salmonella*, *E. coli*, *P. aeruginosa*, *Enterococcus faecalis*, and *S. aureus*-related intestinal diseases. | |
| BACTELIDE™, Phagelux (China) | FDA-approved IND, preclinical | Transdermal | Patches and sprays for pressure ulcers | (194) |
| PhageBank, Adaptive Phage Therapeutics (United States) | FDA-approved IND, Phase 1/2 | Intravenous | Treat diabetes-related foot osteomyelitis, prosthetic joint infections, chronic recurrent urinary tract infections, eye infections, and lung infections related to cystic fibrosis. | (203) |
| crPhage™, Locus Biosciences (Korea) | Phase 1b | Injection | Combined with CRISPR-Cas3 to increase the effectiveness of bactericidal treatment for various bacterial infections, including IBD and UTI. | (204) |
| AcneFree, SciPhage (Republic of Colombia) | Undefined | Transdermal | Fights acne-targeting bacteria | (205) |
| Balancing Phage Serum, Ellis Day Skin Science (United States) | Marketed | Transdermal | Restore the skin's microbiome to balance, and eliminate the bacteria that cause blemishes and acne. | (206) |
| Hydrating Phage Serum, Ellis Day Skin Science (United States)7 | | | | |

eczema (210), diseases that phages could not possibly be successful against. Phage preparations should be provided with detailed, scientifically backed information regarding their effectiveness against specific bacterial pathogens, potential adverse effects, etc.

6. *Failure to establish scientific proof of the efficacy of phage treatment*: Most clinical investigations using therapeutic phages lacked placebo controls (211, 212). Highly pure, lytic phages should be used in well-controlled, double-blinded placebo experiments, and outcomes need to be assessed using patient data and meticulous laboratory testing.
7. *Development of Phage-neutralizing antibodies*: Another issue that could impair the ability of phages to lyse certain bacteria *in vivo* is the emergence of phage-neutralizing antibodies. Indeed, parenteral phage delivery has been associated with the formation of neutralizing antibodies (213). Nevertheless, it is still being determined how substantial of a concern this might be during phage therapy, particularly when administered orally and/or topically. Since the kinetics of phage activity is significantly faster than the host's synthesis of neutralizing antibodies, the production of neutralizing antibodies should not, in theory, pose a substantial challenge during the initial treatment of acute infections (208). A study by Archana et al. (214) reported the appearance of neutralizing antibodies after the third week following immunization. Complete neutralization of bacteriophages was detectable between 3 and 5 weeks after immunization.
8. *Clearance of phages by reticuloendothelial systems*: According to the study by Merril et al. (215), the reticuloendothelial system's removal of phages from the patient could represent a problem because it could lower the number of phages to a level that is insufficient to combat the infecting bacteria. The authors chose phages with greater capacity to remain in mice's bloodstreams using a natural selection process they elegantly referred to as the "serial passage" method. Understanding the mechanisms underlying this characteristic of phages will illuminate critical aspects of how they interact with their bacterial hosts.
9. *The emergence of resistance*: Like antibiotics, bacteria can resist phages over time, leading to the need to develop new phages. Phage therapy's efficiency may also be constrained by the co-evolution of bacteria and phages, which can result in the emergence of bacterial strains that are resistant to phages (9, 55, 173, 216).
10. *Quality control*: Ensuring the quality and purity of phage preparations can be challenging, as it requires careful monitoring of phage production and purification processes (217, 218).
11. *Delivery methods*: The mode of administration can impact the effectiveness of phage therapy since enough phages must be delivered to the infection site for treatment to be effective. This can be challenging in some cases, such as treating deep tissue infections or infections in areas that are difficult to access (22, 24).

Many phages produce virulence factors or toxins, making them ineffective as antimicrobial agents. The precise elimination of potentially dangerous genetic information from viral genomes can tackle this crucial safety concern. The release of bacterial toxins, lipopolysaccharides, and other pathogen-associated molecular patterns (PAMPs) as a result of phage-induced lysis might trigger an innate immune response, increase virulence, or result in additional harm. To lessen these possibly detrimental effects, phage variants that are engineered to destroy target cells without releasing PAMP can be utilized. Phages can be designed to cleave defined nucleotide sequences and genotypes, for example, host cells that carry antibiotic-resistance genes or particular virulence factors, by delivering sequence-specific CRISPR-Cas nucleases via modified genomes or phagemids. Phagemids or modified genomes can be used to deliver sequence-specific CRISPR-Cas nucleases, which can then be programmed to cleave specific genotypes and nucleotide sequences (217).

Phage therapy has not yet been widely adopted in clinical practice, despite increased interest in phages and the collaborative efforts of scientists and clinicians that have led to increased case reports. The fundamental reason for this lack of implementation is the ongoing dearth of reliable clinical evidence on phage therapy, which prevents ethical, actuarial, and governmental authorities from addressing their concerns. To make phage therapy an integral part of clinical practice in the future, a focused and practical regulatory framework for personalized phage therapy techniques and interdisciplinary collaboration between researchers, microbiologists, clinicians, and pharmacists are essential.

## Conclusion

Phage therapy holds the promise of unveiling novel approaches to combat bacterial infections. Particularly noteworthy is the potential of phage cocktails, which could revolutionize the treatment landscape by offering solutions for a wide spectrum of bacterial diseases that have proven resistant even to the latest generations of antibiotics. Nevertheless, several pivotal challenges must be surmounted to harness the full potential of phages as antibacterial agents. These challenges encompass ensuring safety, evaluating effectiveness, and assessing the likelihood of immune responses triggered by administered phages. Additionally, refining phage purification techniques and optimizing their growth are essential hurdles that demand attention.

Within clinical settings, phage therapy has emerged as a beacon of promise, despite the obstacles. Ongoing technological advancements and strides in genetic engineering hold the potential to unlock the creation of more precise and potent phages. The synergistic use of phage therapy in conjunction with other therapeutic modalities, such as immunity modifiers or antibiotics, may further enhance its efficacy. Beyond the realm of human healthcare, phage therapy extends its reach into veterinary medicine, agriculture, and food safety, presenting multifaceted applications with vast potential.

In summation, phage therapy stands as a formidable contender in the battle against antibiotic-resistant bacteria, offering a compelling alternative or adjunct to traditional antibiotic treatments. Nevertheless, these prospects are not without their challenges. Continued research and development efforts are imperative to fully exploit the myriad advantages this promising technology offers and to surmount the remaining obstacles that lie in its path. The journey to harness the full potential of phage therapy is still underway, but the destination promises a brighter future in the fight against bacterial infections.

## Author contributions

SK, MG, and GN planned to compile phage data systematically. SK, RK, and MG collected and analyzed the data from various authentic resources. SK compiled the data and finalized the review article as per the proposed objectives from the database and the major contributor to writing the manuscript. SK, MG, SB, and GN finalized the draft, and the published data was presented in the review article. All authors have read and approved the final manuscript.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## References

1. O'Neill J. *Antimicrobial resistance. Antimicrobial resistance: tackling a crisis for the health and wealth of nations*. (2014).

2. De Kraker MEA, Stewardson AJ, Harbarth S. Will 10 million people die a year due to antimicrobial resistance by 2050? *PLoS Med*. (2016) 13:e1002184. doi: 10.1371/journal.pmed.1002184

3. Bassetti M, Poulakou G, Ruppe E, Bouza E, Van Hal SJ, Brink A. Antimicrobial resistance in the next 30 years, humankind, bugs and drugs: a visionary approach. *Intensive Care Med*. (2017) 43:1464–75. doi: 10.1007/s00134-017-4878-x

4. Tacconelli E, Carrara E, Savoldi A, Harbarth S, Mendelson M, Monnet DL, et al. Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. *Lancet Infect Dis*. (2018) 18:318–27. doi: 10.1016/S1473-3099(17)30753-3

5. Jault P, Leclerc T, Jennes S, Pirnay JP, Que YA, Resch G, et al. Efficacy and tolerability of a cocktail of bacteriophages to treat burn wounds infected by *Pseudomonas aeruginosa* (PhagoBurn): a randomised, controlled, double-blind phase 1/2 trial. *Lancet Infect Dis*. (2019) 19:35–45. doi: 10.1016/S1473-3099(18)30482-1

6. Bakuradze N, Merabishvili M, Makalatia K, Kakabadze E, Grdzelishvili N, Wagemans J, et al. In vitro evaluation of the therapeutic potential of phage va7 against enterotoxigenic *Bacteroides fragilis* infection. *Viruses*. (2021) 13:2044. doi: 10.3390/v13102044

7. Jamal M, Bukhari SMAUS, Andleeb S, Ali M, Raza S, Nawaz MA, et al. Bacteriophages: an overview of the control strategies against multiple bacterial infections in different fields. *J Basic Microbiol*. (2019) 59:123–33. doi: 10.1002/jobm.201800412

8. Hanlon GW. Bacteriophages: an appraisal of their role in the treatment of bacterial infections. *Int J Antimicrob Agents*. (2007) 30:118–28. doi: 10.1016/j.ijantimicag.2007.04.006

9. Górski A, Międzybrodzki R, Węgrzyn G, Jończyk-Matysiak E, Borysowski J, Weber-Dąbrowska B. Phage therapy: current status and perspectives. *Med Res Rev*. (2020) 40:459–63. doi: 10.1002/med.21593

10. Hesse S, Adhya S. Phage therapy in the twenty-first century: Facing the decline of the antibiotic era; is it finally time for the age of the phage? *Annu Rev Microbiol*. (2019) 73:155–74. doi: 10.1146/annurev-micro-090817-062535

11. Łusiak-Szelachowska M, Weber-Dąbrowska B, Górski A. Bacteriophages and Lysins in biofilm control. *Virol Sin*. (2020) 35:125–33. doi: 10.1007/s12250-019-00192-3

12. Veiga-Crespo P, Ageitos JM, Poza M, Villa TG. Enzybiotics: a look to the future, recalling the past. *J Pharm Sci*. (2007) 96:1917–24. doi: 10.1002/jps.20853

13. Pires DP, Oliveira H, Melo LDR, Sillankorva S, Azeredo J. Bacteriophage-encoded depolymerases: their diversity and biotechnological applications. *Appl Microbiol Biotechnol*. (2016) 100:2141–51. doi: 10.1007/s00253-015-7247-0

14. Yang H, Wang M, Yu J, Wei H. Antibacterial activity of a novel peptide-modified lysin against *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. *Front Microbiol*. (2015) 6:1471. doi: 10.3389/fmic,b.2015.01471

15. Sybesma W, Rohde C, Bardy P, Pirnay JP, Cooper I, Caplin J, et al. Silk route to the acceptance and re-implementation of bacteriophage therapy–part II. *Antibiotics*. (2018) 7:35. doi: 10.3390/antibiotics7020035

16. Abdelkader K, Gerstmans H, Saafan A, Dishisha T, Briers Y. The preclinical and clinical progress of bacteriophages and their lytic enzymes: the parts are easier than the whole. *Viruses*. (2019) 11:96. doi: 10.3390/v11020096

17. Yoong P, Schuch R, Nelson D, Fischetti VA. Identification of a broadly active phage lytic enzyme with lethal activity against antibiotic-resistant Enterococcus faecalis and *Enterococcus faecium*. *J Bacteriol*. (2004) 186:4808–12. doi: 10.1128/JB.186.14.4808-4812.2004

18. Fischetti VA. Bacteriophage lytic enzymes: novel anti-infectives. *Trends Microbiol*. (2005) 13:491–6. doi: 10.1016/j.tim.2005.08.007

19. Fischetti VA. 12 the use of phage lytic enzymes to control bacterial infections. In: Kutter E, Sulakvelidze A, *Bacteriophages: Biology and applications*. Boca Raton, FL: CRC Press, p. 321, (2004).

20. Vázquez R, García E, García P. Phage lysins for fighting bacterial respiratory infections: a new generation of antimicrobials. *Front Immunol*. (2018) 9:2252. doi: 10.3389/fimmu.2018.02252

21. Loh B, Gondil VS, Manohar P, Khan FM, Yang H, Leptihn S. Encapsulation and delivery of therapeutic phages. *Appl Environ Microbiol*. (2021) 87:e01979–20. doi: 10.1128/AEM.01979-20

22. Gondil VS, Chhibber S. Evading antibody mediated inactivation of bacteriophages using delivery systems. *J Virol Curr Res*. (2017) 1:555–74. doi: 10.19080/JOJIV.2017.01.55557

23. Malik DJ, Sokolov IJ, Vinner GK, Mancuso F, Cinquerrui S, Vladisavljevic GT, et al. Formulation, stabilisation and encapsulation of bacteriophage for phage therapy. *Adv Colloid Interf Sci*. (2017) 249:100–33. doi: 10.1016/j.cis.2017.05.014

24. Gondil VS, Chhibber S. Bacteriophage and endolysin encapsulation systems: a promising strategy to improve therapeutic outcomes. *Front Pharmacol*. (2021) 12:675440. doi: 10.3389/fphar.2021.675440

25. Leung SSY, Parumasivam T, Gao FG, Carrigy NB, Vehring R, Finlay WH, et al. Production of inhalation phage powders using spray freeze drying and spray drying techniques for treatment of respiratory infections. *Pharm Res*. (2016) 33:1486–96. doi: 10.1007/s11095-016-1892-6

26. Mensink MA, Frijlink HW, van der Voort Maarschalk K, Hinrichs WLJ. How sugars protect proteins in the solid state and during drying (review): mechanisms of stabilization in relation to stress conditions. *Eur J Pharm Biopharm*. (2017) 114:288–95. doi: 10.1016/j.ejpb.2017.01.024

27. Semler DD, Goudie AD, Finlay WH, Dennis JJ. Aerosol phage therapy efficacy in *Burkholderia cepacia* complex respiratory infections. *Antimicrob Agents Chemother*. (2014) 58:4005–13. doi: 10.1128/AAC.02388-13

28. Chang RY, Wong J, Mathai A, et al. Production of highly stable spray dried phage formulations for treatment of *Pseudomonas aeruginosa* lung infection. *Eur J Pharm Biopharm*. (2017) 121:1–13. doi: 10.1016/J.EJPB.2017.09.002

29. Ibaraki H, Kanazawa T, Chien WY, Nakaminami H, Aoki M, Ozawa K, et al. The effects of surface properties of liposomes on their activity against *Pseudomonas aeruginosa* PAO-1 biofilm. *J Drug Deliv Sci Technol*. (2020) 57:101754. doi: 10.1016/j.jddst.2020.101754

30. Rukavina Z, Vanić Ž. Current trends in development of liposomes for targeting bacterial biofilms. *Pharmaceutics*. (2016) 8:18. doi: 10.3390/pharmaceutics8020018

31. Chhibber S, Shukla A, Kaur S. Transfersomal phage cocktail is an effective treatment against methicillin-resistant *Staphylococcus aureus*-mediated skin and soft tissue infections. *Antimicrob Agents Chemother*. (2017) 61:e02146. doi: 10.1128/AAC.02146-16

32. Colom J, Cano-Sarabia M, Otero J, Aríñez-Soriano J, Cortés P, Maspoch D, et al. Microencapsulation with alginate/CaCO3: a strategy for improved phage therapy. *Sci Rep*. (2017) 7:41441. doi: 10.1038/srep41441

33. Rosner D, Clark J. Formulations for bacteriophage therapy and the potential uses of immobilization. *Pharmaceuticals*. (2021) 14:359. doi: 10.3390/ph14040359

34. Anany H, Chen W, Pelton R, Griffiths MW. Biocontrol of listeria monocytogenes and *Escherichia coli* O157: H7 in meat by using phages immobilized on modified cellulose membranes. *Appl Environ Microbiol*. (2011) 77:6379–87. doi: 10.1128/AEM.05493-11

35. Nang SC, Lin YW, Fabijan AP, Chang RYK, Rao GG, Iredell J, et al. Pharmacokinetics/pharmacodynamics of phage therapy: a major hurdle to clinical translation. *Clin Microbiol Infect*. (2023) 29:702–9. doi: 10.1016/j.cmi.2023.01.021

36. Qadir MI, Mobeen T, Masood A. Phage therapy: Progress in pharmacokinetics. Brazilian. *J Pharm Sci*. (2018) 54. doi: 10.1590/s2175-97902018000117093

37. Chernomordik AB. Bacteriophages and their therapeutic-prophylactic use. *Med Sestra*. (1989) 48:44–7.

38. Gupta R, Prasad Y. Efficacy of polyvalent bacteriophage P-27/HP to control multidrug resistant *Staphylococcus aureus* associated with human infections. *Curr Microbiol*. (2011) 62:255–60. doi: 10.1007/s00284-010-9699-x

39. Skurnik M, Pajunen M, Kiljunen S. Biotechnological challenges of phage therapy. *Biotechnol Lett*. (2007) 29:995–1003. doi: 10.1007/s10529-007-9346-1

40. Abedon ST, Thomas-Abedon C. Phage therapy pharmacology. *Curr Pharm Biotechnol*. (2010) 11:28–47. doi: 10.2174/138920110790725410

41. Kutter E, De Vos D, Gvasalia G, Alavidze Z, Gogokhia L, Kuhl S, et al. Phage therapy in clinical practice: treatment of human infections. *Curr Pharm Biotechnol*. (2010) 11:69–86. doi: 10.2174/138920110790725401

42. Sun F, Qu F, Ling Y, et al. Biofilm-associated infections: antibiotic resistance and novel therapeutic strategies. *Future Microbiol*. (2013) 8:877–86. doi: 10.2217/fmb.13.58

43. Drulis-Kawa Z, Maciejewska B. Special Issue: "Bacteriophages and biofilms". *Viruses*. (2021) 13:257. doi: 10.3390/V13020257

44. Seth AK, Geringer MR, Nguyen KT, Agnew SP, Dumanian Z, Galiano RD, et al. Bacteriophage therapy for *Staphylococcus aureus* biofilm-infected wounds: a new approach to chronic wound care. *Plast Reconstr Surg*. (2013) 131:225–34. doi: 10.1097/PRS.0b013e31827e47cd

45. Gurjala AN, Geringer MR, Seth AK, Hong SJ, Smeltzer MS, Galiano RD, et al. Development of a novel, highly quantitative in vivo model for the study of biofilm-impaired cutaneous wound healing. *Wound Repair Regen*. (2011) 19:400–10. doi: 10.1111/J.1524-475X.2011.00690.X

46. Pires DP, Melo LDR, Vilas Boas D, Sillankorva S, Azeredo J. Phage therapy as an alternative or complementary strategy to prevent and control biofilm-related infections. *Curr Opin Microbiol*. (2017) 39:48–56. doi: 10.1016/J.MIB.2017.09.004

47. Chegini Z, Khoshbayan A, Moghadam MT, Farahani I, Jazireian P, Shariati A. Bacteriophage therapy against *Pseudomonas aeruginosa* biofilms: a review. *Ann Clin Microbiol Antimicrob*. 19:45.

48. Chang C, Yu X, Guo W, Guo C, Guo X, Li Q, et al. Bacteriophage-mediated control of biofilm: a promising new Dawn for the future. *Front Microbiol*. (2022) 13:825828. doi: 10.3389/fmicb.2022.825828

49. Hyman P, Abedon ST. Bacteriophage host range and bacterial resistance. *Adv Appl Microbiol*. (2010) 70:217–48. doi: 10.1016/S0065-2164(10)70007-1

50. Carlton RM. Phage therapy: past history and future prospects. *Arch Immunol Ther Exp*. (1999) 47:267–74.

51. Capparelli R, Nocerino N, Iannaccone M, Ercolini D, Parlato M, Chiara M, et al. Bacteriophage therapy of *Salmonella enterica*: a fresh appraisal of bacteriophage therapy. *J Infect Dis*. (2010) 201:52–61. doi: 10.1086/648478

52. Skurnik M, Strauch E. Phage therapy: facts and fiction. *Int J Med Microbiol*. (2006) 296:5–14. doi: 10.1016/j.ijmm.2005.09.002

53. Górski A, Borysowski J, Międzybrodzki R. Bacteriophages in medicine. *Bacteriophage*. (2007):125–58.

54. Mann NH. The potential of phages to prevent MRSA infections. *Res Microbiol*. (2008) 159:400–5. doi: 10.1016/j.resmic.2008.04.003

55. Loc-Carrillo C, Abedon ST. Pros and cons of phage therapy. *Bacteriophage*. (2011) 1:111–4. doi: 10.4161/bact.1.2.14590

56. Clokie MRJ, Kropinski A. *Methods and protocols, volume 1: isolation, characterization, and interactions methods in molecular biology*. Totowa, NJ: Humana Press, pp. 69–81. (2009).

57. Manohar P, AshokJ T, Leptihn S, Ramesh N. Pharmacological and immunological aspects of phage therapy. *Infect Microb Dis*. (2019) 1:34–42. doi: 10.1097/im9.0000000000000013

58. Chopra I, Hodgson J, Metcalf B, Poste G. The search for antimicrobial agents effective against bacteria resistant to multiple antibiotics. *Antimicrob Agents Chemother*. (1997) 41:497–503. doi: 10.1128/AAC.41.3.497

59. Tóthová Ľ, Celec P, Bábíčková J, Gajdošová J, al-Alami H, Kamodyova N, et al. Phage therapy of *Cronobacter*-induced urinary tract infection in mice. *Med Sci Monit*. (2011) 17:BR173–8. doi: 10.12659/msm.>16271

60. Tang F, Zhang P, Zhang Q, Xue F, Ren J, Sun J, et al. Isolation and characterization of a broad-spectrum phage of multiple drug resistant Salmonella and its therapeutic utility in mice. *Microb Pathog*. (2019) 126:193–8. doi: 10.1016/j.micpath.2018.10.042

61. Gu J, Liu X, Li Y, et al. A method for generation phage cocktail with great therapeutic potential. *PLoS One*. (2012) 7:e31698. doi: 10.1371/journal.pone.0031698

62. Tiwari BR, Kim S, Rahman M, Kim J. Antibacterial efficacy of lytic Pseudomonas bacteriophage in normal and neutropenic mice models. *J Microbiol*. (2011) 49:994–9. doi: 10.1007/s12275-011-1512-4

63. Takemura-Uchiyama I, Uchiyama J, Osanai M, Morimoto N, Asagiri T, Ujihara T, et al. Experimental phage therapy against lethal lung-derived septicemia caused by *Staphylococcus aureus* in mice. *Microbes Infect*. (2014) 16:512–7. doi: 10.1016/j.micinf.2014.02.011

64. Hung CH, Kuo CF, Wang CH, Wu CM, Tsao N. Experimental phage therapy in treating *Klebsiella pneumoniae*-mediated liver abscesses and bacteremia in mice. *Antimicrob Agents Chemother*. (2011) 55:1358. doi: 10.1128/AAC.01123-10

65. Pouillot F, Chomton M, Blois H, Courroux C, Noelig J, Bidet P, et al. Efficacy of bacteriophage therapy in experimental Sepsis and meningitis caused by a clone O25b:H4-ST131 *Escherichia coli* strain producing CTX-M-15. *Antimicrob Agents Chemother*. (2012) 56:3568–75. doi: 10.1128/AAC.06330-11

66. Cheng M, Liang J, Zhang Y, Hu L, Gong P, Cai R, et al. The bacteriophage EF-P29 efficiently protects against lethal vancomycin-resistant enterococcus faecalis and alleviates gut microbiota imbalance in a murine bacteremia model. *Front Microbiol*. (2017) 8:837. doi: 10.3389/fmicb.2017.00837

67. Yin S, Huang G, Zhang Y, Jiang B, Yang Z, Dong Z, et al. Phage Abp1 rescues human cells and mice from infection by Pan-drug resistant *Acinetobacter baumannii*. *Cell Physiol Biochem*. (2018) 44:2337–45. doi: 10.1159/000486117

68. Schneider G, Szentes N, Horváth M, Dorn A, Cox A, Nagy G, et al. Kinetics of targeted phage rescue in a mouse model of systemic *Escherichia coli* K1. *Biomed Res Int*. (2018) 2018:7569645. doi: 10.1155/2018/7569645

69. Oechslin F, Piccardi P, Mancini S, et al. Synergistic interaction between phage therapy and antibiotics clears *Pseudomonas aeruginosa* infection in endocarditis and reduces virulence. *J Infect Dis*. (2017) 215:703. doi: 10.1093/INFDIS/JIW632

70. Patel SR, Pratap CB, Nath G. Evaluation of bacteriophage cocktail on septicaemia caused by colistin-resistant *Acinetobacter baumannii* in immunocompromised mice model. *Indian J Med Res*. (2021) 154:141. doi: 10.4103/IJMR.IJMR_2271_18

71. Singh A, Singh AN, Rathor N, Chaudhry R, Singh SK, Nath G. Evaluation of bacteriophage cocktail on septicemia caused by colistin-resistant *Klebsiella pneumoniae* in mice model. *Front Pharmacol*. (2022) 13:778676. doi: 10.3389/fphar.2022.778676

72. Maura D, Galtier M, le Bouguénec C, Debarbieux L. Virulent bacteriophages can target O104:H4 Enteroaggregative *Escherichia coli* in the mouse intestine. *Antimicrob Agents Chemother*. (2012) 56:6235. doi: 10.1128/AAC.00602-12

73. Jaiswal A, Koley H, Ghosh A, Palit A, Sarkar B. Efficacy of cocktail phage therapy in treating *Vibrio cholerae* infection in rabbit model. *Microbes Infect*. (2013) 15:152–6. doi: 10.1016/J.MICINF.2012.11.002

74. Jaiswal A, Koley H, Mitra S, Saha DR, Sarkar B. Comparative analysis of different oral approaches to treat *Vibrio cholerae* infection in adult mice. *Int J Med Microbiol*. (2014) 304:422–30. doi: 10.1016/J.IJMM.2014.02.007

75. Jun JW, Shin TH, Kim JH, Shin SP, Han JE, Heo GJ, et al. Bacteriophage therapy of a *Vibrio parahaemolyticus* infection caused by a multiple-antibiotic-resistant O3:K6 pandemic clinical strain. *J Infect Dis*. (2014) 210:72–8. doi: 10.1093/INFDIS/JIU059

76. Nale JY, Spencer J, Hargreaves KR, Trzepiński P, Douce GR, Clokie MRJ. Bacteriophage combinations significantly reduce *Clostridium difficile* growth in vitro and proliferation in vivo. *Antimicrob Agents Chemother*. (2016) 60:968. doi: 10.1128/AAC.01774-15

77. Galtier M, de Sordi L, Maura D, Arachchi H, Volant S, Dillies MA, et al. Bacteriophages to reduce gut carriage of antibiotic resistant uropathogens with low impact on microbiota composition. *Environ Microbiol*. (2016) 18:2237–45. doi: 10.1111/1462-2920.13284

78. Nikkhahi F, Soltan Dallal MM, Alimohammadi M, Rahimi Foroushani A, Rajabi Z, Fardsanei F, et al. Phage therapy: assessment of the efficacy of a bacteriophage isolated in the treatment of salmonellosis induced by *Salmonella enteritidis* in mice. *Gastroenterol Hepatol Bed Bench*. (2017) 10:131–6.

79. Vahedi A, Soltan Dallal MM, Douraghi M, Nikkhahi F, Rajabi Z, Yousefi M, et al. Isolation and identification of specific bacteriophage against enteropathogenic *Escherichia coli* (EPEC) and in vitro and in vivo characterization of bacteriophage. *FEMS Microbiol Lett*. (2018) 365:fny136. doi: 10.1093/FEMSLE/FNY136

80. Yadav VB, Nath G. Bacteriophage therapy of human-restricted Salmonella species—a study in a surrogate bacterial and animal model. *Lett Appl Microbiol*. (2022) 75:422–30. doi: 10.1111/LAM.13744

81. Dallal MMS, Nikkhahi F, Alimohammadi M, Douraghi M, Rajabi Z, Foroushani AR, et al. Phage therapy as an approach to control *Salmonella enterica* serotype Enteritidis infection in mice. *Rev Soc Bras Med Trop*. (2019) 52:e20190290. doi: 10.1590/0037-8682-0290-2019

82. Kim HJ, Kim YT, Kim HB, Choi SH, Lee JH. Characterization of bacteriophage VVP001 and its application for the inhibition of *Vibrio vulnificus* causing seafood-borne diseases. *Food Microbiol*. (2021) 94:103630. doi: 10.1016/j.fm.2020.103630

83. Chhibber S, Gupta P, Kaur S. Bacteriophage as effective decolonising agent for elimination of MRSA from anterior nares of BALB/c mice. *BMC Microbiol*. (2014) 14:1–13. doi: 10.1186/S12866-014-0212-8/FIGURES/5

84. Drilling A, Morales S, Boase S, Jervis-Bardy J, James C, Jardeleza C, et al. Safety and efficacy of topical bacteriophage and ethylenediaminetetraacetic acid treatment of *Staphylococcus aureus* infection in a sheep model of sinusitis. *Int Forum Allergy Rhinol*. (2014) 4:176–86. doi: 10.1002/ALR.21270

85. Drilling AJ, Ooi ML, Miljkovic D, James C, Speck P, Vreugde S, et al. Long-term safety of topical bacteriophage application to the frontal sinus region. *Front Cell Infect Microbiol*. (2017) 7:49. doi: 10.3389/FCIMB.2017.00049/BIBTEX

86. Fong SA, Drilling AJ, Ooi ML, Paramasivan S, Finnie JW, Morales S, et al. Safety and efficacy of a bacteriophage cocktail in an in vivo model of *Pseudomonas aeruginosa* sinusitis. *Transl Res*. (2019) 206:41–56. doi: 10.1016/J.TRSL.2018.12.002

87. Morello E, Saussereau E, Maura D, Huerre M, Touqui L, Debarbieux L. Pulmonary bacteriophage therapy on *Pseudomonas aeruginosa* cystic fibrosis strains: first steps towards treatment and prevention. *PLoS One*. (2011) 6:e16963. doi: 10.1371/journal.pone.0016963

88. Alemayehu D, Casey PG, McAuliffe O, Guinane CM, Martin JG, Shanahan F, et al. Bacteriophages ϕMR299-2 and ϕNH-4 can eliminate *Pseudomonas aeruginosa* in the murine lung and on cystic fibrosis lung airway cells. *MBio*. (2012) 3:e00029–12. doi: 10.1128/mBio.00029-12

89. Cao F, Wang X, Wang L, Li Z, Che J, Wang L, et al. Evaluation of the efficacy of a bacteriophage in the treatment of pneumonia induced by multidrug resistance *Klebsiella pneumoniae* in mice. *Biomed Res Int*. (2015) 2015:752930. doi: 10.1155/2015/752930

90. Singla S, Harjai K, Katare OP, Chhibber S. Bacteriophage-loaded nanostructured lipid carrier: improved pharmacokinetics mediates effective resolution of *Klebsiella pneumoniae*-induced lobar pneumonia. *J Infect Dis*. (2015) 212:325–34. doi: 10.1093/INFDIS/JIV029

91. Oduor JMO, Onkoba N, Maloba F, Nyachieo A. Experimental phage therapy against haematogenous multi-drug resistant *Staphylococcus aureus* pneumonia in mice. Afr. *J Lab Med*. (2016) 5:435. doi: 10.4102/AJLM.V5I1.435

92. Waters EM, Neill DR, Kaman B, Sahota JS, MRJ C, Winstanley C, et al. Phage therapy is highly effective against chronic lung infections with *Pseudomonas aeruginosa*. *Thorax*. (2017) 72:666–7. doi: 10.1136/thoraxjnl-2016-209265

93. Drilling AJ, Ooi ML, Miljkovic D, et al. Long-term safety of topical bacteriophage application to the frontal sinus region. *Front Cell Infect Microbiol*. (2017):7(FEB). doi: 10.3389/FCIMB.2017.00049

94. Chang RYK, Chen K, Wang J, Wallin M, Britton W, Morales S, et al. Proof-of-principle study in a murine lung infection model of antipseudomonal activity of phage PEV20 in a dry-powder formulation. *Antimicrob Agents Chemother*. (2018) 62:e01714. doi: 10.1128/AAC.01714-17

95. Jeon J, Yong D. Two novel bacteriophages improve survival in *Galleria mellonella* infection and mouse acute pneumonia models infected with extensively drug-resistant *Pseudomonas aeruginosa*. *Appl Environ Microbiol*. (2019) 85:e02900–18. doi: 10.1128/AEM.02900-18

96. Dufour N, Delattre R, Chevallereau A, Ricard JD, Debarbieux L. Phage therapy of pneumonia is not associated with an overstimulation of the inflammatory response compared to antibiotic treatment in mice. *Antimicrob Agents Chemother*. (2019) 63:e00379. doi: 10.1128/AAC.00379-19

97. Horváth M, Kovács T, Koderivalappil S, Ábrahám H, Rákhely G, Schneider G. Identification of a newly isolated lytic bacteriophage against K24 capsular type, carbapenem resistant *Klebsiella pneumoniae* isolates. *Sci Rep*. (2020) 10:5891. doi: 10.1038/s41598-020-62691-8

98. Dhungana G, Regmi M, Paudel P, Parajuli A, Upadhyay E, Gyawali I, et al. Therapeutic efficacy of bacteriophage therapy to treat carbapenem resistant *Klebsiella pneumoniae* in mouse model. *J Nepal Health Res Council*. (2021) 19:76–82. doi: 10.33314/jnhrc.v19i1.3282

99. Prazak J, Valente LG, Iten M, Federer L, Grandgirard D, Soto S, et al. Benefits of aerosolized phages for the treatment of pneumonia due to methicillin-resistant *Staphylococcus aureus*: an experimental study in rats. *J Infect Dis*. (2022) 225:1452–9. doi: 10.1093/INFDIS/JIAB112

100. Hesse S, Malachowa N, Porter AR, Freedman B, Kobayashi SD, Gardner DJ, et al. Bacteriophage treatment rescues mice infected with multidrug-resistant *Klebsiella pneumoniae* ST258. *MBio*. (2021) 12:1–11. doi: 10.1128/MBIO.00034-21

101. Kumari S, Harjai K, Chhibber S. Evidence to support the therapeutic potential of bacteriophage Kpn5 in burn wound infection caused by *Klebsiella pneumoniae* in BALB/c mice. *J Microbiol Biotechnol*. (2010) 20:935–41. doi: 10.4014/JMB.0909.09010

102. Kumari S, Harjai K, Chhibber S. Bacteriophage versus antimicrobial agents for the treatment of murine burn wound infection caused by *Klebsiella pneumoniae* B5055. *J Med Microbiol*. (2011) 60:205–10. doi: 10.1099/jmm.0.018580-0

103. Mendes JJ, Leandro C, Corte-Real S, Barbosa R, Cavaco-Silva P, Melo-Cristino J, et al. Wound healing potential of topical bacteriophage therapy on diabetic cutaneous wounds. *Wound Repair Regen*. (2013) 21:595–603. doi: 10.1111/WRR.12056

104. Chhibber S, Kaur T, Kaur S. Co-therapy using lytic bacteriophage and linezolid: effective treatment in eliminating methicillin resistant *Staphylococcus aureus* (MRSA) from diabetic foot infections. *PLoS One*. (2013) 8:56022. doi: 10.1371/journal.pone.0056022

105. Trigo G, Martins TG, Fraga AG, Longatto-Filho A, Castro AG, Azeredo J, et al. Phage therapy is effective against infection by *Mycobacterium ulcerans* in a murine footpad model. *PLoS Negl Trop Dis*. (2013) 7:e2183. doi: 10.1371/journal.pntd.0002183

106. Dąbrowska K, Kaźmierczak Z, Majewska J, Miernikiewicz P, Piotrowicz A, Wietrzyk J, et al. Bacteriophages displaying anticancer peptides in combined antibacterial and anticancer treatment. *Future Microbiol*. (2014) 9:861–9. doi: 10.2217/fmb.14.50

107. Shivaswamy VC, Kalasuramath SB, Sadanand CK, Basavaraju AK, Ginnavaram V, Bille S, et al. Ability of bacteriophage in resolving wound infection caused by multidrug-resistant *Acinetobacter baumannii* in uncontrolled diabetic rats. *Microb Drug Resist*. (2015) 21:171–7. doi: 10.1089/mdr.2014.0120

108. Basu S, Agarwal M, Nath G. An in vivo wound model utilizing bacteriophage therapy of *Pseudomonas aeruginosa* biofilms. *Ostomy Wound Manage*. (2015) 61:16–23.

109. Chadha P, Katare OP, Chhibber S. In vivo efficacy of single phage versus phage cocktail in resolving burn wound infection in BALB/c mice. *Microb Pathog*. (2016) 99:68–77. doi: 10.1016/j.micpath.2016.08.001

110. Chadha P, Katare OP, Chhibber S. Liposome loaded phage cocktail: enhanced therapeutic potential in resolving *Klebsiella pneumoniae* mediated burn wound infections. *Burns*. (2017) 43:1532–43. doi: 10.1016/j.burns.2017.03.029

111. Albac S, Medina M, Labrousse D, Hayez D, Bonnot D, Anzala N, et al. Efficacy of bacteriophages in a *Staphylococcus aureus* nondiabetic or diabetic foot infection murine model. *Antimicrob Agents Chemother*. (2020) 64:1870. doi: 10.1128/AAC.01870-19

112. Huon JF, Montassier E, Leroy AG, Grégoire M, Vibet MA, Caillon J, et al. Phages versus antibiotics to treat infected diabetic wounds in a mouse model: a microbiological and microbiotic evaluation. *Microb Syst*. (2020) 5:542. doi: 10.1128/msystems.00542-20

113. Kaur S, Chhibber S. A mouse air pouch model for evaluating the anti-bacterial efficacy of phage MR-5 in resolving skin and soft tissue infection induced by methicillin-resistant *Staphylococcus aureus*. *Folia Microbiol (Praha)*. (2021) 66:959–72. doi: 10.1007/s12223-021-00895-9

114. Kifelew LG, Warner MS, Morales S, Vaughan L, Woodman R, Fitridge R, et al. Efficacy of phage cocktail AB-SA01 therapy in diabetic mouse wound infections caused by multidrug-resistant *Staphylococcus aureus*. *BMC Microbiol*. (2020) 20:204. doi: 10.1186/s12866-020-01891-8

115. Hawkins C, Harper D, Burch D, Änggård E, Soothill J. Topical treatment of *Pseudomonas aeruginosa* otitis of dogs with a bacteriophage mixture: a before/after clinical trial. *Vet Microbiol*. (2010) 146:309–13. doi: 10.1016/j.vetmic.2010.05.014

116. Fukuda K, Ishida W, Uchiyama J, Rashel M, Kato SI, Morita T, et al. *Pseudomonas aeruginosa* keratitis in mice: effects of topical bacteriophage KPP12 administration. *PLoS One*. (2012) 7:e47742. doi: 10.1371/journal.pone.0047742

117. Furusawa T, Iwano H, Hiyashimizu Y, Matsubara K, Higuchi H, Nagahata H, et al. Phage therapy is effective in a mouse model of bacterial equine keratitis. *Appl Environ Microbiol*. (2016) 82:5332–9. doi: 10.1128/aem.01166-16

118. Shlezinger M, Friedman M, Houri-Haddad Y, Hazan R, Beyth N. Phages in a thermoreversible sustained-release formulation targeting *E. faecalis* in vitro and in vivo. *PLoS One*. (2019) 14:e0219599. doi: 10.1371/journal.pone.0219599

119. Jiang L, Tan J, Hao Y, Wang Q, Yan X, Wang D, et al. Isolation and characterization of a novel myophage Abp9 against pandrug resistant *Acinetobacter baumannii*. *Front Microbiol*. (2020) 11:506068. doi: 10.3389/fmicb.2020.506068

120. Grygorcewicz B, Roszak M, Golec P, Śleboda-Taront D, Łubowska N, Górska M, et al. Antibiotics act with vB_AbaP_AGC01 phage against *Acinetobacter baumannii* in human heat-inactivated plasma blood and galleria mellonella models. *Int J Mol Sci*. (2020) 21:1–14. doi: 10.3390/IJMS21124390

121. Górski A, Borysowski J, Międzybrodzki R. Phage therapy: towards a successful clinical trial. *Antibiotics*. (2020) 9:1–7. doi: 10.3390/antibiotics9110827

122. Villarroel J, Larsen MV, Kilstrup M, Nielsen M. Metagenomic analysis of therapeutic PYO phage cocktails from 1997 to 2014. *Viruses*. (2017) 9:328. doi: 10.3390/v9110328

123. Abedon ST. Information phage therapy research should report. *Pharmaceuticals*. (2017) 10:328. doi: 10.3390/ph10020043

124. Harper DR. Criteria for selecting suitable infectious diseases for phage therapy. *Viruses*. (2018) 10:177. doi: 10.3390/V10040177

125. Uyttebroek S, Chen B, Onsea J, Ruythooren F, Debaveye Y, Devolder D, et al. Safety and efficacy of phage therapy in difficult-to-treat infections: a systematic review. *Lancet Infect Dis*. (2022) 22:e208–20. doi: 10.1016/S1473-3099(21)00612-5

126. Marza JAS, Soothill JS, Boydell P, Collyns TA. Multiplication of therapeutically administered bacteriophages in *Pseudomonas aeruginosa* infected patients. *Burns*. (2006) 32:644–6. doi: 10.1016/j.burns.2006.02.012

127. Parracho HM, Burrowes BH, Enright MC. The role of regulated clinical trials in the development of bacteriophage therapeutics. *J Mol Genet Med*. (2012) 6:279:286. doi: 10.4172/1747-0862.1000050

128. Fish R, Kutter E, Wheat G, Blasdel B, Kutateladze M, Kuhl S. Bacteriophage treatment of intransigent diabetic toe ulcers: a case series. *Methods Mol Biol*. (2016) 25:S27–33. doi: 10.12968/jowc.2016.25.sup7.s27

129. Fish R, Kutter E, Bryan D, Wheat G, Kuhl S. Resolving digital staphylococcal osteomyelitis using bacteriophage–a case report. *Antibiotics*. (2018) 7:87. doi: 10.3390/antibiotics7040087

130. Fish R, Kutter E, Wheat G, Blasdel B, Kutateladze M, Kuhl S. Compassionate use of bacteriophage therapy for foot ulcer treatment as an effective step for moving toward clinical trials. *Methods Mol Biol*. (2018) 1693:159–70. doi: 10.1007/978-1-4939-7395-8_14

131. Ferry T, Leboucher G, Fevre C, Herry Y, Conrad A, Josse J, et al. Salvage debridement, antibiotics and implant retention ("DAIR") with local injection of a selected cocktail of bacteriophages: is it an option for an elderly patient with relapsing *Staphylococcus aureus* prosthetic-joint infection? Open forum. *Infect Dis*. (2018) 5:ofy269. doi: 10.1093/ofid/ofy269

132. Cano EJ, Caflisch KM, Bollyky PL, van Belleghem JD, Patel R, Fackler J, et al. Phage therapy for limb-threatening prosthetic knee *Klebsiella pneumoniae* infection: case report and in vitro characterization of anti-biofilm activity. *Clin Infect Dis*. (2021) 73:e144:–e151. doi: 10.1093/cid/ciaa705

133. Doub JB, Ng VY, Johnson AJ, Slomka M, Fackler J, Horne B'A, et al. Salvage bacteriophage therapy for a chronic MRSA prosthetic joint infection. *Antibiotics (Basel)*. (2020) 9:241. doi: 10.3390/antibiotics9050241

134. Ramirez-Sanchez C, Gonzales F, Buckley M, Biswas B, Henry M, Deschenes MV, et al. Successful treatment of *Staphylococcus aureus* prosthetic joint infection with bacteriophage therapy. *Viruses*. (2021) 13:1182. doi: 10.3390/V13061182

135. Onsea J, Soentjens P, Djebara S, Merabishvili M, Depypere M, Spriet I, et al. Bacteriophage application for difficult-to-treat musculoskeletal infections: development of a standardized multidisciplinary treatment protocol. *Viruses*. (2019) 11:891. doi: 10.3390/V11100891

136. Chan BK, Turner PE, Kim S, Mojibian HR, Elefteriades JA, Narayan D. Phage treatment of an aortic graft infected with *Pseudomonas aeruginosa*. *Evol Med Public Health*. (2018) 2018:60. doi: 10.1093/emph/eoy005

137. Ujmajuridze A, Chanishvili N, Goderdzishvili M, Leitner L, Mehnert U, Chkhotua A, et al. Adapted bacteriophages for treating urinary tract infections. *Front Microbiol*. (2018) 9:1832. doi: 10.3389/fmicb.2018.01832/full

138. Kuipers S, Ruth MM, Mientjes M, De Sévaux RGL, Van Ingen J. A Dutch case report of successful treatment of chronic relapsing urinary tract infection with bacteriophages in a renal transplant patient. *Antimicrob Agents Chemother*. (2019) 64:e01281. doi: 10.1128/AAC.01281-19

139. Corbellino M, Kieffer N, Kutateladze M, Balarjishvili N, Leshkasheli L, Askilashvili L, et al. Eradication of a multidrug-resistant, Carbapenemase-producing *Klebsiella pneumoniae* isolate following Oral and intra-rectal therapy with a custom made, lytic bacteriophage preparation. *Rev Infect Dis*. (2020) 70:1998–2001. doi: 10.1093/cid/ciz782

140. Khawaldeh A, Morales S, Dillon B, Alavidze Z, Ginn AN, Thomas L, et al. Bacteriophage therapy for refractory *Pseudomonas aeruginosa* urinary tract infection. *J Med Microbiol*. (2011) 60:1697–700. doi: 10.1099/JMM.0.029744-0

141. Qin J, Wu N, Bao J, Shi X, Ou H, Ye S, et al. Heterogeneous *Klebsiella pneumoniae* co-infections complicate personalized bacteriophage therapy. *Front Cell Infect Microbiol*. (2021) 10:608402. doi: 10.3389/FCIMB.2020.608402

142. Bao J, Wu N, Zeng Y, Chen L, Li L, Yang L, et al. Non-active antibiotic and bacteriophage synergism to successfully treat recurrent urinary tract infection caused by extensively drug-resistant *Klebsiella pneumoniae*. *Emerg Microbes Infect*. (2020) 9:771–4. doi: 10.1080/22221751.2020.1747950

143. Rostkowska OM, Międzybrodzki R, Miszewska-Szyszkowska D, Górski A, Durlik M. Treatment of recurrent urinary tract infections in a 60-year-old kidney transplant recipient. The use of phage therapy. *Transpl Infect Dis*. (2021) 23:e13391. doi: 10.1111/tid.13391

144. Johri AV, Johri P, Hoyle N, Pipia L, Nadareishvili L, Nizharadze D. Chronic bacterial prostatitis treated with phage therapy after multiple failed antibiotic treatments. *Front Pharmacol*. (2021) 12:692614. doi: 10.3389/fphar.2021.692614

145. Rhoads DD, Wolcott RD, Kuskowski MA, Wolcott BM, Ward LS. Bacteriophage therapy of venous leg ulcers in humans. *J Wound Care*. (2009) 18:237–8. doi: 10.12968/jowc.2009.18.6.42801

146. Patel DR, Bhartiya SK, Kumar R, Shukla VK, Nath G. Use of customized bacteriophages in the treatment of chronic nonhealing wounds: a prospective study. *Int J Lower Extrem Wounds*. (2021) 20:37–46. doi: 10.1177/1534734619881076

147. Gupta P, Singh HS, Shukla VK, Nath G, Bhartiya SK. Bacteriophage therapy of chronic nonhealing wound: clinical study. *Int J Lower Extrem Wounds*. (2019) 18:171–5. doi: 10.1177/1534734619835115

148. Bhartiya SK, Prasad R, Sharma S, Shukla V, Nath G, Kumar R. Biological therapy on infected traumatic wounds: a case-control study. *Int J Low Extrem Wounds*. (2022):727. doi: 10.1177/15347346211072779

149. Rose T, Verbeken G, De Vos D, Merabishvili M, Vaneechoutte M, Lavigne R, et al. Experimental phage therapy of burn wound infection: difficult first steps. Int J burns. *Trauma*. (2014) 4:66–73.

150. Fadlallah A, Chelala E, Legeais JM. Corneal infection therapy with topical bacteriophage Administration. *Open Ophthalmol J*. (2015) 9:167–8. doi: 10.2174/1874364101509010167

151. Jennes S, Merabishvili M, Soentjens P, Pang KW, Rose T, Keersebilck E, et al. Use of bacteriophages in the treatment of colistin-only-sensitive *Pseudomonas aeruginosa* septicaemia in a patient with acute kidney injury-a case report. *Crit Care*. (2017) 21:129. doi: 10.1186/S13054-017-1709-Y

152. Duplessis C, Biswas B, Hanisch B, Perkins M, Henry M, Quinones J, et al. Refractory Pseudomonas bacteremia in a 2-year-old sterilized by bacteriophage therapy. *J Pediatric Infect Dis Soc*. (2018) 7:253–6. doi: 10.1093/jpids/pix056

153. Petrovic Fabijan A, Lin RCY, Ho J, Maddocks S, Ben Zakour NL, Iredell JR, et al. Safety of bacteriophage therapy in severe *Staphylococcus aureus* infection. *Nat Microbiol*. (2020) 5:465–72. doi: 10.1038/S41564-019-0634-Z

154. Schooley RT, Biswas B, Gill JJ, Hernandez-Morales A, Lancaster J, Lessor L, et al. Development and use of personalized bacteriophage-based therapeutic cocktails to treat a patient with a disseminated resistant *Acinetobacter baumannii* infection. *Antimicrob Agents Chemother*. (2017) 61:e00954. doi: 10.1128/AAC.00954-17

155. Aslam S, Pretorius V, Lehman SM, Morales S, Schooley RT. Novel bacteriophage therapy for treatment of left ventricular assist device infection. *J Heart Lung Transplant*. (2019) 38:475–6. doi: 10.1016/j.healun.2019.01.001

156. Mulzer J, Trampuz A, Trampuz A, Potapov EV. Treatment of chronic left ventricular assist device infection with local application of bacteriophages. *Eur J Cardiothorac Surg*. (2020) 57:1003–4. doi: 10.1093/ejcts/ezz295

157. Maddocks S, Fabijan AP, Ho J, Lin RCY, Ben Zakour NL, Dugan C, et al. Bacteriophage therapy of ventilator-associated pneumonia and empyema caused by *Pseudomonas aeruginosa*. *Am J Respir Crit Care Med*. (2019) 200:1179–81. doi: 10.1164/rccm.201904-0839LE

158. Law N, Logan C, Yung G, Furr CLL, Lehman SM, Morales S, et al. Successful adjunctive use of bacteriophage therapy for treatment of multidrug-resistant *Pseudomonas aeruginosa* infection in a cystic fibrosis patient. *Infection*. (2019) 47:665–8. doi: 10.1007/S15010-019-01319-0

159. Aslam S, Courtwright AM, Koval C, Lehman SM, Morales S, Furr CLL, et al. Early clinical experience of bacteriophage therapy in 3 lung transplant recipients. *Am J Transplant*. (2019) 19:2631–9. doi: 10.1111/ajt.15503

160. Dedrick RM, Guerrero-Bustamante CA, Garlena RA, Russell DA, Ford K, Harris K, et al. Engineered bacteriophages for treatment of a patient with a disseminated drug-resistant *Mycobacterium abscessus*. *Nat Med*. (2019) 25:730–3. doi: 10.1038/S41591-019-0437-Z

161. Gainey AB, Burch AK, Brownstein MJ, Brown DE, Fackler J, Horne B'A, et al. Combining bacteriophages with cefiderocol and meropenem/vaborbactam to treat a pan-drug resistant *Achromobacter* species infection in a pediatric cystic fibrosis patient. *Pediatr Pulmonol*. (2020) 55:2990–4. doi: 10.1002/ppul.24945

162. Hoyle N, Zhvaniya P, Balarjishvili N, Bolkvadze D, Nadareishvili L, Nizharadze D, et al. Phage therapy against *Achromobacter xylosoxidans* lung infection in a patient with cystic fibrosis: a case report. *Res Microbiol*. (2018) 169:540–2. doi: 10.1016/j.resmic.2018.05.001

163. Tan X, Chen H, Zhang M, Zhao Y, Jiang Y, Liu X, et al. Clinical experience of personalized phage therapy against Carbapenem-resistant *Acinetobacter baumannii* lung infection in a patient with chronic obstructive pulmonary disease. *Front Cell Infect Microbiol*. (2021) 11:631585. doi: 10.3389/fcimb.2021.631585

164. Dedrick RM, Freeman KG, Nguyen JA, Bahadirli-Talbott A, Smith BE, Wu AE, et al. Potent antibody-mediated neutralization limits bacteriophage treatment of a pulmonary *Mycobacterium abscessus* infection. *Nat Med*. (2021) 27:1357–61. doi: 10.1038/S41591-021-01403-9

165. Wu N, Dai J, Guo M, Li J, Zhou X, Li F, et al. Pre-optimized phage therapy on secondary *Acinetobacter baumannii* infection in four critical COVID-19 patients. *Emerg Microbes Infect*. (2021) 10:612–8. doi: 10.1080/22221751.2021.1902754

166. Lebeaux D, Merabishvili M, Caudron E, Lannoy D, Van Simaey L, Duyvejonck H, et al. A case of phage therapy against Pandrug-resistant *Achromobacter xylosoxidans* in a 12-year-old lung-transplanted cystic fibrosis patient. *Viruses*. (2021) 13:10060. doi: 10.3390/V13010060

167. Ooi ML, Drilling AJ, Morales S, Fong S, Moraitis S, Macias-Valle L, et al. Safety and tolerability of bacteriophage therapy for chronic rhinosinusitis due to *Staphylococcus aureus*. *JAMA Otolaryngol Head Neck Surg*. (2019) 145:723–9. doi: 10.1001/jamaoto.2019.1191

168. Tamma PD, Souli M, Billard M, Campbell J, Conrad D, Ellison DW, et al. Safety and microbiological activity of phage therapy in persons with cystic fibrosis colonized with Pseudomonas aeruginosa: study protocol for a phase 1b/2, multicenter, randomized, double-blind, placebo-controlled trial. *Trials*. 23:1057. doi: 10.1186/s13063-022-07047-5

169. CYPHY. *CYstic Fibrosis bacterioPHage Study at Yale (CYPHY)–Study Results*. (2023). Available at: https://clinicaltrials.gov/ct2/show/results/NCT04684641 (Accessed June 18, 2023).

170. CTRI. (2023). Available at: https://ctri.nic.in/Clinicaltrials/regtrial.php?modid=1&compid=19&EncHid=37814.12246 (Accessed June 18, 2023).

171. UTI. *Phage Therapy for the Treatment of Urinary Tract Infection*. (2023). Available at: https://clinicaltrials.gov/ct2/show/NCT05537519 (Accessed June 18, 2023).

172. Huang Y, Wang W, Zhang Z, Gu Y, Huang A, Wang J, et al. Phage products for fighting antimicrobial resistance. *Microorganisms*. (2022) 10:1324. doi: 10.3390/microorganisms10071324

173. Kropinski AM. Phage therapy-everything old is new again. *Can J Infect Dis Med Microbiol*. 17:297–306. doi: 10.1155/2006/329465

174. Endersen L, O'Mahony J, Hill C, Ross RP, McAuliffe O, Coffey A. Phage therapy in the food industry. *Annu Rev Food Sci Technol*. (2014) 5:327–49. doi: 10.1146/annurev-food-030713-092415

175. World Medical Association. World Medical Association Declaration of Helsinki: Ethical principles for medical research involving human subjects. *JAMA*. (2013) 310:2191–4. doi: 10.1001/jama.2013.281053

176. Pirnay JP, Verbeken G, Ceyssens PJ, Huys I, De Vos D, Ameloot C, et al. The magistral phage Viruses. *Viruses*. (2018) 10:2. doi: 10.3390/V10020064

177. De Vos D, Verbeken G, Quintens J, Pirnay JP. Phage therapy in Europe: Regulatory and intellectual property protection issues. VosD De, *Phage Therapy: A Practical Approach*. Berlin: Springer, pp. 363–377. (2019).

178. Administration USFD. Food additives permitted for direct addition to food for human consumption; bacteriophage preparation. *Fed Regist*. (2006) 71:47729–32.

179. Patey O, McCallin S, Mazure H, Liddle M, Smithyman A, Dublanchet A. Clinical indications and compassionate use of phage therapy: personal experience and literature review with a focus on osteoarticular infections. *Viruses*. (2019) 11:18. doi: 10.3390/v11010018

180. Voelker R. FDA approves bacteriophage trial. *JAMA*. (2019) 321:638. doi: 10.1001/jama.2019.0510

181. US Food and Drug Administration. *Draft guidance for industry: Development of bacteriophage therapy products*. (2019). Available at: https://www.fda.gov/media/159400/download. (Accessed March 2, 2023).

182. Vaughan TJ, Williams AJ, Pritchard K, Osbourn JK, Pope AR, Earnshaw JC, et al. Human antibodies with sub-nanomolar affinities isolated from a large non-immunized phage display library. *Nat Biotechnol*. (1996) 14:309–14. doi: 10.1038/nbt0396-309

183. Sarhan WA, Azzazy HME. Phage approved in food, why not as a therapeutic? *Expert Rev Anti-Infect Ther*. (2015) 13:91–101. doi: 10.1586/14787210.2015.990383

184. Hamzeh-Mivehroud M, Alizadeh AA, Morris MB, Bret Church W, Dastmalchi S. Phage display as a technology delivering on the promise of peptide drug discovery. *Drug Discov Today*. (2013) 18:1144–57. doi: 10.1016/j.drudis.2013.09.001

185. Morozov VA, Morozov AV, Schürmann D, Jessen H, Kücherer C. Transmembrane protein polymorphisms and resistance to T-20 (Enfuvirtide, Fuzeon) in HIV-1 infected therapy-naive seroconverters and AIDS patients under HAART-T-20 therapy. *Virus Genes*. (2007) 35:167–74. doi: 10.1007/S11262-007-0098-8

186. Takagi T, Arisawa T, Yamamoto K, Hirata I, Nakano H, Sawada M. Identification of ligands binding specifically to inflammatory intestinal mucosa using phage display. *Clin Exp Pharmacol Physiol*. (2007) 34:286–9. doi: 10.1111/J.1440-1681.2007.04563.X

187. Intralytix, Inc. (2023). Available at: http://www.intralytix.com/index.php?page=prod (Accessed March 3, 2023).

188. Alomari MMM, Dec M, Urban-Chmiel R. Bacteriophages as an alternative method for control of zoonotic and foodborne pathogens. *Viruses*. (2021) 13:2348. doi: 10.3390/v13122348

189. Soffer N, Woolston J, Li M, Das C, Sulakvelidze A. Bacteriophage preparation lytic for Shigella significantly reduces *Shigella sonnei* contamination in various foods. *PLoS One*. (2017) 12:e0175256. doi: 10.1371/journal.pone.0175256

190. Zhang X, Niu YD, Nan Y, Stanford K, Holley R, McAllister T, et al. SalmoFresh™ effectiveness in controlling Salmonella on romaine lettuce, mung bean sprouts and seeds. *Int J Food Microbiol*. (2019):108250:305. doi: 10.1016/j.ijfoodmicro.2019.108250

191. Moye ZD, Woolston J, Sulakvelidze A. Bacteriophage applications for Food production and processing. *Viruses*. (2018) 10:205. doi: 10.3390/v10040205

192. Candidate Products–Pherecydes. (2023). Available at: https://www.pherecydes-pharma.com/en/candidate-products/ (Accessed March 3, 2023).

193. Galtier M, De Sordi L, Sivignon A, De Vallée A, Maura D, Neut C, et al. Bacteriophages targeting adherent invasive *Escherichia coli* strains as a promising new treatment for Crohn's disease. *J Crohns Colitis*. (2017) 11:840–7. doi: 10.1093/ecco-jcc/jjw224

194. Figileco. (2023). Available at: http://www.phageluxagrihealth.com/en/ (Accessed March 3, 2023).

195. Bacteriophages. (2023). Available at: https://www.microgen.ru/en/products/bakteriofagi/ (Accessed March 3, 2023).

196. ELIAVA. (2023). Available at: https://phage.ge/?lang=en (Accessed March 3, 2023).

197. Our Pipeline–Biomx. (2023). https://www.biomx.com/our-pipeline/ (Accessed March 3, 2023).

198. Mai V, Ukhanova M, Reinhard MK, Li M, Sulakvelidze A. Bacteriophage administration significantly reduces Shigella colonization and shedding by Shigella-challenged mice without deleterious side effects and distortions in the gut microbiota. *Bacteriophage*. (2015) 5:e1088124. doi: 10.1080/21597081.2015.1088124

199. Phage Products BioChimPharm. (2023). https://biochimpharm.ge/en/products/ (Accessed March 3, 2023).

200. Production. (2023). Available at: https://aziyaimmunopreparat.uz/%D0%BF%D1%80%D0%BE%D0%B4%D1%83%D0%BA%D1%86%D0%B8%D1%8F (Accessed March 3, 2023).

201. Produkty Výzkum, vývoj a výroba MB Pharma. (2023). Available at: https://www.mbph.cz/produkty (Accessed March 3, 2023).

202. About RPC Micromir. (2023). Available at: https://micromir.bio/about-eng (Accessed March 3, 2023).

203. Development Pipeline–Adaptive Phage Therapeutics. (2023). Available at: https://aphage.com/science/pipeline/ (Accessed March 3, 2023).

204. Selle K, Fletcher JR, Tuson H, Schmitt DS, McMillan L, Vridhambal GS, et al. In vivo targeting of Clostridioides difficile using phage-delivered CRISPR-Cas3 antimicrobials. *MBio*. (2020) 11:e00019. doi: 10.1128/MBIO.00019-20

205. Sciphage Products. (2023). Available at: https://sciphage.com/products/ (Accessed March 3, 2023).

206. Ellis Day Skin Science–Balancing Phage Serum. (2023). Available at: https://www.ellisdayskinscience.com/products/balancing-phage-serum (Accessed March 3, 2023).

207. Leptihn S, Loh B. Complexity, challenges and costs of implementing phage therapy. *Future Microbiol*. (2022) 17:643–6. doi: 10.2217/fmb-2022-0054

208. Sulakvelidze A, Alavidze Z, Morris JG. Bacteriophage therapy. *Antimicrob Agents Chemother*. (2001) 45:649–59. doi: 10.1128/AAC.45.3.649-659.2001

209. Alisky J, Iczkowski K, Rapoport A, Troitsky N. Bacteriophages show promise as antimicrobial agents. *J Infect*. (1998) 36:5–15. doi: 10.1016/s0163-4453(98)92874-2

210. Barrow PA, Soothill JS. Bacteriophage therapy and prophylaxis: rediscovery and renewed assessment of potential. *Trends Microbiol*. (1997) 5:268–71. doi: 10.1016/S0966-842X(97)01054-8

211. Eaton MD, Bayne-Jones S. Bacteriophage therapy: review of the principles and results of the use of bacteriophage in the treatment of infections. *J Am Med Assoc*. (1934) 103:1769–76. doi: 10.1001/jama.1934.72750490003007

212. Krueger AP, Scribner EJ. The bacteriophage: its nature and its therapeutic use. *J Am Med Assoc*. (1941) 116:2269–77.

213. Kucharewicz-Krukowska A, Slopek S. Immunogenic effect of bacteriophage in patients subjected to phage therapy. *Arch Immunol Ther Exp*. (1987) 35:553–61.

214. Archana A, Patel PS, Kumar R, Nath G. Neutralizing antibody response against subcutaneously injected bacteriophages in rabbit model. *Virus*. (2021) 32:38–45. doi: 10.1007/s13337-021-00673-8

215. Merril CR, Biswas B, Carlton R, Jensen NC, Creed GJ, Zullo S, et al. Long-circulating bacteriophage as antibacterial agents. *Proc Natl Acad Sci*. (1996) 93:3188–92. doi: 10.1073/PNAS.93.8.3188

216. Bhargava K, Nath G, Bhargava A, Aseri GK, Jain N. Phage therapeutics: from promises to practices and prospectives. *Appl Microbiol Biotechnol*. (2021) 105:9047–67. doi: 10.1007/s00253-021-11695-z

217. Kilcher S, Loessner MJ. Engineering bacteriophages as versatile biologics. *Trends Microbiol*. (2019) 27:355–67. doi: 10.1016/j.tim.2018.09.006

218. Lenneman BR, Fernbach J, Loessner MJ, Lu TK, Kilcher S. Enhancing phage therapy through synthetic biology and genome engineering. *Curr Opin Biotechnol*. (2021) 68:151–9. doi: 10.1016/j.copbio.2020.11.003

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# Dopamine alters phage morphology to exert an anti-infection effect

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Antiviral drug development is important for human health, and the emergence of novel COVID-19 variants has seriously affected human lives and safety. A bacteriophage—a bacterial virus with a small and simple structure—is an ideal experimental candidate for studying the interactions between viruses and their hosts. In this study, the effects and mechanisms of catecholamines on phages were explored, and dopamine (DA) was found to have general and efficient anti-infection effects. A clear dose-dependent effect was observed when different phages were treated with DA, with higher DA concentrations exhibiting stronger anti-phage activity. The half maximal inhibitory concentration values of DA for vB-EcoS-IME167, T4 Phage, and VMY22 were determined as 0.26, 0.12, and 0.73mgmL$^{-1}$, respectively. The anti-phage effect of DA increased with treatment duration. In addition, the anti-infection activities of DA against vB-EcoS-IME167, T4 Phage, and VMY22 were increased by $10^5$, $10^4$, and $10^4$ folds compared to that of the control. This ability of DA was observed only in phages and not in the host bacteria. Morphological changes of phages were observed under transmission electron microscopy following their treatment with DA, and considerable changes in adsorption were confirmed *via* quantitative reverse transcription polymerase chain reaction. These results suggest that the anti-phage effect of DA is primarily due to the destruction of the external structure of the phage. This study, to the best of our knowledge, is the first to report the universal anti-phage infection effect of dopamine, which provides novel information regarding DA and forms a basis for further research and development of antiviral drugs. Moreover, it provides a new perspective for the research about the defense and counter-defense of bacteria and bacteriophages.

KEYWORDS

**anti-infection, anti-phage activity, bacteriophage, catecholamine, dopamine, quantitative RT-PCR**

## 1. Introduction

Viruses infect bacteria, fungi, actinomycetes, and other microorganisms (Bettarel et al., 2004; Ackermann, 2007; De La Higuera and Lázaro, 2022) and represent some of the most abundant members of the biosphere (Davies et al., 2016). The phage performs its own proliferation and progeny reproduction by lysing the host; it is a bacterial virus that specifically infects the host bacteria. Bacteriophages are also the most abundant viruses and possess low toxicity, presenting an ideal alternative biological model for viral studies that can be used in preliminary research for novel antiviral drug development.

Phages can be divided into virulent phages, which lyse the host, and lysogenic phages, which coexist with the host (Clokie et al., 2011; Hampton et al., 2020). Phage infections occur through five steps: adsorption, injection, synthesis, assembly, and release (Manning and Kuehn, 2011; Fortier and Sekulovic, 2013; Reyes-Robles et al., 2018). As bacteria and bacteriophages typically coexist in the biosphere, bacteria have evolved various defense mechanisms, such as adsorption inhibition, injection blocking, abortive infection, and restriction repair systems (Jackson et al., 2017; Tzipilevich et al., 2017; Hille et al., 2018; Gordeeva et al., 2019) to combat phage infection. The bacterial synthesis of dopamine (DA) has recently been reported (Villageliú and Lyte, 2018; Liu and Zhu, 2020). Therefore, studies on the anti-phage mechanisms of DA are necessary to expand our understanding of DA and provide new insights into the defense mechanisms of microbial systems.

Catecholamines are active substances that primarily function as neurotransmitters and hormones (Szopa et al., 2001; Eisenhofer et al., 2004). Sriram et al. found that catecholamines play an important role in the physiological regulation of the cardiovascular, respiratory, metabolic, and immune systems in COVID-19 (Gubbi et al., 2020). DA is an important neurotransmitter that is widely found in animals and plants; it regulates important functions and is the first component of the catecholamine synthesis pathway (Jiang et al., 2006; Juárez Olguín et al., 2016). DA can enhance adaptability to various stimuli (Norcliffe-Kaufmann, 2022) and improve plant resistance to pathogens and environmental stress (Allen, 2003; Skirycz et al., 2005). DA is a catecholamine comprising catechol and amino group side chains. In nature, catecholamines are active substances that have various physiological and biochemical effects on animals and plants (Li P. et al., 2019; Kaufmann et al., 2020); however, their effects on microorganisms have not been reported. Therefore, investigations are required to fill the existing research gap on catecholamines and their functions in microorganisms.

Catecholamine substances, including dopamine, are often present in environments where bacteria and bacteriophages coexist. Previous studies have found that they played important roles in bacterial growth, biofilm formation, etc. However, there have been no reports on the specific effects of dopamine on bacteriophages. The dopamine produced by bacteria itself is limited under normal growth; however, it can produce high concentrations of dopamine under environmental stress, which include biological, physical, and chemical factors. Therefore, it is necessary to explore the defense and counter-defense mechanisms of dopamine against bacteria and bacteriophages (Lyte and Ernst, 1992; Sharaff and Freestone, 2011).

In this study, three catecholamines [DA, norepinephrine (NE), and epinephrine (E)] and three phages (vB-EcoS-IME167, T4 phage, and VMY22) were used to perform a series of anti-phage infection studies. All bacteriophages used in this study were virulent. The results showed, for the first time, that DA had a general and efficient effect on blocking phage infection of host bacteria; the highest anti-infection abilities of vB-EcoS-IME167, T4 Phage, and VMY22 were $10^5$, $10^4$, and $10^4$ times that of the control, respectively. The anti-infection effect of DA was found to target only phages and preliminarily confirmed the anti-infection mechanism: DA decreased the phage adsorption rate, leading to inhibition of the adsorption step during infection. The findings of this study highlight the role of DA and catecholamines in their specific action toward phages and significantly contribute to further studies on antiviral drug development. DA is a kind of hormone produced by the host under stress or stress, which plays an important role in increasing bacterial susceptibility (Fan and Pedersen, 2021). It is an effective method for improving bacterial infections from the perspective of bacteriophages. Our findings provide a new perspective; however, research in this area remains lacking. The study of DA against bacteriophage infection is worth exploring. These results broaden the understanding of catecholamines and lay a foundation for further research and development of antiviral drugs, providing new insights for the in-depth exploration of the defense mechanism of microbial systems. The findings of this study highlight the role of DA and catecholamines in their specific action toward phages and significantly contribute to further studies on antiviral drug development.

## 2. Materials and methods

### 2.1. Bacterial strains, reagents, and culture conditions

DA, NE, and E were purchased from the Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). The raw materials for Luria-Bertani (LB) medium, Minimal Medium (MM), and experimental consumables were obtained from Yunnan Chien Technology Company (Kunming, China). All chemicals used in this study were of analytical grade. Ultrapure water was used to prepare sterile aqueous solvent (Milli-Q system, 18.2 MΩ cm, 25°C).

*Escherichia coli* BW25113, *E. coli* ATCC 11303, and *Bacillus cereus* MYB41-22 were the three host bacteria used in the experiment. The phages vB-Ecos-IME167, T4 phage, and VMY22, belonging to *Siphoviridae, Myoviridae*, and *Podoviridae*, respectively, were selected for this study.

### 2.2. Activation of host bacteria

The preservation strains of the host bacteria, *E. coli* BW25113, *E. coli* ATCC 11303, and *B. cereus* MYB41-22, were removed from the ultra-low temperature refrigerator at −80°C. First, the host bacteria were placed on crushed ice to thaw naturally. Second, a small volume of the bacterial preservation solution was transferred to a solid plate medium *via* plate streaking for bacterial activation. The plates were then marked and placed in the LB medium for bacterial culture (MM medium can only sustain the basic life processes of bacteria). Optimum culture temperatures were selected for the three host bacteria, namely, 37°C for *E. coli* BW25113 and *E. coli* ATCC 11303 and 28°C for *B. cereus* MYB41-22. After transfer, all bacteria were cultured overnight.

### 2.3. Preparation of bacteriophage

A single colony was selected from the activated host bacterial plates using an inoculation ring, and the bacterial solutions were expanded in a conical flask until the $OD_{600}$ reached 0.8. The preserved phages were then added to the respective bacterial solutions and inoculated at a density of 10%. After the solutions turned clear, a sterile 0.22 μm stream filtration membrane was used to obtain each

phage stock solution, which was marked and stored at 4°C for subsequent use.

## 2.4. Anti-phage activity assay

### 2.4.1. Treatment concentration

Sterile water was used as the control, and different concentration gradients (0.01, 0.1, 1, 5, and 10 mg/mL) of each sample solution were mixed with the respective phage dilution at a ratio of 1:2. After 5 min of incubation, the effect of each sample on phage infection was compared *via* the double-layer plate method. The experiment was performed in triplicate. Semi-inhibitory concentration ($IC_{50}$) values indicate half of the amount of a drug or inhibitor that inhibits certain substances, such as enzymes, cell receptors, or microorganisms. The ratio of DA concentration to inhibition rate was used to calculate the $IC_{50}$, which reflects the anti-infective ability of DA more directly.

### 2.4.2. Time of treatment

Sterile water was used as the control, the sample concentrations were determined as described previously, and different treatment times (10, 30, 60, 120, and 180 min) were applied. The differences between groups were statistically analyzed using the double-layer plate method. The experiment was performed in triplicate.

### 2.4.3. Treatment environment

An anti-phage infection experiment was performed on the culture media to test the effects of LB and MM. The experiment was performed in triplicate. Briefly, three strains of host bacteria and three different bacteriophages were cultured in two different media: LB and MM (dilutions: $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$, $10^{-6}$, $10^{-7}$). DA solution at a concentration of 10 mg/mL and bacteriophages at different dilutions were incubated for 60 min at a 1:2 ratio (100 μL DA + 200 μL bacteriophage). After incubation, each mixture was transferred to a 5 mL Eppendorf (EP) tube containing 200 μL of the host bacteria and was incubated for 5 min at 26°C (with three replicates per dilution tested). The sterilized semi-solid medium was dissolved in a microwave oven and then cooled to 55–65°C. After incubation, the bacterial mixture was quickly poured onto the solid medium and then cultured overnight at 37°C/ 28°C. The plaque on the plate was counted, and the pfu/mL was calculated.

## 2.5. Morphological properties based on transmission electron microscope (TEM) imaging

### 2.5.1. Phage purification

The phage stock solution (500 mL) was expanded to obtain highly concentrated purified phage particles by enzymatic hydrolysis, centrifugation, filtration, sedimentation, and separation, as described below.

The plated host bacteria were inoculated in a 5 mL test tube and cultured overnight (with a 3% inoculation amount) in 500 mL LB liquid medium until $OD_{600} = 1.0$ was reached. Next, the host bacteria and corresponding phage were mixed at a ratio of 1:10 and cultured on a shaker at 180 rpm and 28°C/ 37°C until the solution turned clear. After cooling this solution to 20–25°C, 50 μL DNaseI and 50 μL RNase were added to digest the nucleic acids of host bacteria after cleavage. An amount of 29.2 g NaCl granules was dissolved into each sample bottle (placed in an ice bath) *via* stirring with a glass rod for 60 min. Next, the mixture was centrifuged (15 min, 4°C, 11000 × g) using a large cryo centrifuge. The supernatant was collected, its volume measured and placed in a 500 mL beaker. Polyethylene glycol ($PEG_{6000}$; 50 g/500 mL) was added according to the volume of the supernatant and dissolved with a magnetic agitator, and the mixture was allowed to settle overnight in an ice bath. Thereafter, the mixture was centrifuged again (15 min, 4°C, 11000 × g), and the supernatant was discarded. The phage precipitate was retained and resuspended in an SM solution (5.8 g/L NaCl, 2 g/L $MgSO_4 \cdot 7H_2O$, 50 mL/L 1 mol/L Tris-Cl) at a proportion of 500 mL precipitate per 8 mL of SM solution. This suspension was incubated for 60 min at room temperature, and the resuspension liquid was transferred to a 50 mL centrifuge tube. An equal volume of chloroform was added. The remaining $PEG_{6000}$ and host cell fragments were extracted, mixed evenly, and centrifuged at 3000 × g for 15 min. Its organic and hydrophilic phases were separated, retaining the hydrophilic phase containing phage particles. After measuring the volume thereof, cesium chloride was added at a proportion of 0.75 g/mL and gently shaken until dissolved. The mixture was centrifuged at 4°C and 160,000 × *g* for 12 h. Lastly, the ultra-free tube was carefully removed, and the concentrated phage particles were extracted with a syringe and stored at 4°C.

### 2.5.2. Negative dyeing

Using a pipette gun, 20 μL of phage particles was gently aspirated into the same volume of different concentrations of DA solution (0, 0.01, and 10 mg/mL). After incubation for 60 min, 5 μL of the mixture was absorbed and gently added to the carbon-coated layer of a copper mesh, which was then kept static to allow adsorption for 10 min at room temperature under gravity. The remaining liquid on the copper mesh was blotted with filter paper and allowed to dry for 1 min, after which 5 μL of 1% phosphotungstic acid solution was added for 2 min.

### 2.5.3. TEM imaging

TEM images were used to observe changes in the morphology of phages treated with DA. A purified and high concentration of each bacteriophage (20–100 μL) was gently poured into a 1.5 mL EP tube with a liquid transfer gun. It was incubated with 20 μL of $ddH_2O$ and 0.01 or 10 mg/ mL of DA solution for 60 min. Next, 5 μL of the mixture was gently placed on a carbon-coated copper mesh grid and allowed to adsorb statically through self-gravity for 10 min at room temperature. Thereafter, any remaining liquid was removed from the copper mesh with clean filter paper, and the sample was re-dyed with 5 μL of 1% phosphotungstic acid solution for 2 min. The remaining liquid was once again absorbed with a clean filter paper, and the samples were observed under a projection electron microscope (Tecnai G2 TF30 S-Twin; FEI, Hillsboro, OR, United States).

## 2.6. Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

The whole phage genome was extracted using a Viral DNA Kit (Omega Biotek, GA, United States), a recombinant plasmid was prepared, and a standard curve was constructed. Using sterile water as the control and DA as the experimental group, the phage solution

was mixed at a ratio of 9:1, incubated for 30 min, centrifuged, and filtered, with the supernatant being retained. The phage genome was extracted from the supernatant and amplified *via* RT-PCR. The results of the experimental and control groups were compared based on the standard curve. The Ct value was used as the horizontal coordinate, and the logarithm of the standard copy number was used as the vertical coordinate to create the standard curve (Fig. S1). Three genes were selected: the tail fiber protein of vB-EcoS-IME167, a short-tail fiber protein of the T4 phage, and DNA packaging ATPase in VMY22.

The vB-EcoS-IME167 phage was represented as $Y = -0.2968x + 12.94, R^2 = 0.9991$.

The T4 phage was represented as $Y = -0.3754x + 10.218, R^2 = 0.9991$

VMY22 was represented as $Y = -0.3216x + 7.7848, R^2 = 0.9994$

## 2.7. Statistical analysis

Data represent the mean ± standard error (SE) of at least three experiments done in triplicate (n = 3). GraphPad Prism 9.5.1 software was used for all analyses. Paired t-tests were used to compare data between treated and untreated groups and to compare means within the same set of experiments. Results were considered statistically significant at $p < 0.05$ ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$).

# 3. Results

## 3.1. Anti-phage experiment

The anti-phage activities of DA, NE, and E are shown in Figure 1. DA showed a significant anti-phage effect, whereas NE and E had no effect on phage activity compared with the control. Indeed, DA showed a more than $10^3$-fold inhibitory effect on all three phages tested. Thus, DA exhibited a high inhibitory activity against phage infection.

## 3.2. Variation in inhibition based on concentration

Due to the high efficiency of DA against phage infection in host bacteria, we performed gradient experiments to determine the optimal treatment conditions. The three phages exhibited a dose-dependent relationship, and their anti-infection abilities increased with increasing phage concentration. The strongest resistance to vB-EcoS-IME167 was observed at 5 mg/mL DA, and the strongest resistance to the T4 phage and VMY22 was observed at 10 mg/mL DA. Further data mining of DA concentration and anti-infection ability is shown in Figures 2D–F. The semi-inhibitory concentrations ($IC_{50}$) of the three phages were 0.26, 0.12, and 0.73 mg/mL, respectively. DA concentration was found to be the key factor affecting anti-infection ability. At a concentration of 10 mg/mL, the maximum resistance to vB-EcoS-IME167, the T4 phage, and VMY22 were $10^5$-, $10^5$-, and $10^4$-fold, respectively, and the lowest effective concentration of DA was in the range of 0.1–1 mg/mL, revealing the efficient and extensive ability of DA to inhibit phage infection in bacteria.

## 3.3. Variation in inhibition based on treatment duration

Different DA treatment times induced diverse anti-phage activity intensities. The experimental results for DA-induced resistance to phages at incubation times of 10, 3-, 60, 90, and 120 min are shown in Figure 3. Compared to that of the control, the anti-phage activity of DA was enhanced with longer treatments. At 10 min, DA showed an approximately 10-fold ability to block vB-EcoS-IME167 and T4 phage infection in host bacteria. For VMY22, DA instantaneously exhibited a $10^3$-fold resistance at 60 min. From the overall trend depicted in Figure 3, it is evident that DA exhibited rapid anti-infection activity against vB-EcoS-IME167 and T4 phages within a short period; this anti-infection ability increased with time, finally reaching approximately $10^4$-fold resistance. Simultaneously, DA-induced resistance in VMY22 rapidly increased by more than $10^3$-fold between 30 and 60 min and then stabilized. The duration of DA treatment is therefore a key factor in resistance. The maximum inhibitory activity of a variety of phages also corresponded with varied treatment times.

## 3.4. Inhibition effect of environments

Microbial growth generally places strict requirements on culture media. Therefore, we selected LB and MM, which are commonly used for microbial growth, to confirm the influence of the cultivation environment on phage infection. Figure 4 shows that the three phages responded the same after receiving either DA or H2O (control) treatment in LB, indicating that the anti-infection effect of DA was negligible in this environment. However, DA induced a $10^3$-, $10^3$-, and $10^2$-fold higher resistance to the three respective phages in an MM environment than the control treatment, indicating that the DA treatment of phages in MM led to a significant decline in phage infection activity. We speculate that the complex composition of LB media and the complex macromolecules contained in yeast powder and peptone may affect the action of DA on phages, thus resulting in this phenomenon; however, the composition of MM is simpler, mostly comprising inorganic ions and has relatively little influence on the interaction between DA and phages. Therefore, the MM medium may allow the effective inhibition of phages *via* DA.

## 3.5. The illustration of mechanisms involved in phage resistance of DA

DA was found to induce resistance to bacteriophage infections only in the MM environment. The main target of DA remained unclear and could have been the host bacteria or bacteriophages. Hence, we compared the plaque-forming units of host bacteria and bacteriophages treated with either DA or sterile water (control) in MM (Figure 5). No significant difference was observed in the number of plaques between the experimental (DA) and control (sterile water) groups of host bacteria. In contrast, the experimental group of bacteriophages showed a considerable $10^2$-fold decrease in plaque-forming units following DA treatment compared to the

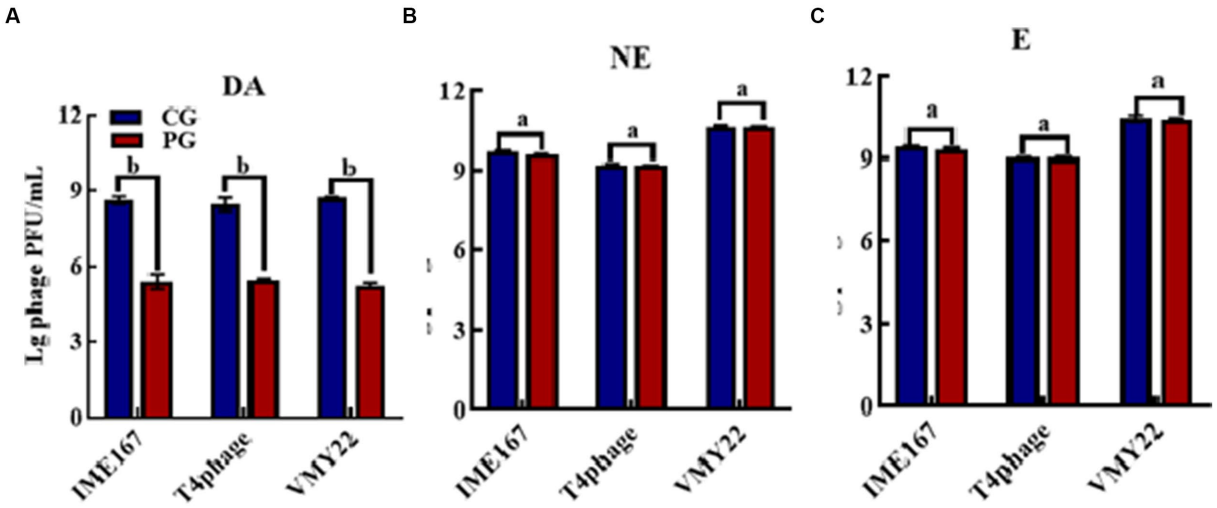


FIGURE 1
Anti-phage activities of different reagents. **(A–C)** indicate resistance to dopamine (DA), norepinephrine (NE), and epinephrine (E). (CG, control group; PG, treatment group). Error bars indicate the standard deviation of three replicate cultures. Different letters indicate significant differences (one-way Prism with Tukey's *post hoc* test; a: $p > 0.05$, $p < 0.05$).

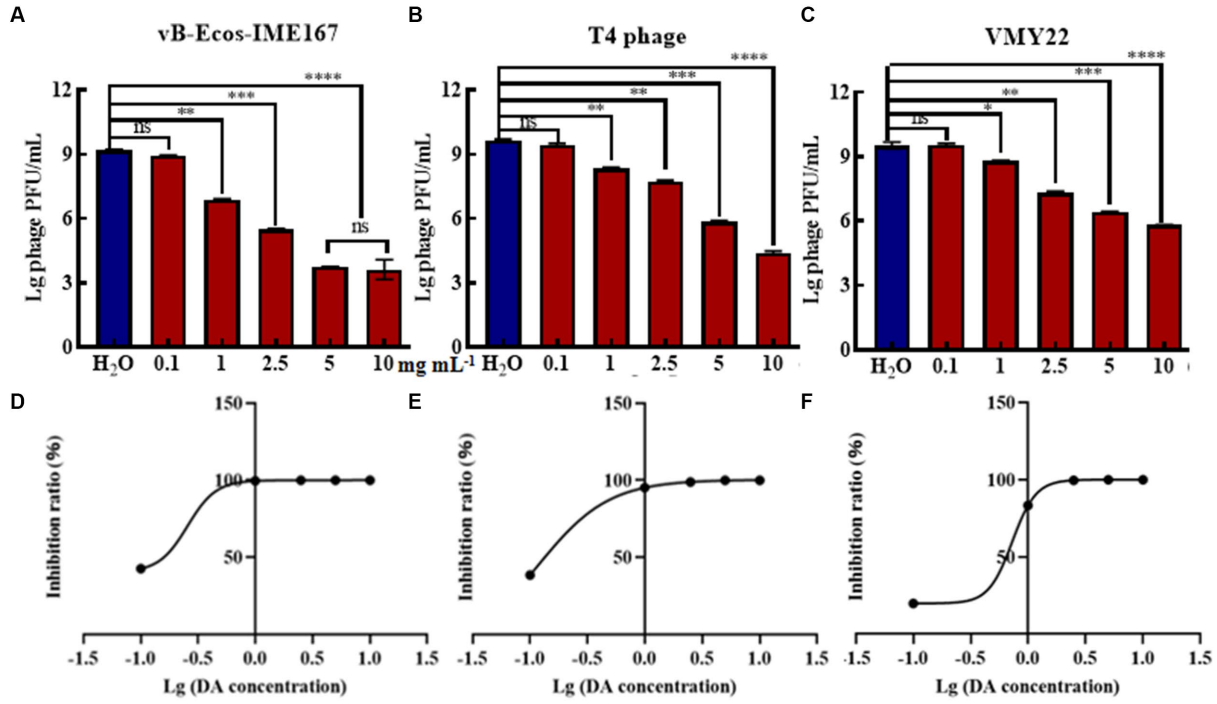


FIGURE 2
The different concentrations of DA and the $IC_{50}$ of DA. **(A–C)** show three different phages, vB-EcoS-IME167, T4, and VMY22, treated with different concentrations of DA. Error bars indicate the standard deviation of three replicate cultures. Different letters indicate significant differences (one-way ANOVA with Tukey's *post hoc* test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$). **(D–F)** indicate the half-maximal inhibitory concentrations ($IC_{50}$) of DA against vB-EcoS-IME167, T4 phage, and VMY22; the $IC_{50}$ value was calculated using the ratio of the logarithm of the DA concentration to the inhibition rate.

control. These results show that the anti-infective effect of DA is seen only during its action on the phage and has no effect on the host bacteria, which further indicates that DA may cause some changes in the phage, leading to a sudden decline in its infection ability.

## 3.6. TEM imaging

Based on the above experiments, we speculated that DA caused changes in phages that resulted in a decrease in the adsorption rate. The morphologies of the three phages (treated with either DA or a

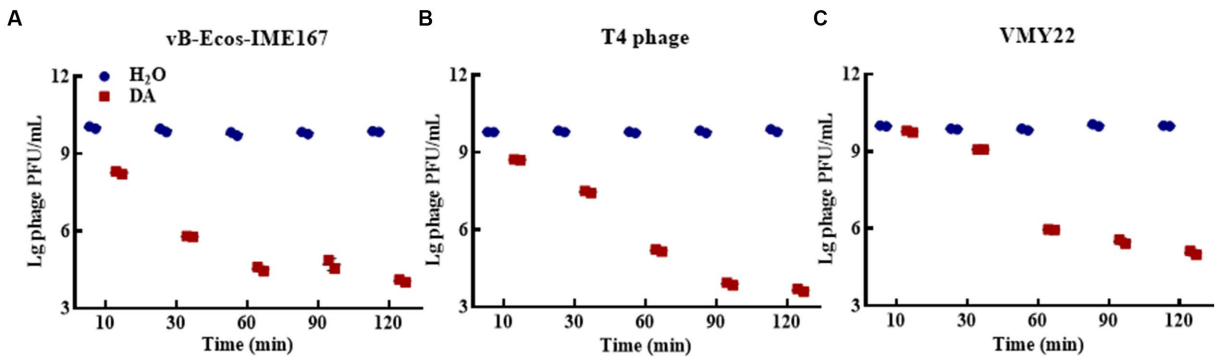


FIGURE 3
The different incubation times of DA. **(A–C)** show anti-phage activity against vB-EcoS-IME167, T4 phage, and VMY22, respectively. Bacteriophages vB-EcoS-IME167, T4 phage, and VMY22 were treated with DA for 10, 30, 60, 90, or 120 min. The plaque number was recorded using a double-layered plate and plaque forming units (pfu/mL) were calculated using the formula (pfu/mL = plaque number × 10 × dilution times); the experiment was performed in triplicate.

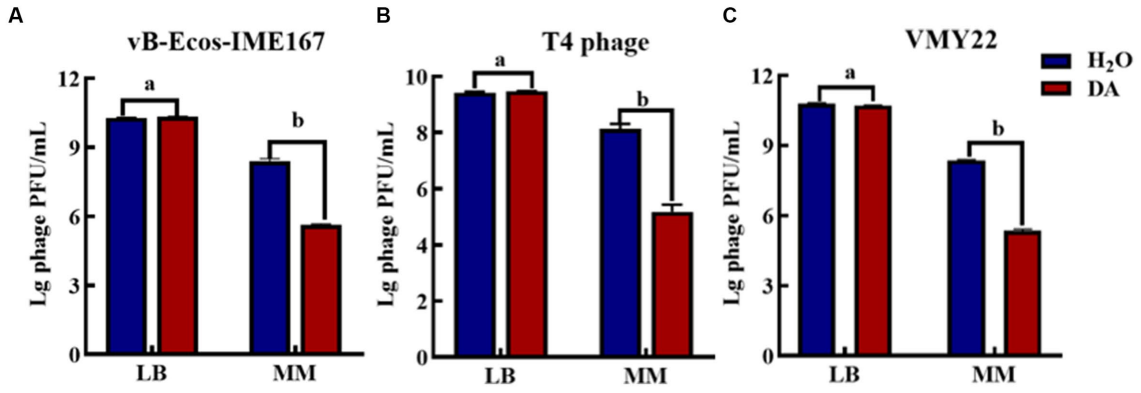


FIGURE 4
The anti-phage effect of DA treatment in different media. **(A–C)** show the anti-phage activity when phages were treated with DA in Luria-Bertani (LB) or minimal (MM) media. Bacteriophage concentrations were diluted in LB and MM media. After receiving DA treatment, these bacteriophages were introduced to a bacterial solution. The plaque number was recorded using the double-layer plate method and the plaque forming units (pfu) were calculated using the following formula: pfu/mL = plaque number × 10 × dilution times. Error bars indicate the standard deviations of three replicate cultures. Different letters indicate significant differences (one-way Prism with Tukey's *post hoc* test; a: $p > 0.05$, b: $p < 0.05$).

sterile water control) were detected using TEM. Figures 6A–C show the resultant images of the structure of vB-EcoS-IME167 treated with sterile water, low DA concentrations, and high DA concentrations, respectively. Figure 6A (sterile water treatment) shows a slender phage with good integrity, with a head measuring approximately 40 × 40 nm and a slender tail approximately 150 nm in length. These characteristics are consistent with those reported in previous studies (Li Y. et al., 2019). Figure 6B (low DA exposure) shows an evidently blurred head and a cross-linked tail of the phage. In Figure 6C (high DA exposure), the phage displays an aggregated structure in which the head is connected, and the tail is tightly wrapped around it. Overall, DA significantly altered the morphological characteristics of vB-EcoS-IME167.

TEM images of the T4 phage exposed to similar treatments (Figures 6D–F) showed that, although the phages were fixed in the contraction state of the caudal sheath, they still had complete components of the T4 phage following treatment with sterile water (Figure 6D), including the caudal sheath, caudal tube, substrate, caudal tail, and caudal filaments of the head, neck, and tail (Ye et al., 2019). At low DA concentrations (Figure 6E), the head of the phage showed evident deformation, and the tail filaments and original complete morphology were lost. At high DA concentrations (Figure 6F), an image of a completely deformed T4 phase was obtained. The head was completely deformed and broken to release inclusions, the head and tail began to separate, most of the tail protein was deformed and fell off, and the phage decomposed. The morphological characteristics of the T4 phage were significantly altered by DA, similar to the results observed for vB-EcoS-IME167.

Lastly, TEM images for VMY22 are presented in Figures 6G–I. Figure 6G (sterile water treatment) shows the distinguishable hexagonal prism-like head, collar-like neck, and short straight tail of VMY22. The head size is 60 × 35 nm, the neck collar protein size is approximately 10 nm, and the tail length is approximately 40 nm (Fang et al., 2013). As shown in Figure 6H, following low DA exposure, the short, straight tail of most VMY22 phages was missing, the head and neck proteins remained intact, and the morphology was incomplete. Figure 6I shows that, after exposure

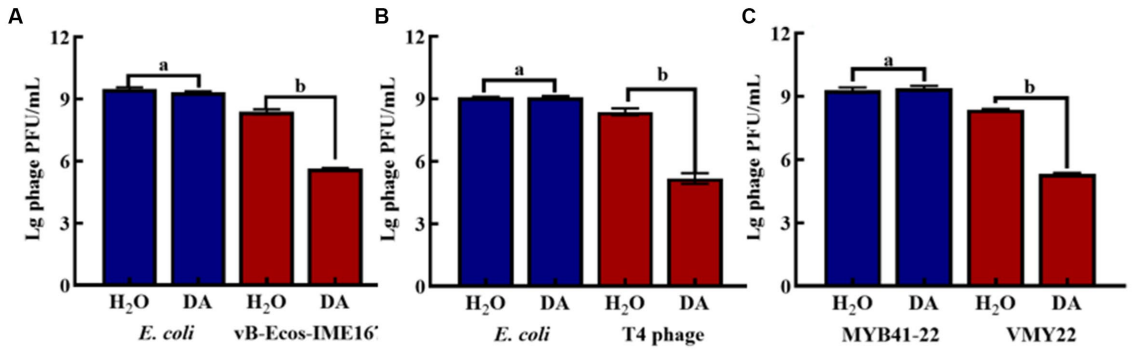


FIGURE 5
The effect of anti-phage activity of DA under different cultivation conditions. **(A–C)** show results for host bacteria and phages that were treated with either DA or sterile water (control). Phage spots were recorded using the double-layer plate method, and plaque forming units (pfu) were calculated using the following formula: pfu/mL = plaque number × 10 × dilution times. Error bars indicate the standard deviations of three replicate cultures. Different letters indicate significant differences (one-way Prism with Tukey's *post hoc* test; a: $p > 0.05$, b: $p < 0.05$).

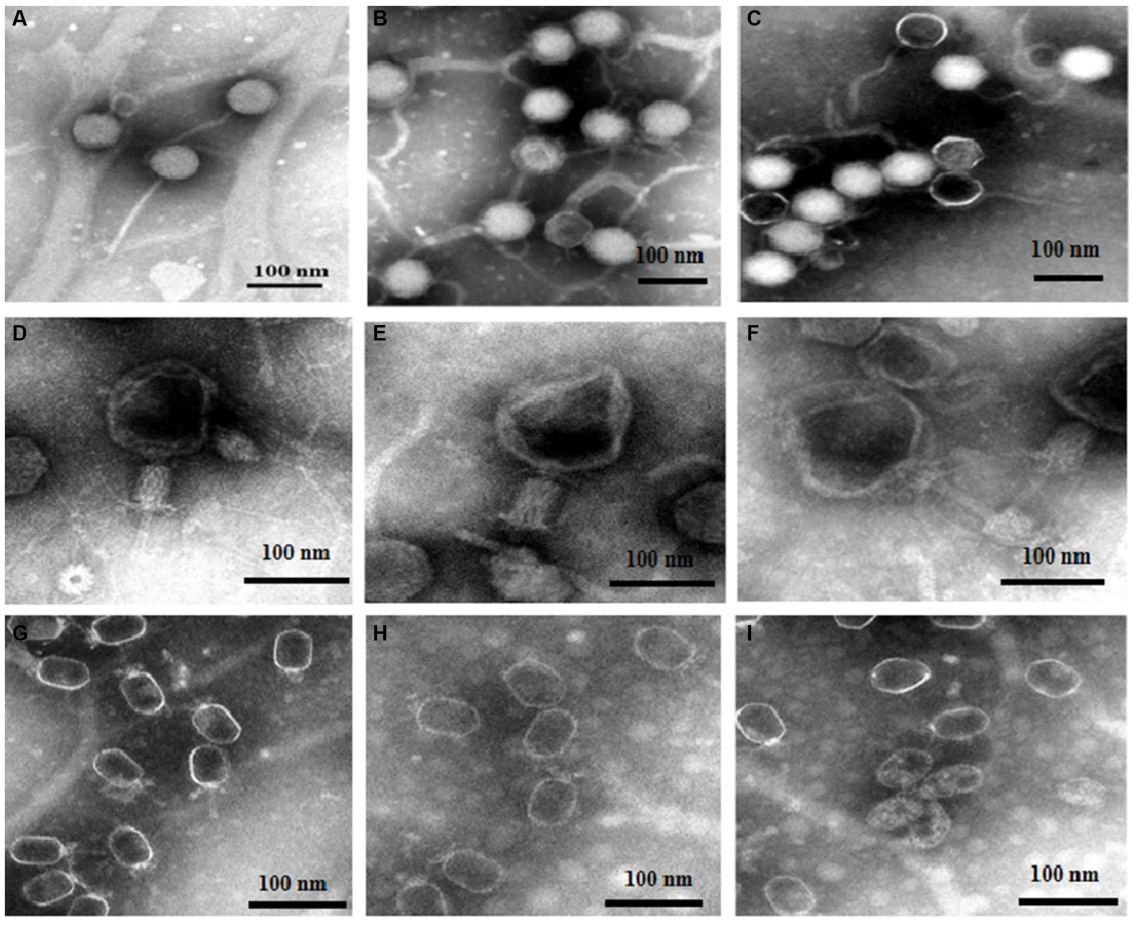


FIGURE 6
The morphological properties of different phages treated with DA under TEM. Scanning of the morphology and appearance of the three bacteriophages in different treatment groups was performed using transmission electron microscopy. **(A–C)** TEM images of vB-EcoS-IME167; **(D–F)** TEM images of the T4 phage; and **(G–I)** TEM images of VMY22. **(A)**, **(D)**, and **(G)** TEM images of phages in sterile water, **(B)**, **(E)**, and **(H)** at low concentrations of DA, and **(C)**, **(F)**, and **(I)** at high concentrations of DA.

to high DA concentrations, only the deformed head shell of the VMY22 remained. Even the deformed head shell was gathered, shrunk, and broken, and no original morphological characteristics of the VMY22 phage were evident. These three images revealed that DA significantly affected the morphological characteristics of VMY22 phages.

TEM images of vB-EcoS-IME167, the T4 phage, and VMY22 showed that the phages were not resistant to DA. We speculate that the anti-phage activity of DA first affects the complete morphology of the phages and then obstructs the specific adsorption step during phage infection.

## 3.7. Quantitative RT-PCR analysis

The accuracy of the hypothesized DA mechanism was further verified by comparing the changes in the adsorption rate using RT-PCR. As shown in Figure 7A, a significant difference was observed in the adsorption rate between DA-treated samples and the control. The copy number in DA-treated samples was lower than that in sterile water controls at each dilution, initially confirming that the anti-infection mechanism of DA on vB-EcoS-IME167 was caused by the destruction of specific adsorption. T4 phage and VMY22 also exhibited a similar phenomenon, with much lower DA than that of sterile water, indicating that DA could inhibit the specific adsorption of T4 phages and VMY22. These trends were consistent. Due to the resistance of DA to these three phages, the anti-phage activity of DA shows potential for application in the fermentation industry to control phage contamination.

# 4. Discussion

DA is an essential neurotransmitter in humans and a hormone-like reagent in plants and fungi. In this study, a potential anti-phage function of DA was discovered, and its mechanism was illustrated. This is the first report of the significant anti-phage activity of DA. The anti-phage activity of DA was found to be strictly dependent on its treatment time and dosage, and the lowest effective concentration of DA was 0.1–1 mg/mL. The half-maximal inhibitory concentrations of DA against phages vB-EcoS-IME167, T4, and VMY22 were 0.26, 0.12, and 0.73 mg $mL^{-1}$, respectively. The anti-infection ability of DA was more than $10^4$-fold, and the resistance was significantly higher than that of previously reported antiviral agents (Ferro et al., 2018; Liao et al., 2020).

Currently, research on the relationship between catecholamines and microorganisms is primarily based on an increase in bacterial susceptibility to catecholamines (Halang et al., 2015); however, the anti-phage activity of DA has not been previously reported. In this study, we demonstrate, for the first time, that DA can significantly block phage infection in host bacteria. The TEM and RT-PCR results revealed that DA primarily affected bacteriophage morphology, thus reducing their adsorption rate onto bacteria. Most antiviral drugs achieve anti-infective effects by specifically acting on the adsorption process of the virus to host cells (Astani et al., 2012, 2014; Denzler et al., 2016). Approximately 50–70% of human DA originates from the digestive tract and is produced in large quantities by the intestinal flora. Studies have shown high concentrations of DA in the intestinal cavity: $10^{-7}$ to $10^{-5}$ mol/L in the pancreas, $10^{-9}$ to $10^{-5}$ mol/L in the small intestine, and $10^{-8}$ to $10^{-4}$ mol/L in the colon (Asano et al., 2012). In this study, a DA concentration of approximately $10^{-4}$ to $10^{-3}$ mol/L effectively blocked phage infection in bacterial hosts, which is slightly higher than the existing DA concentration in the intestinal environment; however, under stress, the local concentration may be much higher (Bansal et al., 2007). Upon infection, the local concentration of pathogens may be several orders of magnitude higher than the normal concentration, consistent with the effective concentration determined in this study (Lyte and Ernst, 1992; Lyte, 2004).

DA mainly affects the adsorption of bacteriophages to host bacteria by disrupting their structure. From a chemical perspective, the pH value of the DA solution is approximately 7.0–8.5. In this case, -$NH_2$ on DA molecules mainly exists in the form of $NH_3^{3+}$, and the presence of a certain ion strength may cause the protein shell of bacteriophages to deform (Wang et al., 2022), thereby affecting adsorption. Another possibility is that a certain concentration of DA monomers self-polymerize to obtain polydopamine, which exhibits strong adhesion and forms a strong interaction force with the protein shell of the bacteriophage. This force is between covalent and non-covalent bonds, affecting the assembly of the bacteriophage protein shell and making it difficult to adsorb onto the bacterial surface (Lin et al., 2007). In Figure 8, we predicted the mechanism by which DA affects phage infection based on existing knowledge and results. As DA can facilitate resistance to bacteriophage infection in the host, further studies are

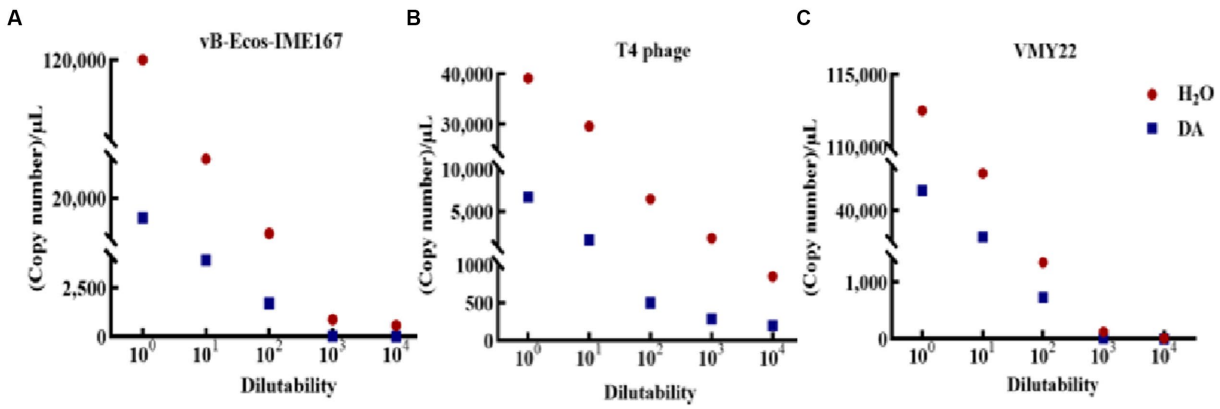


FIGURE 7
RT-PCR detection of the change of phage adsorption rate under DA treatment. The Ct values of the different treatment groups (sterile water: control group; DA: experimental group) were obtained using reverse transcription polymerase chain reaction (RT-PCR), and the corresponding copy numbers were obtained by substituting the standard curves of different phage recombinant plasmids (not shown). A lower copy number indicated a lower adsorption rate. **(A–C)** Changes in vB-EcoS-IME167, T4 phage, and VMY22 cells treated with DA and sterile water.

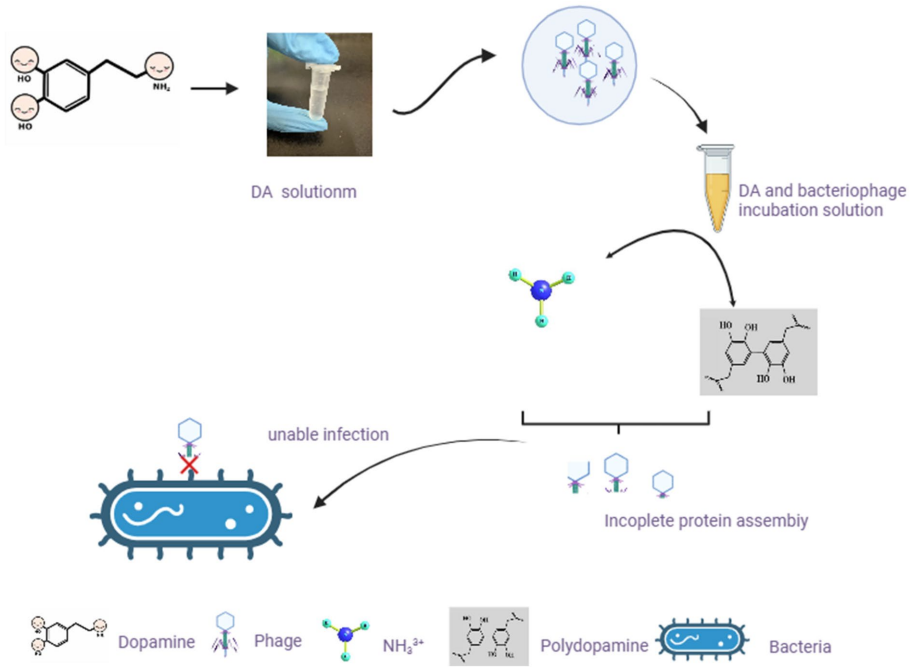


FIGURE 8
Hypothetical diagram of DA changing the morphology of bacteriophages and exerting their anti-infection mechanisms. In our study, after incubating bacteriophages with DA solution, the morphology of bacteriophages changes. Based on existing knowledge, we predicted two possible scenarios to affect the morphology of bacteriophages, resulting in the inability of bacteriophages to adsorb on the bacterial surface.

required to investigate the application of DA to treat inflammation caused by disorders of the intestinal microbial system.

Recently, research on dopamine has not been limited to neurotransmitters, and several studies have focused on its role in microorganisms. Microorganisms produce, modify, and respond to the same neurochemicals utilized in various signaling pathways in their mammalian hosts. This is the mechanism by which the host and microbiota interact to influence the progression of infectious diseases and behavior through the microbiota-gut-brain axis (Samson et al., 2013; Van Houte et al., 2016). Bacteriophages exist in the presence of bacteria; however, the effects of catecholamines on bacteriophages have not been reported. The relationship between catecholamines and bacteriophages is very complex, with much remaining unknown, and the role of DA in the defense and anti-defense of bacteriophage systems similarly remains unclear, which warrants further exploration.

## Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

## Author contributions

XH: Conceptualization, Data curation, Methodology, Supervision, Writing – original draft, Formal analysis. CZ: Formal analysis, Methodology, Supervision, Writing – original draft. YJ: Methodology, Supervision, Formal Analysis, Project administration, Writing – review & editing. XL: Conceptualization, Investigation, Writing – review & editing. SZ: Conceptualization, Investigation, Data curation, Methodology, Supervision, Writing – original draft. YW: Conceptualization, Funding acquisition, Project administration, Resources, Validation, Visualization, Writing – original draft, Data curation.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## References

Ackermann, H. W. (2007). 5500 phages examined under an electron microscope. *Arch. Virol.* 152, 227–243. doi: 10.1007/s00705-006-0849-1

Allen, J. F. (2003). Superoxide as an obligatory catalytic intermediate in the photosynthetic reduction of oxygen by adrenaline and dopamine. *Antioxid. Redox Signal.* 5, 7–14. doi: 10.1089/152308603321223496

Asano, Y., Hiramoto, T., Nishino, R., Aiba, Y., Kimura, T., Yoshihara, K., et al. (2012). Critical role of gut microbiota in the production of biologically active, free catech olamines in the gut lumen of mice. *Am. J. Physiol-Gastr L.* 11: G1288–G95. doi: 10.1152/ajpgi.00341.2012

Astani, A., Navid, M. H., and Schnitzler, P. (2014). Attachment and penetration of acyclovir-resistant herpes simplex virus are inhibited by *Melissa officinalis* extract. *Phytother. Res.* 28, 1547–1552. doi: 10.1002/ptr.5166

Astani, A., Reichling, J., and Schnitzler, P. (2012). *Melissa officinalis* extract inhibits attachment of herpes simplex virus in vitro. *Chemotherapy* 58, 70–77. doi: 10.1159/000335590

Bansal, T., Englert, D., Lee, J., Hegde, M., Wood, T. K., and Jayaraman, A. (2007). Differential effects of epinephrine, norepinephrine, and indole on *Escherichia coli* O157:H7 chemotaxis, colonization, and gene expression. *Infect. Immun.* 75, 4597–4607. doi: 10.1128/IAI.00630-07

Bettarel, Y., Sime-Ngando, T., Amblard, C., and Dolan, J. (2004). Viral activity in two contrasting lake ecosystems. *Appl. Environ. Microbiol.* 70, 2941–2951. doi: 10.1128/AEM.70.5.2941-2951.2004

Clokie, M. R., Millard, A. D., Letarov, A. V., and Heaphy, S. (2011). Phages in nature. *Bacteriophage* 1, 31–45. doi: 10.4161/bact.1.1.14942

Davies, E. V., Winstanley, C., Fothergill, J. L., and James, C. E. (2016). The role of temperate bacteriophages in bacterial infection. *FEMS Microbiol. Lett.* 363:fnw015. doi: 10.1093/femsle/fnw015

De La Higuera, I., and Lázaro, E. (2022). Viruses in astrobiology. *Front. Microbiol.* 13:1032918. doi: 10.3389/fmicb.2022.1032918

Denzler, K., Moore, J., Harrington, H., Morrill, K., Huynh, T., Jacobs, B., et al. (2016). Characterization of the physiological response following in vivo administration of Astragalus membranaceus. *Evid. Based Complement. Alternat. Med.* 2016:6861078. doi: 10.1155/2016/6861078

Eisenhofer, G., Kopin, I. J., and Goldstein, D. S. (2004). Catecholamine metabolism: a contemporary view with implications for physiology and medicine. *Pharmacol. Rev.* 56, 331–349. doi: 10.1124/pr.56.3.1

Fan, Y., and Pedersen, O. (2021). Gut microbiota in human metabolic health and disease. *Nat. Rev. Microbiol.* 19, 55–71. doi: 10.1038/s41579-020-0433-9

Fang, Y., Ji, X., Zhang, Q., Lin, L., and Wei, Y. (2013). Isolation and characterization of a *Bacillus cereus* cold-active bateriophage phage. *Chin. J. Microecol.* 25, 745–749. doi: 10.13381/j.cnki.cjm.2013.07.001

Ferro, S., Gitto, R., Buemi, M. R., Karamanou, S., Stevaert, A., Naesens, L., et al. (2018). Identification of influenza PA-Nter endonuclease inhibitors using pharmacophore- and docking-based virtual screening. *Bioorg. Med. Chem.* 26, 4544–4550. doi: 10.1016/j.bmc.2018.07.046

Fortier, L. C., and Sekulovic, O. (2013). Importance of prophages to evolution and virulence of bacterial pathogens. *Virulence* 4, 354–365. doi: 10.4161/viru.24498

Gordeeva, J., Morozova, N., Sierro, N., Isaev, A., Sinkunas, T., Tsvetkova, K., et al. (2019). BREX system of *Escherichia coli* BREX system distinguishes self from non-self by methylation of a specific DNA site. *Nucleic Acids Res.* 47, 253–265. doi: 10.1093/nar/gky1125

Gubbi, S., Nazari, M. A., Taieb, D., Klubo-Gwiezdzinska, J., and Pacak, K. (2020). Catecholamine physiology and its implications in patients with COVID-19. *Lancet Diabetes Endocrinol.* 8, 978–986. doi: 10.1016/S2213-8587(20)30342-9

Halang, P., Toulouse, C., Geißel, B., Michel, B., Flauger, B., Müller, M., et al. (2015). Response of *Vibrio cholerae* to the catecholamine hormones epinephrine and norepinephrine. *J. Bacteriol.* 197, 3769–3778. doi: 10.1128/JB.00345-15

Hampton, H. G., Watson, B. N. J., and Fineran, P. C. (2020). The arms race between bacteria and their phages. *Nature* 577, 327–336. doi: 10.1038/s41586-019-1894-8

Hille, F., Richter, H., Wong, S. P., Bratovič, M., Ressel, S., and Charpentier, E. (2018). The biology of CRISPR-Cas: backward and forward. *Cells* 172, 1239–1259. doi: 10.1016/j.cell.2017.11.032

Jackson, S. A., Mckenzie, R. E., Fagerlund, R. D., Kieper, S. N., Fineran, P. C., and Brouns, S. J. (2017). CRISPR-Cas: adapting to change. *Science* 356:aal5056. doi: 10.1126/science.aal5056

Jiang, J. L., Qiu, Y. H., Peng, Y. P., and Wang, J. J. (2006). Immunoregulatory role of endogenous catecholamines synthesized by immune cells. *Sheng Li Xue Bao: [Sheng Li Xue Bao]* 58, 309–317.

Juárez Olguín, H., Calderón Guzmán, D., Hernández García, E., and Barragán Mejía, G. (2016). The role of dopamine and its dysfunction as a consequence of oxidative stress. *Oxidative Med. Cell. Longev.* 2016:9730467. doi: 10.1155/2016/9730467

Kaufmann, H., Norcliffe-Kaufmann, L., and Palma, J. A. (2020). Baroreflex dysfunction. *N. Engl. J. Med.* 382, 163–178. doi: 10.1056/NEJMra1509723

Li, P., Lin, H., Mi, Z., Xing, S., Tong, Y., and Wang, J. (2019). Screening of polyvalent phage-resistant *Escherichia coli* strains based on phage receptor analysis. *Front. Microbiol.* 10:850. doi: 10.3389/fmicb.2019.00850

Li, Y., Zhang, Y., Zhang, X. L., Feng, X. Y., Liu, C. Z., Zhang, X. N., et al. (2019). Dopamine promotes colonic mucus secretion via the dopamine D5 receptor in rats. *Am. J. Physiol. Cell Physiol.* 316, C393–C403. doi: 10.1152/ajpcell.00261.2017

Liao, Y., Ye, Y., Li, S., Zhuang, Y., Chen, L., Chen, J., et al. (2020). Synthesis and SARs of dopamine derivatives as potential inhibitors of influenza virus PAN endonuclease. *Eur. J. Med. Chem.* 189:112048. doi: 10.1016/j.ejmech.2020.112048

Lin, Q., Gourdon, D., Sun, C., Holten-Andersen, N., Anderson, T. H., Waite, J. H., et al. (2007). Adhesion mechanisms of the mussel foot proteins mfp-1 and mfp-3. Proc. *Natl. Acad. Sci.* 104, 3782–3786. doi: 10.1073/pnas.0607852104

Liu, C. Z., and Zhu, J. X. (2020). Source, metabolism and function of dopamine in digestive tract. *Sheng Li Xue Bao: [Acta Physiol. Sin.]* 72, 336–346. doi: 10.13294/j.aps.2020.0036

Lyte, M. (2004). Microbial endocrinology and infectious disease in the 21st century. *Trends Microbiol.* 12, 14–20. doi: 10.1016/j.tim.2003.11.004

Lyte, M., and Ernst, S. (1992). Catecholamines induced growth of gram negative bacteria. *Life Sci.* 50, 203–212. doi: 10.1016/0024-3205(92)90273-r

Manning, A. J., and Kuehn, M. J. (2011). Contribution of bacterial outer membrane vesicles to the innate bacterial defense. *BMC Microbiol.* 11:258. doi: 10.1186/1471-2180-11-258

Norcliffe-Kaufmann, L. (2022). Stress the baroreflex. *Auton. Neurosci.* 238:102946. doi: 10.1016/j.autneu.2022.102946

Reyes-Robles, T., Dillard, R. S., Cairns, L. S., Silva-Valenzuela, C. A., Housman, M., Ali, A., et al. (2018). *Vibrio cholerae* outer membrane vesicles inhibit bacteriophage infections. *J. Bacteriol.* 200:e00792–17. doi: 10.1128/JB.00792-17

Samson, J. E., Magadán, A. H., Sabri, M., and Moineau, S. (2013). Revenge of the phages: defeating bacterial defences. *Nat. Rev. Microbiol.* 11, 675–687. doi: 10.1038/nrmicro3096

Sharaff, F., and Freestone, P. (2011). Microbial endocrinology. Cent. Eur. *J. Biol.* 6, 685–694. doi: 10.2478/s11535-011-0067-z

Skirycz, A., Swiedrych, A., and Szopa, J. (2005). Expression of human dopamine receptor in potatoes (*Solanum tuberosum*) results in altered tuber carbon metabolism. *BMC Plant Biol.* 5:1. doi: 10.1186/1471-2229-5-1

Szopa, J., Wilczyński, G., Fiehn, O., Wenczel, A., and Willmitzer, L. (2001). Identification and quantification of catecholamines in potato piants (*Solanum tuberosum*) by GC-MS. *Phytochemistry* 58, 315–320. doi: 10.1016/s0031-9422(01)00232-1

Tzipilevich, E., Habusha, M., and Ben-Yehuda, S. (2017). Acquisition of phage sensitivity by bacteria through exchange of phage receptors. *Cells* 168, 186–199.e12. doi: 10.1016/j.cell.2016.12.003

Van Houte, S., Buckling, A., and Westra, E. R. (2016). Evolutionary ecology of prokaryotic immune mechanisms. *Microbiol. Mol. Biol. Rev.* 80, 745–763. doi: 10.1128/MMBR.00011-16

Villageliú, D., and Lyte, M. (2018). Dopamine production in *Enterococcus faecium*: a microbial endocrinology-based mechanism for the selection of probiotics based on neurochemical-producing potential. *PLoS One* 13:e0207038. doi: 10.1371/journal.pone.0207038

Wang, Z. J., Zeng, L., Fu, L. W., Chen, Q. M., He, Z. Y., Zeng, M. M., et al. (2022). Effect of ionic strength on heat-induced gelation behavior of soy protein isolates with ultrasound treatment. *Molecules* 27:8221. doi: 10.3390/MOLECULES27238221

Ye, L., Zhong, Y., Wang, P., and Song, Z. (2019). Advances in the study of T4-like bacteriophages of the Myoviridae family. *J. Pathog. Biol.* 14, 606–609. doi: 10.13350/jcjpb.190525

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# Characterizations of novel broad-spectrum lytic bacteriophages *Sfin-2* and *Sfin-6* infecting MDR *Shigella* spp. with their application on raw chicken to reduce the *Shigella* load

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The evidence and prevalence of multidrug-resistant (MDR) *Shigella* spp. poses a serious global threat to public health and the economy. Food- or water-borne MDR *Shigella* spp. demands an alternate strategy to counteract this threat. In this regard, phage therapy has garnered great interest from medical practitioners and researchers as a potential way to combat MDR pathogens. In this observation, we isolated *Shigella* phages from environmental water samples and tested against various clinically isolated MDR *Shigella* spp. In this study, we have defined the isolation and detailed physical and genomic characterizations of two phages *Sfin-2* and *Sfin-6* from environmental water samples. The phages exhibited potent lytic activity against *Shigella flexneri*, *Shigella dysenteriae*, and *Shigella sonnei*. They showed absorption within 5–10 min, a burst size ranging from ~74 to 265 PFU/cell, and a latent period of 5–20 min. The phages were stable at a broad pH range and survived an hour at 50°C. The purified phages *Sfin-2* and *Sfin-6* belong to the *Siphoviridae* family with an isometric head (64.90 ± 2.04 nm and 62.42 ± 4.04 nm, respectively) and a non-contractile tail (145 ± 8.5 nm and 148.47 ± 14.5 nm, respectively). The *in silico* analysis concluded that the size of the genomic DNA of the *Sfin-2* phage is 50,390 bp with a GC content of 44.90%, while the genome size of the *Sfin-6* phage is 50,523 bp with a GC content of 48.30%. A total of 85 and 83 putative open reading frames (ORFs) were predicted in the *Sfin-2* and *Sfin-6* phages, respectively. Furthermore, a comparative genomic and phylogenetic analysis revealed that both phages represented different isolates and novel members of the T1-like phages. *Sfin-2* and *Sfin-6* phages, either individually or in a cocktail form, showed a significant reduction in the viable *Shigella* count on raw chicken samples after 72 h of incubation. Therefore, these results indicate that these phages might have a potential role in therapeutic approaches designed for shigellosis patients as well as in the biological control of MDR *Shigella* spp. in the poultry or food industry during the course of meat storage.

KEYWORDS

**bacteriophage, *Shigella* spp., phage therapy, genome sequencing, large terminase**

# 1 Introduction

Shigellosis or bacillary dysentery is an acute inflammatory diarrheal disease in most of the developing countries affecting nearly 165 million people each year (WHO, 2013). Though the number of reported deaths has decreased, shigellosis still causes high morbidity and mortality, particularly among children and young adults (Sur et al., 2004). The genus *Shigella* having four pathogenic serogroups (*Shigella dysenteriae, Shigella flexneri, Shigella boydii,* and *Shigella sonnei*) is mainly associated with Shigellosis (Kotloff et al., 1999; Yang et al., 2018). The main mode of transmission is via the fecal-oral route due to the intake of contaminated food and water (Baird-Parker, 1994; Shahin et al., 2019b; Pakbin et al., 2021). The World Health Organization (WHO) recommends antibiotics for the treatment of Shigellosis; nonetheless, the extensive use of antibiotics can lead to the development of multidrug-resistant (MDR) *Shigella* species (Sivapalasingam et al., 2006; von Seidlein et al., 2006; Muthuirulandi Sethuvel et al., 2017; Puzari et al., 2018). Although, recently, there have been some antibiotics suggested for the treatment, including ciprofoxacin [a fuoroquinolone (FQ)], pivmecillinam, azithromycin, and ceftriaxone (a third-generation cephalosporin) (Nandy et al., 2010; Tariq et al., 2012; Azmi et al., 2014), drug-tolerant persister *S. flexneri* and FQ-resistant *Shigella* spp. have still been identified in many Asian countries, including India (Taneja and Mewara, 2016; Puzari et al., 2018; Sethuvel et al., 2019). Hence, the repetitive transition in the antimicrobial resistance behavior of *Shigella* hinders the development of standard drugs against shigellosis. The potential ability of these bacteria to gain and disperse exogenous genes through mobile genetic elements, such as plasmids, transposons, insertion sequences, and genomic islands, is mainly responsible for the emergence of their multidrug-resistant strains (Muthuirulandi Sethuvel et al., 2019; Ranjbar and Farahani, 2019).

Bacteriophages are specific viruses that have the capability to infect and kill their target bacterial cells (Ayariga et al., 2018; Li et al., 2020; Ayariga Joseph et al., 2021; Gildea et al., 2022a,b; Ibrahim et al., 2023). The characteristics of bacteriophages, such as ubiquitous nature, host specificity, safety, antimicrobial activity, and surface decontamination ability, make them a suitable agent for therapeutic purposes (Peng et al., 2019). Currently, antibiotic resistance is a difficult problem to overcome, and due to the host specificity, phages are not ideal for broad-spectrum use; however, a combination of different bacteriophages, known as phage cocktail, can be an ideal means to combat antibiotic-resistant bacterial strains, since the bacterial cocktail increases the host range of the phages (Lin et al., 2017).

There have been recent reports of several bacteriophages against *Shigella* spp. The lytic *Shigella* phages vB_SflS-ISF001, vB_SsoS-ISF002, and pSf-1 infect both *S. flexneri* and *S. sonnei* (Jun et al., 2013; Shahin et al., 2018, 2019a). The lytic phage Sfk20 infects *S. flexneri* serotypes1b, 2a, 3a, *S. sonnei*, and *S. dysenteriae* 1 but is ineffective against *S. flexneri* serotypes *4*, *6,* and *S. boydii* (Mallick et al., 2021). The novel lytic phage *Sfin-1* infects MDR *S. flexneri*, *S. dysenteriae,* and *S. sonnei* along with *Escherichia coli* C (Ahamed et al., 2019). The microviridae phage SGF3 has been reported to infect *S. flexneri* (Lu et al., 2022). Moreover, a number of phages that are involved in the serotype conversion of *S. flexneri* have been discovered, which includes SfII, Sf6, SfV, and SfX (Allison and Verma, 2000).

In addition to water, food can also serve as a possible indirect mode of transmission of *Shigella*. There are reports of the isolation of *Shigella* from different foods, including fresh vegetables, cooked chicken meat, salads, fruits, and dairy products, ultimately leading to Shigellosis outbreaks (Shahin and Bouzari, 2018; Pakbin et al., 2022). Globally, foodborne *Shigella* is estimated to cause 1–3 million disability-adjusted life years (DALYs) (Havelaar et al., 2015). Moreover, a recent analysis of *Shigella* isolates from more than 1,600 food samples, such as seafoods, fresh vegetables, and meats, revealed that 89% of the isolated *Shigella* strains were multidrug-resistant (Marami et al., 2018; Pakbin et al., 2021). Hence, effective measures are necessary to reduce *Shigella*-associated food-borne outbreaks and prevent the spread of resistant bacteria. One potential solution to this issue is the use of bacteriophages. In addition, using a mixture of several different phages, i.e., phage cocktail, provides a highly collaborative effect for antibacterial strength and a broad host range compared to using a single phage (Chan et al., 2018; Costa et al., 2019; Shahin et al., 2021).

Thus, the current study reports the isolation and detailed physical and genomic characterizations of two novel lytic bacteriophages *Sfin-2* and *Sfin-6*. In addition, the efficacy of the novel bacteriophage cocktail consisting of these two *Shigella* phages was investigated based on their ability to reduce *Shigella* loads on raw chicken ready-to-eat meat.

# 2 Materials and methods

## 2.1 Bacterial strains and multidrug resistance test

The study analyzed 50 MDR clinical isolates of *S. flexneri*, *S. dysenteriae*, *S. sonnei*, *S. boydii*, and *Salmonella enterica* serovar Typhi, as well as various strains of *E. coli* such as AG100, K12, XL1 Blue, and *E. coli* C. The stool samples of patients were collected at the Bacteriology Division of National Institute of Cholera and Enteric Diseases (NICED), Kolkata and Christian Medical College (CMC), Vellore, India to obtain all *Shigella* and *Salmonella* strains, which have been reported earlier (Table 1) (Muthuirulandi Sethuvel et al., 2017; Ahamed et al., 2019; Sethuvel et al., 2019). For the purpose of conducting various experiments, the Luria broth (LB) with or without antibiotics was used for growing bacterial strains at 37°C. Then, the growth of these strains was checked by measuring the absorbance at 600 nm.

## 2.2 Isolation, amplification, and purification of bacteriophages

Environmental water samples were collected from the Ganga river, near Barrackpore, North 24 Parganas district and Sreerampore, Hoogly district, which is ~25 km from Kolkata, West Bengal, India. The collected water samples were then filtered through filter paper (Whatman 1) to remove the particulate matter. The log phase *S. flexneri* 2a culture was added to the

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TABLE 1 Host specificity test for several clinically isolated MDR strains to *Sfin-2* and *Sfin-6* phages isolated from the water samples of Ganga river in Kolkata, West Bengal, India.

| Sl. No. | Strain ID | Bacterial isolates with different serotypes | Antimicrobial resistance profile by disc diffusion method | Lysis by Sfin-2 | Lysis by Sfin-6 |
|---|---|---|---|---|---|
| 1. | BCH5722 | *Shigella flexneri 2a (1A)* | ACTQNaCipNorOfx | + | + |
| 2. | BCH4025 | *Shigella flexneri 2a (2A)* | ACQ | + | + |
| 3. | BCH3651 | *Shigella flexneri 2a (3A)* | ACTQ | + | + |
| 4 | BCH3557 | *Shigella flexneri 2a (4A)* | CTQNa | + | + |
| 5 | BCH7151 | *Shigella flexneri 2a (5A)* | ACTQNaCipNorOfx | + | + |
| 6 | CMCFC2181 | *Shigella flexneri (1)* | AQCipCefSxt | + | + |
| 7 | BCH5762 | *Shigella dysenteriae 1(1A)* | ACTQNaCipNor | + | + |
| 8 | BCH5848 | *Shigella dysenteriae 1(2A)* | ACTQNaCipN | + | + |
| 9 | BCH5859 | *Shigella dysenteriae 1(3A)* | ACTQNaCipNorOfxAzm | + | + |
| 10 | BCH5912 | *Shigella dysenteriae 1(4A)* | ACTQNaCipNorOfx | + | + |
| 11 | BCH5946 | *Shigella dysenteriae 1(5A)* | ACTQNaCipNorOfxCef | + | + |
| 12 | CMCFC2358 | *Shigella dysenteriae (1)* | AQCipCef | + | + |
| 13 | BCH7084 | *Shigella sonnei (1)* | TQNa | + | + |
| 14 | BCH7264 | *Shigella sonnei (2)* | TQNa | + | + |
| 15 | CMCFC87 | *Shigella sonnei* | AQNaCipCefSxt | + | + |
| 16 | CMCFC1799 | *Shigella sonnei* | AQNaCipCefSxt | + | + |
| 17 | BCH3143 | *Shigella boydii (1)* | TQNa | – | – |
| 18 | BCH4324 | *Shigella boydii (2)* | TQNa | – | – |
| 19 | CMCFC2293 | *Shigella boydii* | AQNaCipCefSxt | – | – |
| 20 | BCR62 | *Salmonella enterica serovar* Typhi *(1)* | NaCipNorAzm | – | – |
| 21 | BCR43 | *Salmonella enterica serovar* Typhi (2) | NaAzm | – | – |
| 22 | | *Escherichia coli* K12 | | – | – |
| 23 | | *Escherichia coli* C | | – | – |
| 24 | | *Escherichia coli* AG100 | | – | – |
| 25 | | XL1 Blue | | – | – |

BCH, Bidhannagar Children Hospital; BCR, Bidhan Chandra Roy Hospital; CMC, Christian Medical College, Vellore; A, ampicillin; C, chloramphenicol; T, tetracycline; Q, cotrimoxazole; Na, nalidixic acid; Cip, ciprofloxacin; Nor, norfloxacin; Ofx, ofloxacin; Azm, azithromycin; Cef, cefixime; Sxt, sulfamethxazol.

water sample with 10% (w/v) peptone following the incubation at 37°C for 24 h with shaking. To remove the bacterial debris, 1% (w/v) chloroform was mixed with the culture and then shaken properly. Furthermore, after the centrifugation of the mixture, the supernatant was collected and filtered through a 0.22-μm pore membrane (Millipore, USA). A volume of 10 μl of the filtrate was inoculated as a spot on a *Shigella* spp. plate, with subsequent formation of a clear zone around the spot indicating the presence of the bacteriophage against *S. flexneri* 2a. In addition, the other *Shigella* spp. serotypes were also included in the study.

The water samples were then used for plaque assay; 200 μl *Shigella* culture (OD600 = 0.3) and 100 μl filtrate were mixed together with 3.5 ml soft agar (0.9%), and finally, LB hard agar plate was used for plating. After the incubation of the plate at 37°C for 24 h, clear distinct plaques developed on the plate, which was then transferred to a separate *Shigella* plate. An individual plaque was shifted into a 500-μl phage dilution medium (0.85% sodium chloride and 0.1% tryptone). An additional round of plaque assay was done using the above suspended phage solution. In this way, each plaque was transferred three times for the purification of the bacteriophage.

Further, the dilutions and assaying of the phage were done to obtain a confluent lysis plate. The scrapping of the layer of soft agar was carried out and dissolved in a cold phage dilution medium (0.85% sodium chloride, 0.1% tryptone), which was retained on ice for 24 h. The supernatant was then collected after centrifugation at 5,000 × g, and the phage lysate obtained was pelleted at 68,000 g for 2 h at 4°C in an ultracentrifuge, which resulted in a higher phage titer value. Moreover, cesium chloride (CsCl) density gradient centrifugation was performed ($\rho$ = 1.3, 1.5, 1.7 g/ml) at 100,000 g

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TABLE 2 Characteristics of the protein coding sequences of phage *Sfin-2* and *Sfin-6* according to the homology to protein database.

| Predicted functional CDSs | Best blastp match and identity (%) and protein family | CDS | Start | Stop | Length (bp) | CDS | Start | Stop | Length (bp) |
|---|---|---|---|---|---|---|---|---|---|
| | | *Sfin-2* | | | | *Sfin-6* | | | |
| Tail fiber protein | *Sfin-1*, 100% pfam09327COG4733 | 1 | 3,807 | 358 | 3,450 | 8 | 10,302 | 6,853 | 3,450 |
| Tail fiber | *Sfin-1*100% | 5 | 6,425 | 6,072 | 354 | 2 | 1,050 | 2,834 | 1,784 |
| Tail fiber | Escherichia phage vB_EcoS_Chao | 10 | 10,756 | 10,088 | 669 | 12 | 12,920 | 12,567 | 354 |
| Tail fiber | *Sfin-1* 98% | 78 | 44,946 | 45,809 | 864 | 16 | 17,252 | 16,584 | 669 |
| Tail assembly protein | *Sfin-1*, 100 % cl01945 | 2 | 4,483 | 3,884 | 600 | 9 | 10,978 | 10,379 | 600 |
| Tail assembly protein | *Sfin-1*, 100 % cd08073 | 3 | 5,214 | 4,480 | 735 | 10 | 11,709 | 10,975 | 735 |
| Minor tail protein | phi2457T, 100 % cl01908 | 4 | 5,993 | 5,211 | 783 | 11 | 12,488 | 11,706 | 783 |
| Minor tail protein | *Sfin-1*, 100 % cl01940 | 79 | 45,923 | 46,729 | 807 | | | | |
| Tail length tape-measure protein | phi2457T, 100 % COG4942 | 6 | 7,871 | 6,492 | 1,380 | 13 | 15,795 | 12,988 | 2,808 |
| Tail length tape-measure protein | phi2457T, 100 % pfam06791 | 7 | 9,298 | 7,916 | 1,383 | | | | |
| Capsid and scaffold protein | phi2457T, 100 % COG2369 | 19 | 16,224 | 15,112 | 1,113 | 25 | 22,721 | 21,609 | 1,113 |
| Minor capsid protein | Shigella phage phi2457T, 100% | 20 | 16,988 | 16,227 | 762 | 26 | 23,485 | 22,724 | 762 |
| Terminase large subunit | *Sfin-1*, 100 % COG5410 | 22 | 19,886 | 18,318 | 1,569 | 28 | 26,383 | 24,815 | 1,569 |
| Terminase small subunit | Shfl1, 100 % pfam16677 | 23 | 20,450 | 19,926 | 525 | 29 | 26,947 | 26,423 | 525 |
| 3′-phosphatase, 5′-polynucleotide kinase | Sfin-3, 97% | 32 | 23,523 | 22,990 | 534 | 38 | 30,021 | 29,488 | 534 |
| Holin protein | Echerichia phageADB-2, 100% | 65 | 37,499 | 37,284 | 216 | 72 | 43,999 | 43,784 | 216 |
| DNA adenine methyltransferase | ADB-2, 100 % cl05442 | 72 | 40,805 | 40,092 | 714 | 79 | 47,306 | 46,593 | 714 |
| DNA helicase | *Sfin-1*, 100 % COG1061 | 74 | 43,076 | 41,286 | 1,791 | 81 | 49,576 | 47,786 | 1,791 |
| DNA helicase | Escherichia phage ADB-2, 95% cl28899 | 75 | 43,303 | 43,067 | 237 | 82 | 49,803 | 49,567 | 237 |
| DNA primase | VbEcoS SA12KD, 99% smart00778 | 77 | 43,925 | 44,845 | 921 | 1 | 30 | 992 | 963 |
| Recombinase | Escherichia phage vB_EcoS_SA30RD, 99% pfam04404 | 81 | 47,865 | 47,218 | 648 | 4 | 3,970 | 3,323 | 648 |
| Exonuclease | *Sfin-1*, 100% cl00641 | 82 | 49,004 | 47,940 | 1,065 | 5 | 5,109 | 4,045 | 1,065 |

for 3 h at 4°C to obtain increased purification. The phage band captured between 1.7 and 1.5g/ml was gathered and then dialyzed against Tris-HCl magnesium sulfate (TM) buffer (50 mM Tris-Cl, pH 8.0 with 10 mM $MgSO_4$). Finally, the phage was stored at 4°C.

## 2.3 Host range determination

The different strains of *Shigella*, *Salmonella*, and *E. coli* were used for determining the host range of isolated phages (Table 1). After growing them through the night in nutrient broth at 37°C, 200 μl of the bacterial cell culture was mixed with 3.5 ml of the molten soft agar (0.7% w/v) and overspread onto the surface of solid basal LB agar (1.5% w/v). A suspension phage of 10 μl (about $1.0 \times 10^{10}$ PFU/ml) was used for spotting onto the bacterial lawn, which was then incubated overnight at 37°C. Clear lysis of the spot where the phage suspension was inoculated indicated the sensitivity of the bacteria. Each test was repeated three times. There were two categories of spots according to the degree of clarity: clear (+) and no reaction (–).

## 2.4 Thermal and pH stability

The thermal stability testing was performed using 1 ml of phage particles (~$16 \times 10^{13}$ pfu/ml for *Sfin-2* and $15 \times 10^{15}$ pfu/ml for *Sfin-6)*, which were incubated at 4, 37, 50, 60, 70, 80, and 90°C, with aliquots (100 μl) taken for each temperature after 5, 15, 40, and 60 min and titered by the double-layered plaque assay against *Shigella* spp. Similarly, the pH stability testing was performed on phage particles (about $16 \times 10^{12}$ pfu/ml*)* that were placed in 1 ml of TM buffer at different pH ranges between 2 and 12 (modified using HCl or NaOH for acidic or alkaline range, respectively) for 1 h at 37°C. The aliquots (100 μl) from each pH were then titered by the double-layered plaque assay against *Shigella* spp. (Wei et al., 2021).

## 2.5 Transmission electron microscopy

Ultrapure phages obtained from CsCl purification were used for electron microscopic imaging. The imaging was done at the Electron Microscopy Laboratory, University of Burdwan, West Bengal. The bacteriophage suspension (~$1 \times 10^{22}$ pfu/ml) was transferred onto the grid using a Gilson pipette and negatively stained with a 2% (w/v) uranyl-acetate solution. Then, it was examined under a JEOL JEM-1400Plus transmission electron microscope with an operating voltage of 200 kV.

## 2.6 Lytic activity of *Sfin-2* and *Sfin-6*

According to the CLSI guidelines, the isolated *Shigella* strains were extensively drug-resistant (CLSI, 2021). The method described by Wang et al. (2016) with some modifications was used for determining the bacteriolytic activity of phages. In the presence of various antibiotics, such as ampicillin (32 μg/ml), chloramphenicol (32 μg/ml), tetracycline (16 μg/ml), cotrimoxazole (25 μg/ml), nalidixic acid (32 μg/ml), ciprofloxacin (4 μg/ml), norfloxacin (16 μg/ml), and ofloxacin (8 μg/ml), the cells of *S. flexneri* 2a (strain IDBCH5722, Table 1) and *S. dysenteriae* 1 (strain IDBCH5762, Table 1) were grown. Similarly, in the presence of tetracycline (16 μg/ml), cotrimoxazole (25 μg/ml), and nalidixic acid (32 μg/ml), *S. sonnei* (strain IDBCH7084, Table 1) was grown. After centrifugation, 20 ml of cultures (OD600 = 0.3) were resuspended in 1 ml of freshly prepared LB. Furthermore, after adding phages at different multiplicity of infection (MOI) of 0.1, 0.01, and 0.001, they were allowed to adsorp for 5 min (*S. flexneri* 2a and *S. dysenteriae* 1) or 10 min (*S. sonnei* 1) at 37°C. Thereafter, the individual suspension was transferred to 20 ml of freshly made LB. At specific time intervals of 5 h duration, aliquots were taken and the bacterial cell count was recorded using the spread plate technique. The bacterial cultures inoculated only with phage dilution medium and respective antibiotics were used as the negative control.

## 2.7 One-step growth curve

A one-step growth curve experiments was executed by a procedure stated by Malek et al. (2009) with an alteration. Concisely, *Shigella* spp. (*S. flexneri* 2a, *S. dysenteriae* 1, and *S. sonnei* 1) were cultured in LB medium at 37°C with respective antibiotics. After centrifuging 20 ml of *Shigella* culture (OD600 = 0.3) at 5,000 × g at 4°C for 10 min, the resulting pellet was resublimed in 1 ml of fresh LB. Then, the phage particles at an MOI of 0.01 were mixed with *Shigella* culture. Thereafter, the suspension was incubated for enhanced adsorption (5 min for *S. flexneri* 2a and *S. dysenteriae* 1, 7 min for *S. sonnei* 1) at 37°C pursued by $10^4$-fold of dilution with 10 ml as final volume. Subsequently, during the incubation process at 37°C, 100 μl of aliquots were taken at different time intervals up to 100 min. These samples were then mixed with 200 μl of *Shigella* culture, and a double-layered agar plate assay to determine the phage titration was performed. The above experiments were carried out three times for each *Shigella* spp. The determination of the burst size was calculated as a ratio of the average bacteriophage particles produced after the burst and the average number of phage particles adsorbed.

## 2.8 Genome sequencing and analysis

The phage samples were allowed for ultra-purification just before DNA extraction as described. A sterile 2 ml centrifuge tube (Tarsons, India) was filled with 450 μl of phage lysate. After adding 1 μl of DNase I (2,000 units/ml, NEB, USA) and 5 μl of RNaseA (10 mg/ml, Thermo Scientific, USA) to the solution, it was incubated at 37°C for 1 h. Each centrifuge tube was treated with 5 mM EDTA and then incubated at 78–80°C for 20 min to denature DNase I. Then, 250 μg of Proteinase K (SRL, Mumbai, India) was added with incubation for 2 h at 55°C. After the primary treatment of the phage sample, the genomic DNA was isolated using the phage DNA isolation kit (Norgen "Canada") as per the manufacturer's instruction with modifications (Berg et al., 2016).

The kit ION Xpress (S5-00205) version 5.0.4. was utilized for accomplishing whole genome sequencing of phages. The quality of the sequence data was checked using PRINSEQ, and the reads were quality-trimmed/filtered. The filtered sequence was converted into a single contig using SPAdes 3.8.0 (Bankevich et al., 2012). Rapid Annotation Subsystem Technology (RAST) was used for the accomplishment of genome annotation (Aziz et al., 2008). The resulting nucleotide sequence of the phage genome was submitted at GenBank under accession numbers MK972831 (*Sfin-2*) and MN393473 (*Sfin-6*), respectively. By using the BLASTp program and conserved domain search (http://www.ncbi.nlm.nih.gov/), the function of the proteins encoded by various coding sequences (CDSs) was speculated (Table 2). The possible origin of replication was predicted by GeneSkew program (http://genskew.csb.univie.ac.at/). The Neural Network Promoter Prediction tool of the Berkeley Drosophila Genome Project was used to predict putative promoter regions (minimum promoter score: 0.9, http://www.fritfly.org/seq_tools/promoter.html). The ARNOLD terminator finding program was used for determining Rho-independent transcription terminators (Lesnik et al., 2001). The tRNA scan-SE search program (http://lowelab.ucsc.edu/tRNAscan-SE/) was used for identifying putative tRNAs, if any of them was present (Lowe and Chan, 2016). The Mauve procedure was conducted for whole genome comparisons (http://asap.ahabs.wisc.edu/mauve/).

## 2.9 Genome end determination of isolated phages

A comparative analysis of the phylogenetic relationships between amino acid sequences of phage terminase large subunit and those of the other phages of a familiar packaging system can be performed for recognizing the procedure of phage packaging and determining the bacteriophage genome ends (Amarillas et al., 2017). Hence, the recreation of the phylogenetic tree was done using the phages with the amino acid sequences of the large terminase. In addition, the relationships between *Sfin-2* and *Sfin-6* phages and the other phages were analyzed. For accomplishing the phylogenetic analysis, the predicted amino acid sequences of the large terminase subunit genes of the phages were retrieved from National Center for Biotechnology Information (NCBI). In this study, molecularly analyzed bacteriophages are implicated containing well-characterized dsDNA, which has different types of packaging strategies that are dependent on terminase actions (headful, 5′-extended cos ends, 3′-extended cos ends, and direct terminal repeats). ClustalW in MEGAX with default parameters were used for aligning all the sequences. The neighbor-joining method was used to construct a phylogenetic tree, and phylogenies were determined by the bootstrap value of 1,000 replicates in MEGA X.0 version (Filipski et al., 2014). Furthermore, the genome ends were recognized as shown by Amarillas and Leon-Felix (Amarillas et al., 2017). Approximately 1 μg bacteriophage DNA was digested with separate restriction enzymes (BglII, MluI) as per the manufacturer's guidelines (NEB, USA) for identifying the presence of terminally redundant genome ends that were circularly permutated. The digests produced were then heated to 80°C for 15 min followed by cooling quickly in ice or slowly at ambient temperature. Then, the digests were loaded and run on agarose gel (0.8% w/v) in TAE electrophoresis buffer after which the gel was stained with ethidium bromide (EtBr) and visualized with UV illumination. Lastly, as a DNA molecular weight marker, GeneRuler 1 kb Plus DNA Ladder (Thermo Fisher Scientific, USA) was used.

## 2.10 Characterization of the phage receptor

To determine the receptor features of *Sfin-2* and *Sfin-6* for phage host interaction, the following experiments were performed as described earlier with certain alterations (Kiljunen et al., 2011). To determine the proteinase K effect on the adsorption of phages, *S. flexneri* 2a (OD600 = 0.3) was used. The host was subjected to proteinase K treatment (250 mg/ml, SRL, Mumbai, India) for 2 h at 55°C and was left for adsorption analysis at an MOI of 0.0001. Furthermore, *S. flexneri* 2a cells were centrifuged at 5,000 × g for 5 min to determine the inhibitory action of periodate on the phage–host interaction. The pellets so obtained were dissolved into 50 mM sodium acetate (pH 5.2) solution in the presence or absence of 200 mM $NaIO_4$ and then incubated for 2 h in the dark. An adsorption assay was carried out with the washed cells following the incubation. Again, for *Sfin-2*, the *S. flexneri* 2a cell was primarily treated with proteinase K and allowed for a secondary treatment with periodate. Moreover, without proteinase K and sodium acetate, a control experiment was also performed to confirm that the probable effect is not the result of sodium acetate and host cell incubation at 55°C. For both of these assays, as a non-absorbing control, LB medium was used. In the control supernatant, the phage titer value was adjusted to 100%.

## 2.11 Efficacy of the isolated phages to reduce the *S. flexneri* 2a load on raw chicken samples by a single phage and cocktail phages

In the area where the present study was carried out, chicken is considered as a primary meat source among the meat-based food, thereby increasing the risk of *Shigella* spp contamination. Raw chicken was used in this experiment (Shahin and Bouzari, 2018). The chicken was collected from a local shop and sliced aseptically under a biosafety cabinet. The pieces were then placed on sterile petridishes and stored at 4°C until further use. *Shigella flexneri* 2a was grown in antibiotics containing LB broth at 37°C. Aseptically *S. flexneri* 2a cells ($\pm 10^9$ cfu) were carefully spread on the surface of the chicken pieces. The phage suspension of a single or cocktail of the two phages were put on to the surface of the inoculated chicken piece at a MOI of 0.1, followed by adsorption at room temperature for 10 min. Phage cocktail was prepared by adding an equal ratio of each phage. As a control, only phage suspension medium was used. After that, the treated and control samples were incubated at 4°C up to 96 h (Zhang et al., 2013). The number of viable *S. flexneri* 2a cells and the number of phages were measured at 0, 2, 24, 48, 72, and 96 h.

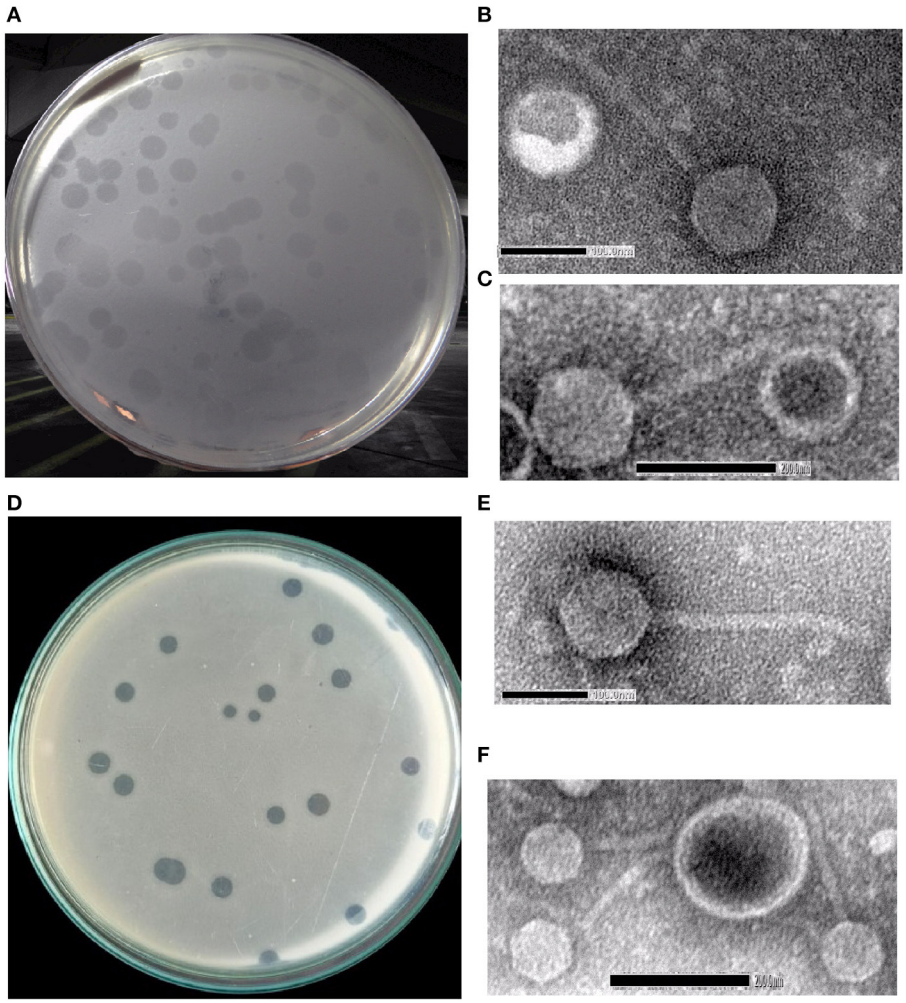


FIGURE 1
*Shigella* spp.-specific phages *Sfin-2* and *Sfin-6*. **(A, D)** Plaques of *Sfin-2* and *Sfin-6* in the lawn of *Shigella* spp. Ultra-purified phages were negatively stained and examined under electron microscope as described in Section 2. **(B, C, E, F)** The electronmicrograph broad view of the phages in 100 and 200 nm scales.

At each sampling time, the pieces of chicken were transferred to a sterile tube containing 5 ml of sodium magnesium (SM) buffer solution or 0.85% NaCl. Then, the samples were shaken at ambient temperature for half an hour. In order to harvest, the suspensions after transfer were centrifuged at 5,000 g for 10 min at room temperature. For phage-treated samples, the supernatant was collected in another microcentrifuge tube to ascertain the number of phages. In case of only host control, the pellet was washed thrice and resuspended in an equal volume of 0.85% NaCl solution. The bacterial cells were measured on HEA or XLD agar by the spread plate method, and the phage number was measured by plaque assay as mentioned previously.

## 2.12 Statistical analysis

To test the thermal stability, the titer value difference taken between 0 and 60 min were estimated for individual temperature. Student's *t*-test was applied for comparing the difference in the titer value for individual temperature to 4°C. To evaluate the details of bactericidal activity, two-way ANOVA test was performed. To analyze the phage receptor on the host cells, student's *t*-test was performed. To perform all statistical analysis, software GraphPad Prism 7.0 was used.

# 3 Results and discussion

## 3.1 Isolation of bacteriophages

The water samples from River Ganga were collected from different regions in and around Kolkata, and *Shigella*-specific phages were determined by the methods as described in Section 2. Two phages named *Sfin-2* and *Sfin-6* were isolated from the waters of the River Ganga that could proliferate in various strains of clinically isolated MDR *Shigella* spp., and they formed clear

plaques of size ranging from 1.3 to 1.9 mm in diameter with well-defined boundaries in the bacterial lawn after overnight incubation at 37°C (Figures 1A, D). The absence of more than one gene of specific phage proteins such as tail tape measure protein and large terminase subunit suggests the presence of a single type of phage in the sample.

## 3.2 Phage morphology

The morphology of purified *Sfin-2* and *Sfin-6* phages were observed using transmission electron microscopy (TEM), which revealed that *Sfin-2* and *Sfin-6* phages had an isometric head (64.90 ± 2.04 nm and 62.42 ± 4.04 nm, respectively) and a non-contractile tail (145 ± 8.5 nm and 148.47 ± 14.5 nm, respectively) anchored with a basal tuft (Figures 1B, C, E, F). The mature phage lacks a neck, base plate, spikes, or fiber. The structure of the phages according to the guidelines of the International Committee on Taxonomy of Viruses (ICTV) suggested that both of them belong to the family *Siphoviridae* and grouped into *Caudovirales* (Fauquet and Fargette, 2005).

The vast majority (over 95%) of the reported phages belong to the order *Caudovirales*, which are tailed phages. According to the Ackermann (1998), ~60% of the phages are classified under the family *Siphoviridae,* which have flexible and long tails.

## 3.3 Phage host range

Lytic spectrum of *Sfin-2* and *Sfin-6* phages were determined by spot test of pure phages on the lawn of different clinically isolated *S. flexneri*, *S. dysenteriae*, *S. boydii*, and *S. sonnei* with other enteropathogens such as *Salmonella typhi* and various *E. coli* strains, including XL1 Blue, AG100, K12, and *E. coli* C. The *Shigella* strains used in this study were resistant to various antibiotics such as amoxicillin, tetracycline, chloramphenicol, norfloxacin, ciprofloxacin, nalidixic acid, ofloxacin, cotrimoxazole, and azithromycin, which are frequently used for therapeutic purposes (Amezquita-Lopez et al., 2014) (Table 1). Spot tests revealed that both the phage suspensions, *Sfin-2* and *Sfin-6*, produced clear zones of inhibition against various serotypes of *S. flexneri*, *S. dysenteriae*, and *S. sonnei* but did not show activity against other bacterial species. This phenomenon clearly indicated that phages are polyvalent in nature.

While phages are usually very much specific, infecting only one species of bacteria, there has been a report of some polyvalent phages (Hamdi et al., 2017; Ahamed et al., 2019). The ability to lyse multiple *Shigella* strains highlighted that these phages could be explored for phage therapies against shigellosis. The wide host range of both the phages determined that the CDSs that encode host specific protein and tail component would be valuable. The main mode of transmission of *Shigella* spp. to humans is through the fecal-oral route; hence, the isolation of *Sfin-2* and *Sfin-6* phages indicated fecal contamination of the river.

## 3.4 *In vitro* bacterial challenge test

*In vitro* bacterial challenge tests were performed using both the phages, *Sfin-2* and *Sfin-6*, individually by adding the phage at an MOI of 0.1, 0.01, and 0.001 to mid-exponential phase cells (OD600 = 0.3) in the presence of multiple antibiotics chloramphenicol, ampicillin, tetracyclin, ciprofloxacin, cotrimoxazole, norfloxacin, and ofloxacin. For every single experiment, host strains were grown in the presence of respective antibiotics, whereas phage suspension medium was taken as control. Killing curves were generated by counting the viable colonies. For *Sfin-2*, the viability of bacterial cells was significantly decreased when infected with an MOI of 0.1, 0.01, and 0.001 and complete lysis occurred within 3.5 h in the case of *S. flexneri* 2a cells. For *S. dysenteriae*1, complete lysis occurred after 3.5 h of infection at an MOI of 0.1, while almost complete lysis occurred after 4.5 h at an MOI of 0.01 and 0.001. *Shigella sonnei* 1 cells were also significantly decreased, and complete lysis occurred after 3 h at an MOI of 0.1, while in the case of MOIs of 0.01 and 0.001, complete lysis occured after 3.5 h and 4.5 h of infections, respectively ($p < 0.005$; Figures 2A–C). In the case of *S. flexneri* 2a, complete lysis occurred after 3.5 h at an MOI of 0.1 and 4.5 h at MOIs of 0.01 and 0.001. The viability of bacterial cells were moderately decreased when *S. dysenteriae* 1 was infected with the phage *Sfin-6* at different MOI. Complete lysis occurred within 4.5 h at an MOI of 0.1, whereas complete lysis occurred at 5 h at MOIs of 0.01 and 0.001. The viable count of *S. sonnei* 1 cells were also decreased at an MOI of 0.1 and complete lysis occurred within 3.5 h, while MOIs of 0.01 and 0.001 showed complete lysis after 4.5 h ($p < 0.005$; Figures 2D–F). Determining the mean differences between all three MOIs and control was done by the two-way ANOVA test, which showed that they are significant ($p < 0.0001$).

The *in vitro* challenge tests established that the phages could be used to inactivate the MDR pathogenic strains of *Shigella* and, therefore, these phages could be useful as a bio control agent. The efficacy of those phages in controlling *Shigella* infection however has to be determined by *in vivo* studies. It is worth noting that a host population may resist long phage treatment, resulting in the emergence of bacterial insensitive mutants (BIMs). To combat this issue, a cocktail of phages may be used instead of a single phage (Amarillas et al., 2017). The use of phage cocktail with more than one phage that follows different infection mechanisms may solve this problem (Yamaki et al., 2014). The analysis of host cell lysis suggests that the MOI is directly dependent on cell death. The application of a higher number of phages on cells causes destabilization of its outer membrane, resulting in cell lysis. As this type of lysis are not due to the replication of phage and its release, it is called "lysis from without" (Brown and Bidle, 2014).

## 3.5 Infectivity of *Sfin-2* and *Sfin-6*

The thermal stability test was performed to investigate the heat-resistant properties of *Sfin-2* and *Sfin-6* phages. When the *Sfin-2* phage was warmed at 37 or 50°C for 5 min, the activity remained unchanged. Then, the activity slowly decreased to 0.1–0.01% when incubated at 60 or 70°C for 5 min, and only 0.0001% activity was present when heated to 80 or 90°C for 5 min. In the case of *Sfin-6*

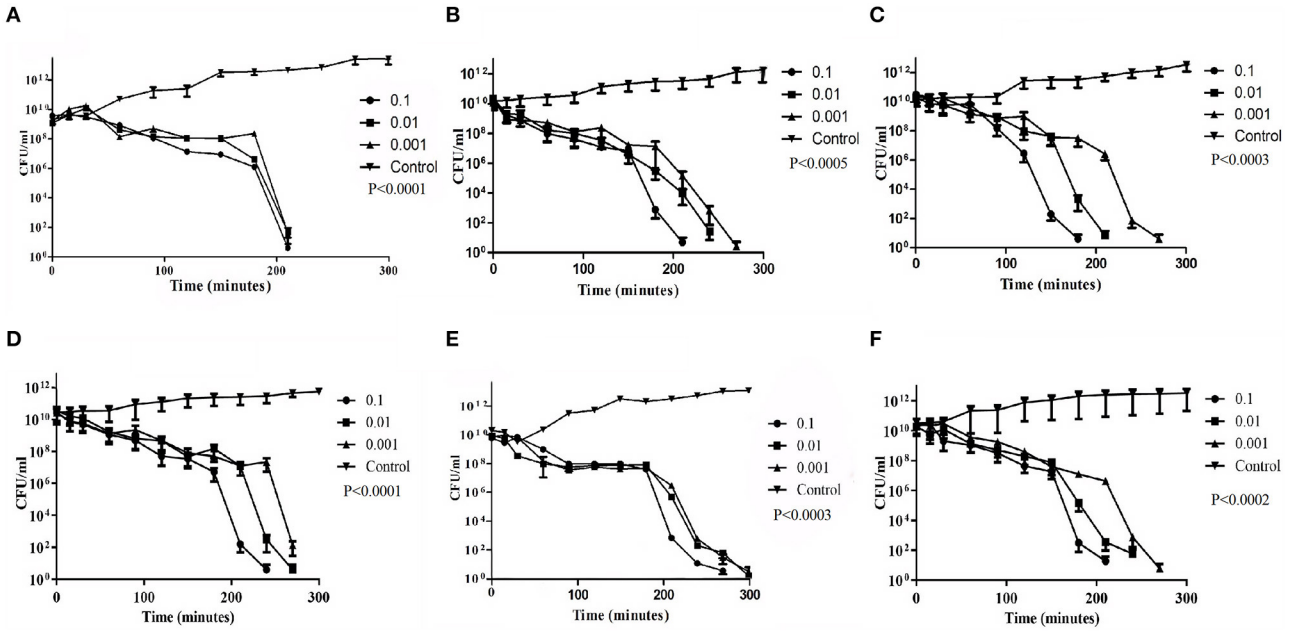


FIGURE 2
Bacterial challenge test of phage *Sfin-2* and *Sfin-6* on different clinical isolates of *Shigella* spp. Clinically isolated species of **(A, D)** *Shigella flexneri* 2a **(B, E)** *Shigella dysenteriae* 1 and **(C, F)** *Shigella sonnei* 1 were grown (OD600 = 0.3) in 20 ml of LB medium in the presence of several antibiotics. They were harvested by centrifugation, resuspended in 1 ml of LB medium, and infected with both phages at MOIs of 0.1, 0.01, and 0.001. After adsorption, the cultures were diluted 21-fold in LB medium and incubated for 5 h with shaking at 37°C. At different time intervals, viability of *Shigella* spp. was determined by the spread plate method. As the negative control, *Shigella* spp. were grown only in the presence of antibiotics. The two-way ANOVA indicated significant difference between control and phage-infected sets ($p < 0.0001$, $n = 3$).

phage, 0.01%−0.001% activity was present when incubated at 50 or 60°C for 5 min and only 0.0001% activity was retained in each case when heated at 70, 80, or 90°C for 5 min. The thermal stability of both the phages was determined by monitoring the changes of titer at different temperatures (Figures 3A, B).

The *Shigella* infection usually occurred in the intestine at acidic pH conditions (Gorden and Small, 1993). Therefore, it is essential to know the pH stability of *Sfin-2* and *Sfin-6* for controlling *Shigella* spp. For both the phages, highest activity was observed after an incubation period of 1 h at pH 7.0 at 37°C. Approximately 30% or 17% recovery of the *Sfin-2* phage and 5% or 12% recovery of the *Sfin-6* phage was found at pH 4.0 and pH 12.0, respectively ($p < 0.005$; Figures 3C, D).

Although the activity of the above phages was affected by higher and lower temperature or pH levels, remarkable activity remained at wide temperature and pH ranges. Thus, the result concluded that *Sfin-2* and *Sfin-6* phages have moderate thermal stability and a wide pH tolerance, which suggests that these phages may be used for therapeutic purposes.

## 3.6 One-step growth curve

Lytic development of *Sfin-2* and *Sfin-6* phages were investigated in one-step growth curve experiments. The adsorption above 90% for both the phages were completed within ∼5–20 min. The growth curve study of *Sfin-2* phage showed a latent period of ∼7 min with the average burst size of 105 PFU/cell against *S. flexneri* 2a. In the case of *S. dysenteriae* 1 and *S. sonnei* 1, latent periods were ∼5 and 10 min with the average burst size of 74 and 101 PFU/cell, respectively (Figures 4A–C). *Sfin-6* exhibited a latent period of ∼5 and 13 min with the average burst size of 71 PFU/cell and 163 PFU/cell for *S. flexneri* 2a and *S. dysenteriae* 1, respectively, whereas against *S. sonnei* 1, *Sfin-6* exhibited a latent period of ∼13 min with the average burst size of 265 PFU/cell (Figures 4D–F).

## 3.7 Whole genome sequencing and synteny study of *Sfin-2* and *Sfin-6* phages

The genome sequencing is essential to understand the phage biology. The genome of *Sfin-2* has 50,390 bp (GenBank accession number: MK972831) with 44.9% GC content. Among the 85 CDSs, 22 are rightward in orientation while others are leftward (Figure 5A) and 25 CDSs had annotated functions. The putative origin of replication and terminus location is ∼201 nt and 43,001 nt, respectively, which could be predicted from the GC-skew analysis (Supplementary Figure S1A). The genome of *Sfin-6* also possesses a circular genome of 50,523 bp (GenBank accession number: MN393473) with a GC content of 48.3%. Out of the 83 CDSs, 16 are rightward in orientation, while others are leftward (Figure 5B). Among them, 23 have annotated functions. The GC skew analysis suggested that the putative origin of replication and the terminus location of phage *Sfin-6* is ∼7,001 nt and 49,501 nt,

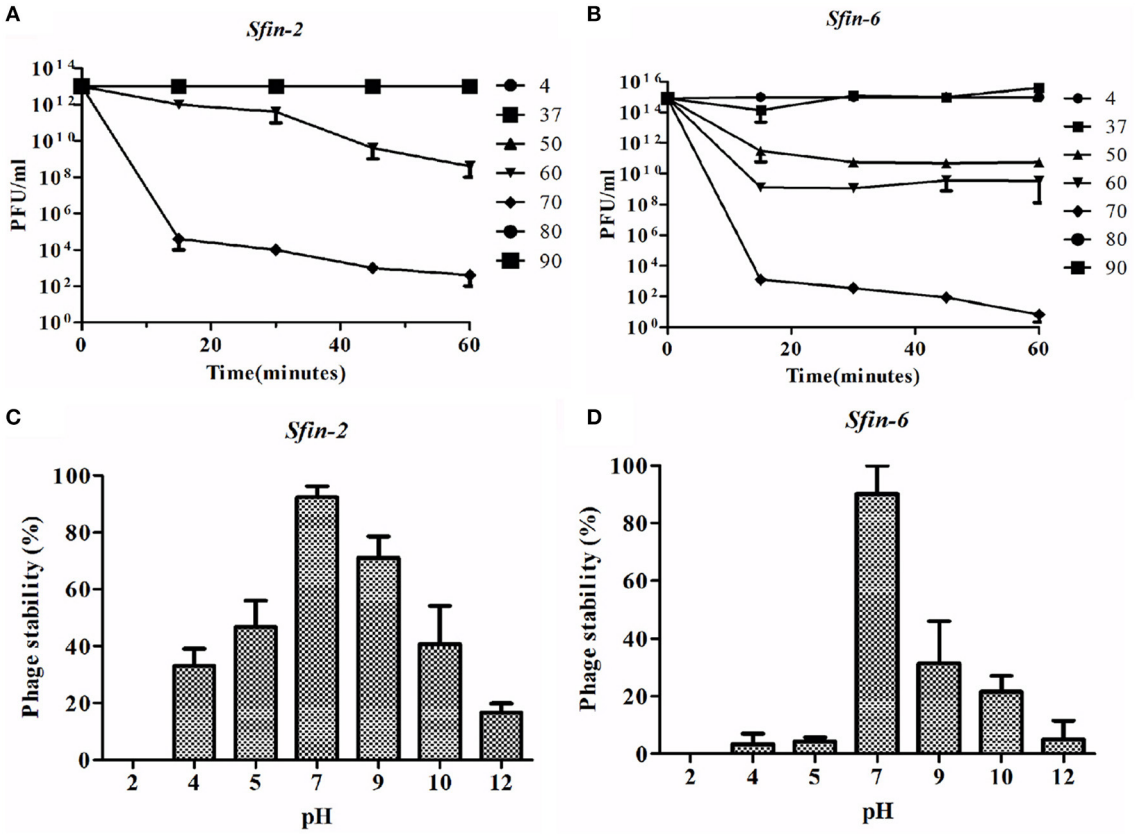


FIGURE 3
Stability of phage *Sfin-2* and *Sfin-6* in wide temperature and pH ranges. **(A, C)** Thermal stability of *Sfin-2* and *Sfin-6* phages at various temperatures. *Sfin-2* ($16 \times 10^{13}$) and *Sfin-6* ($15 \times 10^{15}$) phage particles were incubated at different temperatures in 1 ml of LB medium, and for each temperature, the number of infectious phage particles was determined using 100 μl aliquots from various time points by plaque assay against *S. flexineri* 2a. The result was plotted as mean ± SD ($n = 3$). **(B, D)** pH stability of phage *Sfin-2 Sfin-6*. In 1 ml of TM buffer having different pH, *Sfin-2* ($14 \times 10^{9}$) and *Sfin-6* ($16 \times 10^{9}$) phage particles were incubated at 37°C for 1 h, and the number of infectious phage particles from each sample was determined using 100 μl aliquots by plaque assay against *S. flexineri* 2a. The result was plotted as mean ± SD ($n = 3$).

respectively (Supplementary Figure S1B). No tRNA was found in both the genomes.

The whole genome BLAST analysis of *Sfin-2* and *Sfin-6* against the NCBI database showed that they are related to two phages, i.e., pSf-2 (GenBank accession number: KP085586) and phi2457T (GenBank accession number: MH917278). The genome of the *Sfin-2* phage showed 91.89% similarity with psf-2 and 98.8% similarity with phi2457T, while the genome of the *Sfin-6* phage showed 92.16% similarity with psf-2 and 99% similarity with phi2457T. The Mauve alignment of *Sfin-2, Sfin-6*, phi2457T, and pSf-2 resulted in one large LCB of 29,977 bp (green) and three small LCB of 5,338 bp (blue), 8,492 bp (red), and 6,478 bp (fluorescent green) indicating DNA regions that are homologous among the genomes. The gaps in the graphs indicate the non-identical region of the genome. Furthermore, the alignment of these phages showed some highly homologous regions with major rearrangements, which indicates that the phages share a common genome organization with different positions of genes (Supplementary Figure S2).

## 3.8 Module analysis

The comparative genome study of the two phages showed that genome sequence, genome size, GC contents, number of transcription terminator sequences, and CDSs are close to each other. Although gene sequences of predicted structural and functional proteins share high degree of homology, they are differently arranged and sometimes oppositely oriented. Maximum differences are present in the hypothetical proteins that are yet to be characterized. Approximately 72–75% genes of *Sfin-2* and *Sfin-6* are of unknown functions, and most of them have >78%−80% homology with their counterparts in pSf-2 and phi2457T genomes. The high degree of similarity among these phages may be due to complex evolutionary relationship, though they have been isolated from different geographical locations.

After annotation, the *Sfin-2* and *Sfin-6* proteins can be categorized into following functional groups: DNA metabolism and replication proteins; the downstream gene of *Sfin-2* mostly contains DNA metabolism and replication proteins, which includes 3′-phosphatase, 5′-polynucleotidekinase/CDS33, phage-associated N-6-DNA adenine-methyl transferase/CDS73, DNA helicase/CDS75, 76, DNA primase/CDS78, phage-associated recombinase/CDS82, and phage exonuclease/CDS83, while the upstream and downstream parts of the *Sfin-6* genome contains all of these proteins. The 3′-phosphatase, 5′-polynucleotide kinase belongs to the family pfam03767 that includes the C-terminal domain of the bifunctional enzyme T4 polynucleotide kinase/phosphatase PNKP. The role of The PNKP phosphatase

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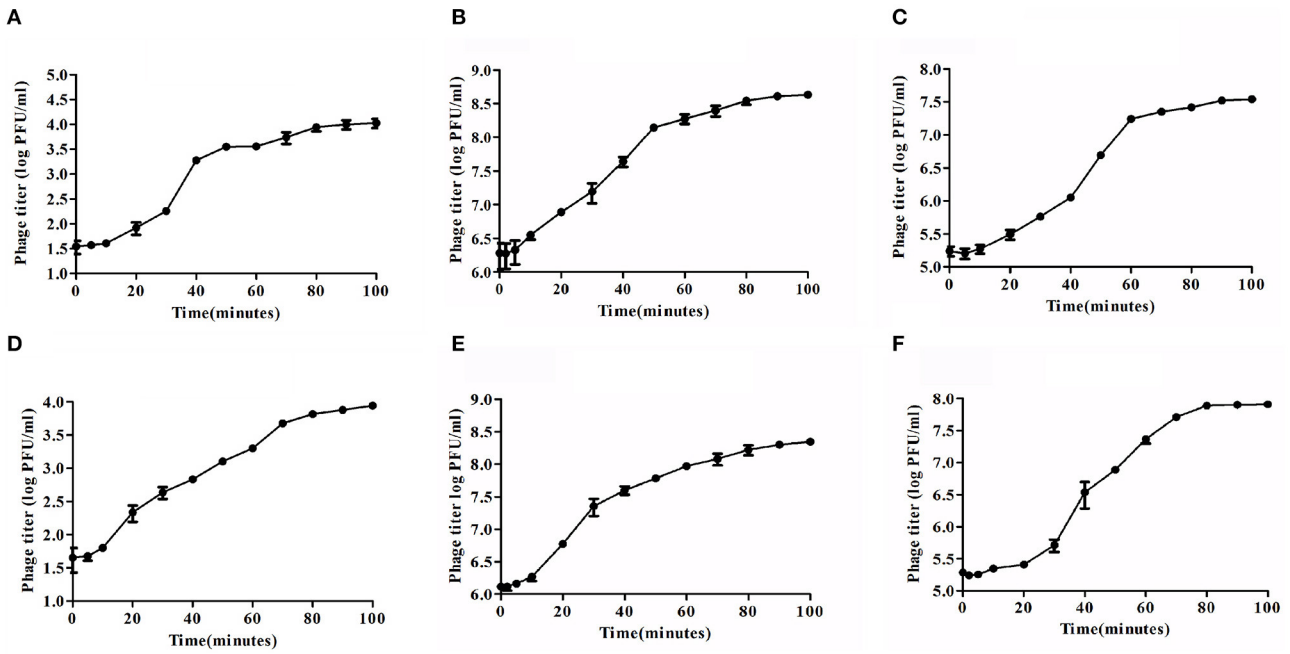


FIGURE 4
One-step growth curve of phage *Sfin-2* and *Sfin-6*. *Shigella flexneri* 2a, *Shigella dysenteriae* 1, and *Shigella sonnei* 1 were infected at an MOI of 0.01 at 37°C. After phage absorption, the cultures were diluted to $10^4$-fold and incubated at 37°C, and the titers in PFU per ml from the infected cultures at different time points were determined. The result was plotted as mean ± SD ($n = 3$). **(A, D), (B, E),** and **(C, F)** Present one-step growth curves of *Sfin-2* and *Sfin-6* in *S. flexneri* 2a, *S. dysenteriae* 1, and *S. sonnei* 1, respectively.

domain is to catalyze the elimination of the 3′-phosphoryl group of DNA, RNA, and deoxynucleoside 3′-monophosphates. The enzyme N-6-DNA adenine-methyl transferase (DAM) is a member of pfam05869 which methylates GATC sequence of its own DNA to protect it from exonuclease. The counterpart of this enzyme is present in the Escherichia phage ADB-2, which shares 99% identity with *Sfin-2* and *Sfin-6*. Both the phages have helicase coding genes that belong to the pfam04851 and are involved in ATP-dependent RNA or DNA unwinding. The primase encoded by phages belongs to pfam08273. The zinc finger motif and ATP binding region of the primase/helicase at N-terminal and C-terminal, respectively, have the origin recognition property. The ERF superfamily's pfam 04404 has the phage-associated recombinase domain that contains several single-stranded annealing proteins (SSAPs) such as Red-beta, Rad 52, ERF, and RecT, which may function as Rec-A dependent and independent DNA recombination pathways. This type of recombinase encoded by the phages promotes horizontal gene transfer by homologous recombination to accelerate the evolution by intra-phage gene shuffling. The recombinase in association with phage exonuclease takes part in the replication process from fork to nucleotide metabolism. The exonuclease encoding gene of both phages encodes an exonuclease VIII that is related to pfam12684 of the PDDEXK superfamily. Thus, 3′-phosphatase, 5′-polynucleotide kinase, phage recombinase, exonuclease are involved in DNA metabolism and recombination process of the phage genome after entering the host cells.

The sequence-based prediction of the *Sfin-2* phage showed that upstream cluster genes are involved in viral head morphogenesis and tail component formation while upstream and downstream cluster genes of *Sfin-6* are involved in viral head morphogenesis and tail component formation. CDS21 of *Sfin-2* and CDS25, CDS26 of *Sfin-6* are likely to produce phage capsid and scaffold protein belonging to Phage Mu protein F-like family which are required for viral head morphogenesis. Head and tail junction proteins, known as portal proteins, allow the phage genome into the pro head as a part of the packaging motor (Lokareddy et al., 2017). CDS23 and CDS24 of *Sfin-2* and CDS28 and CDS29 of *Sfin-6* encode phage large and small terminase subunits, which are involved in the packaging of concatameric DNA in phage capsids (Mobberley et al., 2008). CDS1, CDS4, CDS5, CDS10, CDS78, and CDS79 probably encode the tail component for *Sfin-2*, whereas CDS2 and CDS3 direct the synthesis of the protein responsible for tail assembly. CDS2, CDS8, CDS11, CDS12, and CDS16 encode the tail component for *Sfin-6*, whereas CDS9 and CDS10 direct the synthesis of the protein responsible for tail assembly. CDS6 and CDS7 for *Sfin-2* and CDS13 for *Sfin-6* encode tail tape measure protein which are the second largest genes of the phage genome. The tail length of the lambdoid phages may be hypothetically determined by the total amino acid residue of tail tape measure protein where a single amino acid is corresponding to ∼0.15 nm (Katsura, 1990). According to this hypothesis, the probable tail lengths of *Sfin-2* and *Sfin-6* phages are 140 and 138 nm long, respectively, which are much closed to the measured length of 145 and 148 nm, respectively.

CDS22 and CDS23 of *Sfin-2* and CDS28 and CDS29 of *Sfin-6* encode the large and small terminase subunit, respectively. These are mainly involved in ATP-dependent DNA packaging system.

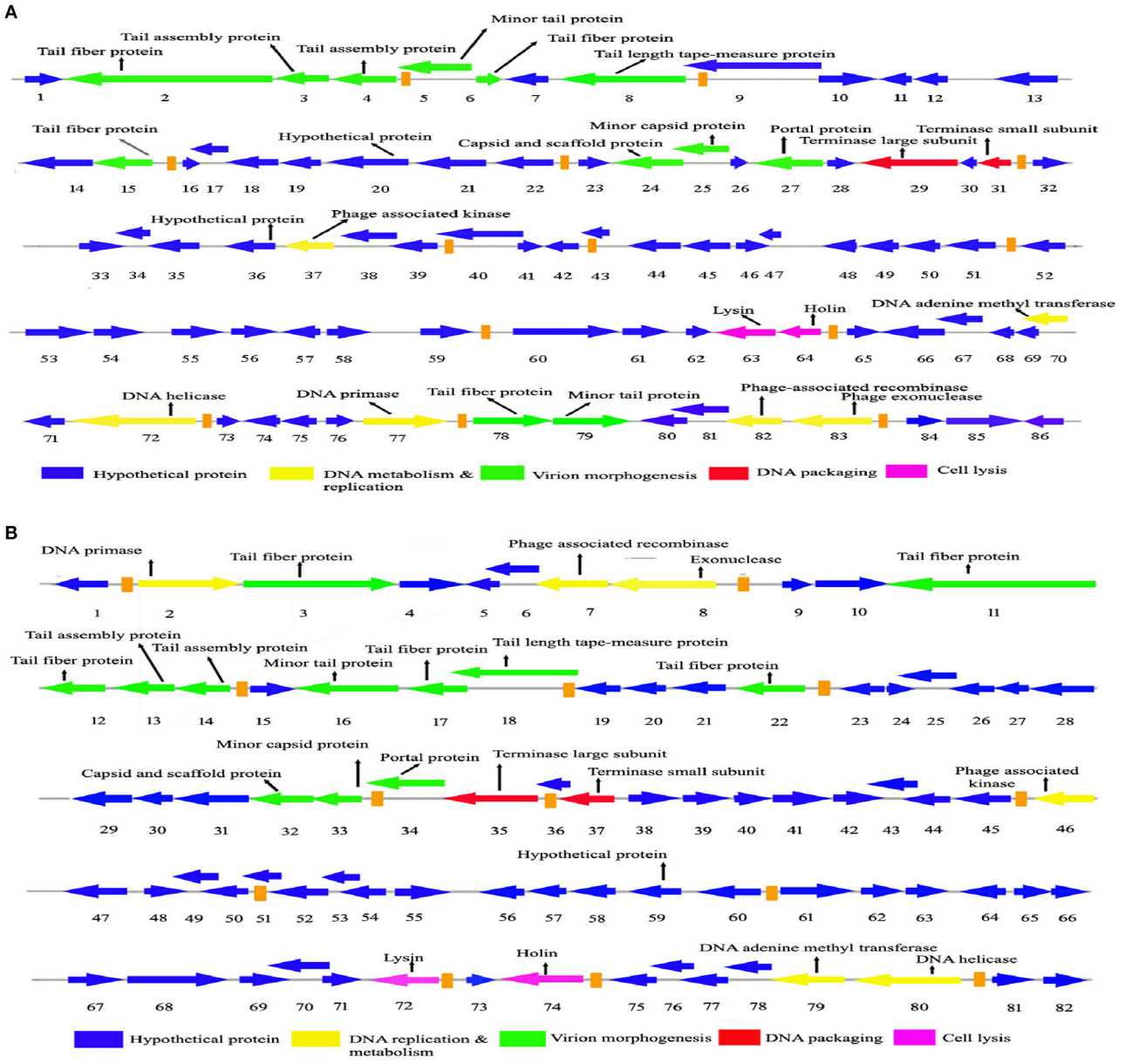


FIGURE 5
Genome organization and comparative genome analysis of *Sfin-2* and *Sfin-6*. The *Sfin-2* **(A)** and *Sfin-2* **(B)** genome maps were schematically presented. The arrows indicate the predicted CDSs and the orientation of the transcription. Predicted molecular functions of CDS were indicated by different colors: Virion morphogenesis (green arrows), DNA metabolism and replication (red arrows), DNA packaging (violet arrows), cell lysis (gray arrows), and hypothetical proteins (blue arrows).

CDS64 of *Sfin-2* and CDS71 of *Sfin-6* encode cell lysis protein lysin while CDS65 for *Sfin-2* and CDS72 for *Sfin-6* encode holins, which play an important role in host cell destruction during the burst step of the phage life cycle. After the assembly of new progeny of phages, the host cell lysed by a dual lysis system followed by a pore-forming holin protein and a cell wall degrading enzyme known as phage lysozyme or endolysin. Both lysin and holin encoding genes are located contagiously at the terminal part of *Sfin-2* and *Sfin-6* genomes. The lysin-coding gene encodes 162 amino acids along with phage lysozyme/endolysin belonging to the pfam00959 family found in dsDNA phages. Holin in association with other members of pfam 00959 cleaves the ß1,4-glycosidic linkage of polysaccharide present in the bacterial membrane (Ziedaite et al., 2005). CDS76 of *Sfin-2* and CDS83 of *Sfin-6* encode transcriptional regulatory cro protein that belongs to the HTH_XRE superfamily. Phages may use this protein to regulate transcriptional timing in the gene expression. Therefore, the presence of lysis genes and the absence of lysogeny-related genes in both the genomes clearly indicate that the phages are potent lytic phages.

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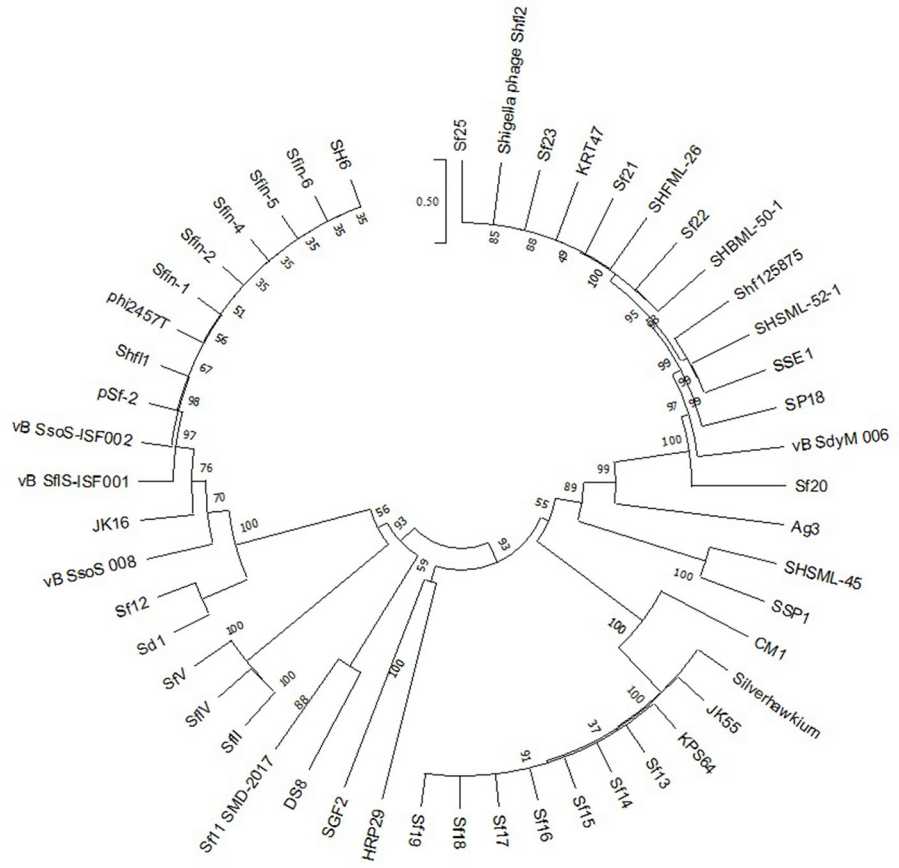


FIGURE 6
Phylogenetic study of *Sfin-2* and *Sfin-6* phages with related phages. The phylogenetic analysis based on the large terminase subunit of known packaging mechanisms phages. The bootstrap analysis was performed with 1,000 repetitions. The terminase large subunits were compared in the MEGA 7.0 version using the neighbor-joining method.

## 3.9 Determination of genome ends

Whole genome sequencing followed by the assembly of both phages revealed that they had a double-stranded DNA genome. In tailed bacteriophages, a linear genome is expected within the channel of the portal protein where only one dsDNA can pass. Therefore, the head contains a linear genome with different types of ends. However, PCR with the primers designed at the two ends of the whole genome sequence confirmed the circular nature of the *Sfin-2* and *Sfin-6* phage genomes (Supplementary Table S1, Supplementary Figure S3). Consequently, two PCRs at the adjacent of the 5′ and 3′ end of the genome were taken as the positive control (data not shown).

Phage terminase is one of the most conserved protein that creates the virion end, and this enzyme is one of the most conserved phage proteins within the group. Therefore, the comparative analysis of terminase amino acid sequence of a phage results in the same clusters with others that generate similar ends. According to the phylogenetic analysis of the large terminase subunit, *Sfin-2* and *Sfin-6* clustered with the terminase of *Shigella* phages ISF002, Shfl1, psf-2, ISF001, and *E. coli* phage ADB-2 which belong to T1 family of phage (Figure 6). According to the cluster, it is predicted that both the genomes have direct terminal repeats with possible circular permutation. In such a circularly permutated headful packaging phage category, the site of initiation cleavage is not precise and several initiation cuts are spread on concatamers. Thus, for this reason, the chromosome length of individual virions are not precise. The abovementioned types of phages are expected to contain all the fragments of the restriction digestion of the circular phage genome as well as of undigested phage DNA along with submolarpac fragment-like P22 genome (Casjens et al., 2004). The pac fragments, such as phage sf6 and ES18, may not be detected for imprecise series initiation cleavage. Hence, as a result, a blur background will be observed due to variable lengths of terminal fragments.

Restriction digests of *Sfin-2* and *Sfin-6* phage genomes by *Bgl*II and *Mlu*I were warmed at 80°C and then cooled down slowly or rapidly, and no difference was noticed between slow- and fast-cooled sets for both the enzymes. However, instead, longer fragments were observed which indicated the absence of cohesive ends in both the phage genomes. Additionally, blur background was also observed in electrophoresis gel. For the phages that contain cohesive ends are expected to anneal and appear as a longer fragment in gel electrophoresis. This result indicates that both *Sfin-2* and *Sfin-6* phages are the T1-like headful packaging phage (Figure 7).

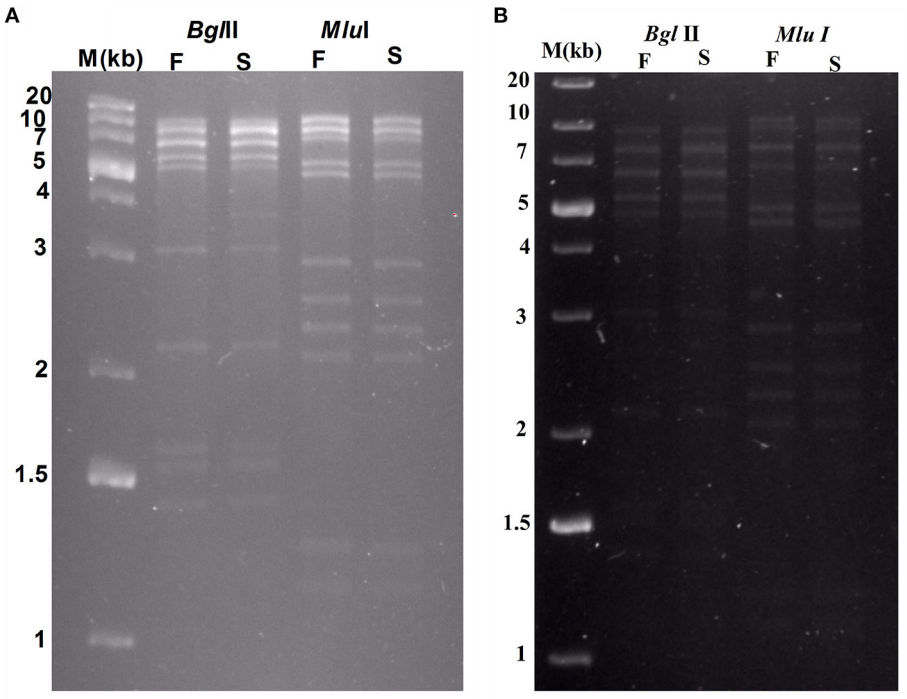


FIGURE 7
Enzymatic analysis of *Sfin-2* and *Sfin-6* genomic DNA. Phage *Sfin-2* **(A)** and *Sfin-6* **(B)** DNA was completely digested with *Bgl*II and *Mlu*I and the products were analyzed by 0.8% agarose gel electrophoresis. Lane M indicates the 1 kb Plus DNA Ladder. Lanes F and S indicate that the digests were heated to 80°C for 15 min and then cooled fast on ice or slow at room temperature, respectively.

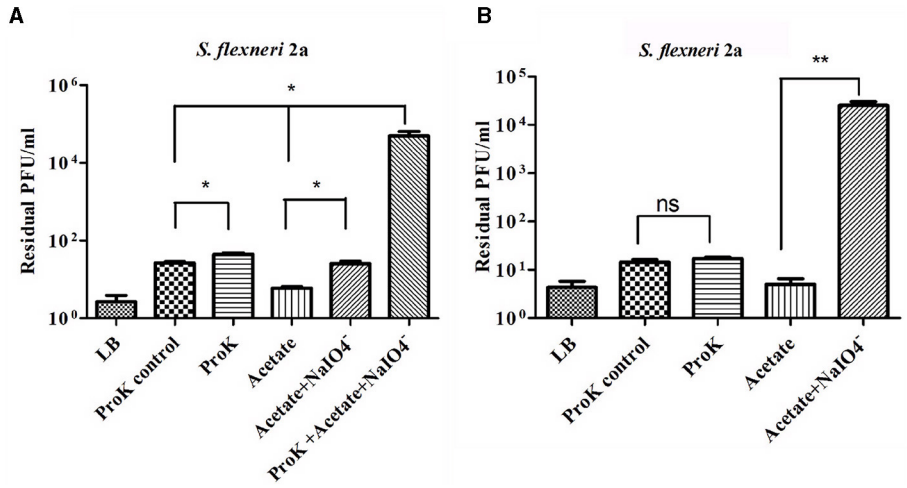


FIGURE 8
*Sfin-2* and *Sfin-6* infections on proteinase K and periodate-treated host. The effect of proteinase K and sodium periodate with proteinase K on *Sfin-2* **(A)**. The effect of sodium periodate and sodium periodate with proteinase K on *Sfin-6* **(B)**. *Shigella flexneri* 2a culture (OD600 = 0.3 U) was treated with proteinase K (250 mg/ml), sodium periodate (200 mM $NaIO_4^-$), and sodium periodate with proteinase K followed by infection at an MOI of 0.0001. Upon centrifugation, the phage titer in the supernatant was determined by plaque assay. Cells suspended in LB medium, cells incubated at 55°C in LB medium, and cells in acetate buffer were used as control. The results are shown as residual PFU percentages. The phage titer in the control supernatant was set to 100%. The mean ± SD of three independent experiments is indicated. To determine the significance of the differences between group means, unpaired *t*-tests were performed between the controls and the tests. Asterisks indicate the significance levels (ns, $p > 0.05$; *$p \leq 0.05$; **$p \leq 0.005$).

## 3.10 Characterization of the host receptor

The important aspect of phage infection is the identification of host cell surface receptor for adsorption. The nature and location of the host cell receptors vary greatly depending on the phage and host (Stone et al., 2019). They range from peptide sequences to polysaccharide moieties. In fact, bacterial capsules or slime layer appendages may also act as the receptor of the

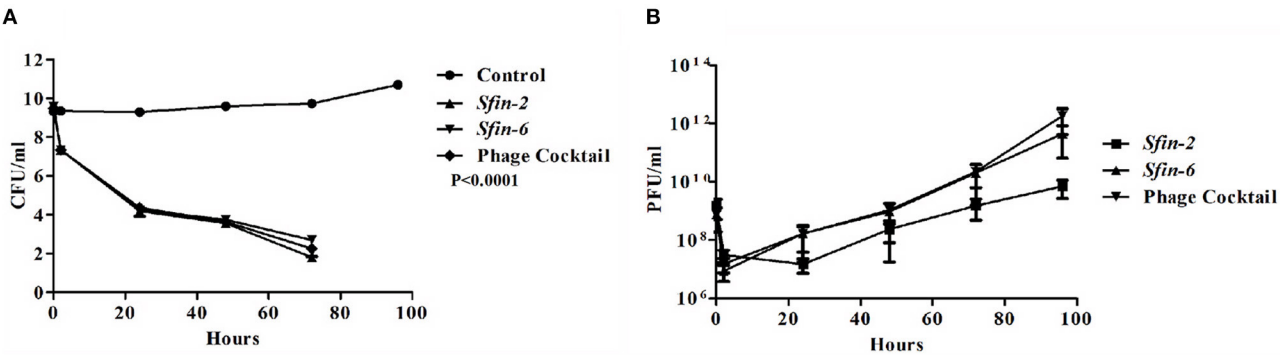


FIGURE 9
Inactivation of *Shigella flexneri* 2a by the single phage or cocktail of phages on chicken. Mid-log phase culture of *S. flexneri* 2a was inoculated on the surface of the chicken and allow for incubation of 10 min. Afterward, a single phage or cocktail form of phages was added (MOI of 0.1) and kept at 4°C up to 96 h. As the negative control, chicken pieces were inoculated with *S. flexneri* 2a without any phages. At different time intervals, the viability of *S. flexneri* 2a was determined by the spread plate method, and the number of phages was measured by plaque assay. **(A)** Reduction in viable count of cells showed after 48 h of incubation. A two-way ANOVA test indicated significant difference between control and phage infected sets ($p < 0.0001$, $n = 3$). **(B)** The number of phages decreased initially for 2 h but after that, the number increased gradually.

phages (Sorensen et al., 2011; Bae and Cho, 2013; Mahony and van Sinderen, 2015; Dowah and Clokie, 2018; Ha et al., 2019; Leprince and Mahillon, 2023).

*Shigella* spp. belong to gram-negative bacteria and exhibit complex LPS and protein in their outer membrane structures. So, either LPS or protein or both of them may involve in phage host interaction (Cohen et al., 2019; Qasim et al., 2022). Therefore, it is very much essential to identify the actual component which serves as the receptor of the phages. Based on the strategy of Kiljunen et al., the outer membrane LPS and protein of *S. flexneri* 2a were degraded by periodate and proteinase K before the infection (Kiljunen et al., 2011; Stone et al., 2019). The *Sfin-2* phage showed no changes in infection efficiency with or without proteinase K and periodate-treated host. In contrast, a high number of phage particles remained unabsorbed when hosts were pre-treated with proteinase K and periodates at a time. Thus, this experiment suggests that the adsorption of phage *Sfin-2* phage to the host is mediated either by the outer membrane of the protein or complex LPS structure (Figure 8A). In the case of *Sfin-6* phage, a high number of residual phage were present when *S. flexneri* 2a cells were pre-treated with periodates whereas no significant change in efficacy of infection was observed when the host cell was pre-treated with proteinase K. Therefore, this result suggests that the adsorption of *Sfin-6* phage to the host is mediated by the outer membrane LPS structure but not the protein (Figure 8B).

## 3.11 Inactivation of *S. flexneri* 2a cells with *Sfin-2* and *Sfin-6* by singly or cocktail of two phages in raw chicken sample

Foodborne infections are major threats to food safety in the present times. Recently, nearly two billion individuals are suffering from foodborne illnesses, resulting in 1 million deaths around the world (Kirk et al., 2015). Traditional food sanitation techniques can be effective in reducing the presence of pathogens in food with varying degrees. However, these methods have plenty of disadvantages, including the damage of organoleptic qualities of foods, and most importantly, chemicals used in food safety eliminate "good" microbes that are beneficial in the natural preservation of foods (Moye et al., 2018). Therefore, it is preferable to use bacteriophages as an alternative tool to combat the problems, as the bacteriophages are host-specific and kill their respective hosts without changing organoleptic properties of foods with low-cost large scale production, self-replicating nature, and low toxicity (Loc-Carrillo and Abedon, 2011; Perera et al., 2015). The use of bacteriophages to control MDR pathogens is gaining more interest in recent times (Rogovski et al., 2021). Zhang et al. (2013) reduced the *Shigella* load on ready-to-eat spiced chicken by at least $2\log_{10}$ after using *Shigella*-specific phages. Shahin et al. (2018) reported significant reduction of *Shigella* contamination in food items after the uses of *Shigella*-specific phages (Shahin and Bouzari, 2018). In this study, the two polyvalent *Shigella* phages, *Sfin-2* and *Sfin-6*, were used either individually or in a cocktail form to reduce the *Shigella* load on raw chicken samples. The result showed significant differences in the number of viable bacterial cells between the control and single phage or cocktail-treated chicken sample. No *Shigella* cells were found in control. The concentration of viable bacterial cells on the treated chicken sample by both single and cocktail of phages decreased by $\sim 2\log_{10}$ of the initial count. The major reduction in cell concentration occurred after 48 h of incubation, and almost complete lysis occurred after 72 h. At 96 h of incubation, the viability of cells reduced below the level of detection (Figure 9A).

The number of active phages were also measured at each time point after treatment. The number of phage decreased by $\sim 2\log_{10}$ of the initial value, 2 h after the addition of single or cocktail phages. Afterward, the number gradually increased with time in both single and cocktail of phages (Figure 9B).

## 4 Conclusion

Shigellosis is still one of the major threats in developing countries, and multidrug resistance of *Shigella* spp. has made the situation even worse. Therefore, to combat the situation, phages are gaining more popularity as an alternative therapeutic agent to resist pathogenic bacterial infection. Other than that, phages are also useful to treat foods infected with MDR bacterial pathogens. In the present study, we have characterized two novel thermostable and wide pH-tolerant *Siphoviridae* phages, *Sfin-2* and *Sfin-6*, that have specificity and lytic properties against important enteropathogenic MDR *Shigella* spp. The article represents the complete physical as well as genomic characterizations of the *Sfin-2* and *Sfin-6* phages that include sequence analysis, genome annotations, and differences between gene rearrangements among the other closely related phages. Genome analysis is very crucial for the study and use of phages to regulate host bacterial machinery. Phylogenetic analysis confirms that *Sfin-2* and *Sfin-6* belong to the T1-like phage family, which may be packaged by the headful packaging method. The phage–host interaction study through specific receptor molecules suggested that the phage *Sfin-2* can interact with both LPS-O antigen and protein, while *Sfin-6* only interacts with the LPS-O antigen of the outer cell membrane of the host cells. Further studies of the activity of *Sfin-2* and *Sfin-6* phages on *Shigella*-infected raw chicken meat either in a single or cocktail form ensure that both the phages have the potential to reduce the number of MDR *Shigella* load from the meat samples.

From the present study, it can be concluded that the *Sfin-2* and *Sfin-6* phages can be satisfactory therapeutic agents either in a single or cocktail form, and further studies on these two phages will be helpful to apply it for the treatment of shigellosis as well as for the preservation of meat.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

## Author contributions

SA, SR, CG, and NG conceived and designed the entire study, performed the experiments, analyzed the results, and prepared the manuscript. DM and VB supplied the clinical samples. SA, NG, and SD analyzed the phage structure. RJ helped in genome analysis. All authors wrote, read, and approved the final manuscript.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1240570/full#supplementary-material

SUPPLEMENTARY FIGURE S1
GC-skew plot. The cumulative graph displays the global minimum and maximum. The window size of 1,000 bp and a step size of 100 bp were used to calculate the global minimum and maximum. The blue and red lines represent the GC-skew and the cumulative GC-skew, respectively. **(A, B)** The putative origin of replication (201 nt) and the putative terminus location (43,001 nt) of *Sfin-2* and putative origin of replication (7,001 nt) and the putative terminus location (4,9501 nt) of *Sfin-6* can be predicted from the minimum and maximum of a GC-skew plot.

SUPPLEMENTARY FIGURE S2
Genome comparison of phage *Sfin-2* and *Sfin-6* with closely related phage spSf-2 and phi2457T were constructed using the Mauve progressive alignments. Boxes with identical colors represent LCBs. White rectangles represent the CDSs. Rectangles below the horizontal line indicate opposite orientations. The graph below the horizontal line indicates relative inverted genome segments.

SUPPLEMENTARY FIGURE S3
PCR primers were designed to confirm the nature of the genome end of *Sfin-2* **(A)** and *Sfin-6* **(B)**. At the 5′ (1A, 1B for *Sfin-2* and 3A, 3B for *Sfin-6*) and 3′ (2A, 2B for *Sfin-2* and 4A, 4B for *Sfin-6*) end primers were designed for positive control. Same primers from 5′ end (1B for *Sfin-2*, 3B for *Sfin-6*) and from 3′ end (2A for *Sfin-2*, 4A for *Sfin-6*) were used for PCR to charecterize the circular or linear nature of phage DNA. As per design, 410 bp PCR product would be expected for *Sfin-2*, 328 bp for *Sfin-6* if the genomes are circular **(C)** PCR products were run in 1.5% agarose gel. The lane M indicates the 100 bp DNA Ladder. 1B and 2A lane and 3B and 4A lane indicate the PCR product using 1B, 2A, and 3B, 4A primers for *Sfin-2* and *Sfin-6* respectively. Control lane indicates the non-template PCR control by using the same primers for each phage separately.

SUPPLEMENTARY TABLE S1
List of primers used for PCR of the genome ends *Sfin-2* and *Sfin-6*.

# References


Ackermann, H. W. (1998). Tailed bacteriophages: the order caudovirales. *Adv. Virus Res.* 51, 135–201. doi: 10.1016/S0065-3527(08)60785-X

Ahamed, S. T., Roy, B., Basu, U., Dutta, S., Ghosh, A. N., Bandyopadhyay, B., et al. (2019). Genomic and proteomic characterizations of Sfin-1, a novel lytic phage infecting multidrug-resistant *Shigella* spp. and *Escherichia coli* C. *Front. Microbiol.* 10, 1876. doi: 10.3389/fmicb.2019.01876

Allison, G. E., and Verma, N. K. (2000). Serotype-converting bacteriophages and O-antigen modification in *Shigella flexneri*. *Trends Microbiol.* 8, 17–23. doi: 10.1016/S0966-842X(99)01646-7

Amarillas, L., Rubi-Rangel, L., Chaidez, C., Gonzalez-Robles, A., Lightbourn-Rojas, L., Leon-Felix, J., et al. (2017). Isolation and characterization of phiLLS, a novel phage with potential biocontrol agent against multidrug-resistant *Escherichia coli*. *Front. Microbiol.* 8, 1355. doi: 10.3389/fmicb.2017.01355

Amezquita-Lopez, B. A., Quinones, B., Lee, B. G., and Chaidez, C. (2014). Virulence profiling of Shiga toxin-producing *Escherichia coli* recovered from domestic farm animals in Northwestern Mexico. *Front. Cell. Infect. Microbiol.* 4, 7. doi: 10.3389/fcimb.2014.00007

Ayariga Joseph, A., Abugri Daniel, A., Bedi, D., and Derrick, D. (2021). "Tuning phage for cartilage regeneration," in *Bacteriophages in Therapeutics*, ed. B. Sonia Bhonchal (Rijeka: IntechOpen), 7. doi: 10.5772/intechopen.97362

Ayariga, J., Venkatesan, K., Ward, R., Wu, H., Jackson, D., Villafane, R., et al. (2018). *Initiation of P22 Infection at the Phage Centennial. Front. Sci. Technol. Eng.* 2, 64–81.

Aziz, R. K., Bartels, D., Best, A. A., DeJongh, M., Disz, T., Edwards, R. A., et al. (2008). The RAST server: rapid annotations using subsystems technology. *BMC Genomics* 9, 75. doi: 10.1186/1471-2164-9-75

Azmi, I. J., Khajanchi, B. K., Akter, F., Hasan, T. N., Shahnaij, M., Akter, M., et al. (2014). Fluoroquinolone resistance mechanisms of *Shigella flexneri* isolated in Bangladesh. *PLoS ONE* 9, e102533. doi: 10.1371/journal.pone.0102533

Bae, H. W., and Cho, Y. H. (2013). Complete genome sequence of pseudomonas aeruginosa podophage MPK7, which requires type IV pili for infection. *Genome Announc.* 1, e00744-13. doi: 10.1128/genomeA.00744-13

Baird-Parker, A. C. (1994). 1993 Fred Griffith review lecture. Foods and microbiological risks. *Microbiology* 140, 687–695. doi: 10.1099/00221287-140-4-687

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., et al. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* 19, 455–477. doi: 10.1089/cmb.2012.0021

Berg, J. A., Merrill, B. D., Crockett, J. T., Esplin, K. P., Evans, M. R., Heaton, K. E., et al. (2016). Characterization of five novel brevibacillus bacteriophages and genomic comparison of brevibacillus phages. *PLoS ONE* 11, e0156838. doi: 10.1371/journal.pone.0156838

Brown, C. M., and Bidle, K. D. (2014). Attenuation of virus production at high multiplicities of infection in *Aureococcus* anophagefferens. *Virology* 466–467, 71–81. doi: 10.1016/j.virol.2014.07.023

Casjens, S., Winn-Stapley, D. A., Gilcrease, E. B., Morona, R., Kuhlewein, C., Chua, J. E., et al. (2004). The chromosome of *Shigella flexneri* bacteriophage Sf6: complete nucleotide sequence, genetic mosaicism, and DNA packaging. *J. Mol. Biol.* 339, 379–394. doi: 10.1016/j.jmb.2004.03.068

Chan, B. K., Turner, P. E., Kim, S., Mojibian, H. R., Elefteriades, J. A., Narayan, D., et al. (2018). Phage treatment of an aortic graft infected with *Pseudomonas aeruginosa*. *Evol Med Public Health*. 2018, 60–66. doi: 10.1093/emph/eoy005

CLSI (2021). *Performance Standards for Antimicrobial Susceptibility Testing. M100.* Wayne, PA: CLSI

Cohen, D., Meron-Sudai, S., Bialik, A., Asato, V., Goren, S., Ariel-Cohen, O., et al. (2019). Serum IgG antibodies to Shigella lipopolysaccharide antigens - a correlate of protection against shigellosis. *Hum. Vaccin. Immunother.* 15, 1401–1408. doi: 10.1080/21645515.2019.1606971

Costa, P., Pereira, C., Gomes, A., and Almeida, A. (2019). Efficiency of single phage suspensions and phage cocktail in the inactivation of *Escherichia coli* and *Salmonella* Typhimurium: an *in vitro* preliminary study. *Microorganisms* 7, 94. doi: 10.3390/microorganisms7040094

Dowah, A. S. A., and Clokie, M. R. J. (2018). Review of the nature, diversity and structure of bacteriophage receptor binding proteins that target Gram-positive bacteria. *Biophys. Rev.* 10, 535–542. doi: 10.1007/s12551-017-0382-3

Fauquet, C. M., and Fargette, D. (2005). International committee on taxonomy of viruses and the 3,142 unassigned species. *Virol. J.* 2, 64. doi: 10.1186/1743-422X-2-64

Filipski, A., Murillo, O., Freydenzon, A., Tamura, K., and Kumar, S. (2014). Prospects for building large timetrees using molecular data with incomplete gene coverage among species. *Mol. Biol. Evol.* 31, 2542–2550. doi: 10.1093/molbev/msu200

Gildea, L., Ayariga, J. A., and Robertson, B. K. (2022a). Bacteriophages as biocontrol agents in livestock food production. *Microorganisms* 10, 2126. doi: 10.3390/microorganisms10112126

Gildea, L., Ayariga, J. A., Robertson, B. K., and Villafane, R. (2022b). P22 phage shows promising antibacterial activity under pathophysiological conditions. *Arch. Microbiol. Immunol.* 6, 81–100. doi: 10.26502/ami.93650078

Gorden, J., and Small, P. L. (1993). Acid resistance in enteric bacteria. *Infect. Immun.* 61, 364–367. doi: 10.1128/iai.61.1.364-367.1993

Ha, E., Chun, J., Kim, M., and Ryu, S. (2019). Capsular polysaccharide is a receptor of a clostridium perfringens bacteriophage CPS1. *Viruses* 11, 1002. doi: 10.3390/v11111002

Hamdi, S., Rousseau, G. M., Labrie, S. J., Tremblay, D. M., Kourda, R. S., Ben Slama, K., et al. (2017). Characterization of two polyvalent phages infecting Enterobacteriaceae. *Sci. Rep.* 7, 40349. doi: 10.1038/srep40349

Havelaar, A. H., Kirk, M. D., Torgerson, P. R., Gibb, H. J., Hald, T., Lake, R. J., et al. (2015). World Health Organization global estimates and regional comparisons of the burden of foodborne disease in 2010. *PLoS Med.* 12, e1001923. doi: 10.1371/journal.pmed.1001923

Ibrahim, I., Ayariga, J. A., Xu, J., Adebanjo, A., Robertson, B. K., Samuel-Foo, M., et al. (2023). CBD resistant Salmonella strains are susceptible to epsilon 34 phage tailspike protein. *Front. Med.* 10, 1075698. doi: 10.3389/fmed.2023.1075698

Jun, J. W., Kim, J. H., Shin, S. P., Han, J. E., Chai, J. Y., Park, S. C., et al. (2013). Characterization and complete genome sequence of the *Shigella* bacteriophage pSf-1. *Res. Microbiol.* 164, 979–986. doi: 10.1016/j.resmic.2013.08.007

Katsura, I. (1990). Mechanism of length determination in bacteriophage lambda tails. *Adv. Biophys.* 26, 1–18. doi: 10.1016/0065-227X(90)90004-D

Kiljunen, S., Datta, N., Dentovskaya, S. V., Anisimov, A. P., Knirel, Y. A., Bengoechea, J. A., et al. (2011). Identification of the lipopolysaccharide core of *Yersinia pestis* and *Yersinia pseudotuberculosis* as the receptor for bacteriophage phiA1122. *J. Bacteriol.* 193, 4963–4972. doi: 10.1128/JB.00339-11

Kirk, M. D., Pires, S. M., Black, R. E., Caipo, M., Crump, J. A., Devleesschauwer, B., et al. (2015). Correction: World Health Organization estimates of the global and regional disease burden of 22 foodborne bacterial, protozoal, and viral diseases, 2010: a data synthesis. *PLoS Med.* 12, e1001940. doi: 10.1371/journal.pmed.1001940

Kotloff, K. L., Winickoff, J. P., Ivanoff, B., Clemens, J. D., Swerdlow, D. L., Sansonetti, P. J., et al. (1999). Global burden of Shigella infections: implications for vaccine development and implementation of control strategies. *Bull. World Health Organ.* 77, 651–666.

Leprince, A., and Mahillon, J. (2023). Phage adsorption to gram-positive bacteria. *Viruses* 15, 196. doi: 10.3390/v15010196

Lesnik, E. A., Sampath, R., Levene, H. B., Henderson, T. J., McNeil, J. A., Ecker, D. J., et al. (2001). Prediction of rho-independent transcriptional terminators in *Escherichia coli*. *Nucleic Acids Res.* 29, 3583–3594. doi: 10.1093/nar/29.17.3583

Li, M., Lin, H., Jing, Y., and Wang, J. (2020). Broad-host-range *Salmonella* bacteriophage STP4-a and its potential application evaluation in poultry industry. *Poult. Sci.* 99, 3643–3654. doi: 10.1016/j.psj.2020.03.051

Lin, D. M., Koskella, B., and Lin, H. C. (2017). Phage therapy: an alternative to antibiotics in the age of multi-drug resistance. *World J. Gastrointest. Pharmacol. Ther.* 8, 162–173. doi: 10.4292/wjgpt.v8.i3.162

Loc-Carrillo, C., and Abedon, S. T. (2011). Pros and cons of phage therapy. *Bacteriophage* 1, 111–114. doi: 10.4161/bact.1.2.14590

Lokareddy, R. K., Sankhala, R. S., Roy, A., Afonine, P. V., Motwani, T., Teschke, C. M., et al. (2017). Portal protein functions akin to a DNA-sensor that couples genome-packaging to icosahedral capsid maturation. *Nat. Commun.* 8, 14310. doi: 10.1038/ncomms14310

Lowe, T. M., and Chan, P. P. (2016). tRNAscan-SE On-line: integrating search and context for analysis of transfer RNA genes. *Nucleic Acids Res.* 44, W54–W57. doi: 10.1093/nar/gkw413

Lu, H., Xiong, W., Li, Z., Yan, P., Liu, R., Liu, X., et al. (2022). Isolation and characterization of SGF3, a novel microviridae phage infecting *Shigella flexneri*. *Mol. Genet. Genomics* 297, 935–945. doi: 10.1007/s00438-022-01883-5

Mahony, J., and van Sinderen, D. (2015). Gram-positive phage-host interactions. *Front. Microbiol.* 6, 61. doi: 10.3389/fmicb.2015.00061

Malek, W., Wdowiak-Wrobel, S., Bartosik, M., Konopa, G., and Narajczyk, M. (2009). Characterization of phages virulent for *Robinia pseudoacacia* Rhizobia. *Curr. Microbiol.* 59, 187–192. doi: 10.1007/s00284-009-9421-z

Mallick, B., Mondal, P., and Dutta, M. (2021). Morphological, biological, and genomic characterization of a newly isolated lytic phage Sfk20 infecting *Shigella flexneri*, *Shigella sonnei*, and *Shigella dysenteriae1*. *Sci. Rep.* 11, 19313. doi: 10.1038/s41598-021-98910-z

Marami, D., Hailu, K., and Tolera, M. (2018). Prevalence and antimicrobial susceptibility pattern of Salmonella and Shigella species among asymptomatic food handlers working in Haramaya University cafeterias, Eastern Ethiopia. *BMC Res. Notes*. 11, 74. doi: 10.1186/s13104-018-3189-9

Mobberley, J. M., Authement, R. N., Segall, A. M., and Paul, J. H. (2008). The temperate marine phage PhiHAP-1 of Halomonas aquamarina possesses a linear plasmid-like prophage genome. *J. Virol.* 82, 6618–6630. doi: 10.1128/JVI.00140-08

Moye, Z. D., Woolston, J., and Sulakvelidze, A. (2018). Bacteriophage applications for food production and processing. *Viruses* 10, 205. doi: 10.3390/v10040205

Muthuirulandi Sethuvel, D. P., Anandan, S., Devanga Ragupathi, N. K., Gajendiran, R., Kuroda, M., Shibayama, K., et al. (2019). IncFII plasmid carrying antimicrobial resistance genes in *Shigella flexneri*: vehicle for dissemination. *J. Glob. Antimicrob. Resist.* 16, 215–219. doi: 10.1016/j.jgar.2018.10.014

Muthuirulandi Sethuvel, D. P., Devanga Ragupathi, N. K., Anandan, S., and Veeraraghavan, B. (2017). Update on: *Shigella* new serogroups/serotypes and their antimicrobial resistance. *Lett. Appl. Microbiol.* 64, 8–18. doi: 10.1111/lam.12690

Nandy, S., Mitra, U., Rajendran, K., Dutta, P., and Dutta, S. (2010). Subtype prevalence, plasmid profiles and growing fluoroquinolone resistance in *Shigella* from Kolkata, India (2001-2007): a hospital-based study. *Trop. Med. Int. Health* 15, 1499–1507. doi: 10.1111/j.1365-3156.2010.02656.x

Pakbin, B., Amani, Z., Allahyari, S., Mousavi, S., Mahmoudi, R., Bruck, W. M., et al. (2021). Genetic diversity and antibiotic resistance of *Shigella* spp. isolates from food products. *Food Sci. Nutr.* 9, 6362–6371. doi: 10.1002/fsn3.2603

Pakbin, B., Basti, A. A., Khanjari, A., Bruck, W. M., Azimi, L., Karimi, A., et al. (2022). Development of high-resolution melting (HRM) assay to differentiate the species of Shigella isolates from stool and food samples. *Sci. Rep.* 12, 473. doi: 10.1038/s41598-021-04484-1

Peng, C., Hanawa, T., Azam, A. H., LeBlanc, C., Ung, P., Matsuda, T., et al. (2019). Silviavirus phage $\phi$MR003 displays a broad host range against methicillin-resistant *Staphylococcus aureus* of human origin. *Appl. Microbiol. Biotechnol.* 103, 7751–7765. doi: 10.1007/s00253-019-10039-2

Perera, M. N., Abuladze, T., Li, M., Woolston, J., and Sulakvelidze, A. (2015). Bacteriophage cocktail significantly reduces or eliminates *Listeria monocytogenes* contamination on lettuce, apples, cheese, smoked salmon and frozen foods. *Food Microbiol.* 52, 42–48. doi: 10.1016/j.fm.2015.06.006

Puzari, M., Sharma, M., and Chetia, P. (2018). Emergence of antibiotic resistant *Shigella* species: a matter of concern. *J. Infect. Public Health* 11, 451–454. doi: 10.1016/j.jiph.2017.09.025

Qasim, M., Wrage, M., Nuse, B., and Mattner, J. (2022). Shigella outer membrane vesicles as promising targets for vaccination. *Int. J. Mol. Sci.* 23, 994. doi: 10.3390/ijms23020994

Ranjbar, R., and Farahani, A. (2019). Shigella: antibiotic-resistance mechanisms and new horizons for treatment. *Infect. Drug Resist.* 12, 3137–3167. doi: 10.2147/IDR.S219755

Rogovski, P., Cadamuro, R. D., da Silva, R., de Souza, E. B., Bonatto, C., Viancelli, A., et al. (2021). Uses of bacteriophages as bacterial control tools and environmental safety indicators. *Front. Microbiol.* 12, 793135. doi: 10.3389/fmicb.2021.793135

Sethuvel, D. P. M., Anandan, S., Michael, J. S., Murugan, D., Neeravi, A., Verghese, V. P., et al. (2019). Virulence gene profiles of *Shigella* species isolated from stool specimens in India: its association with clinical manifestation and antimicrobial resistance. *Pathog. Glob. Health* 113, 173–179. doi: 10.1080/20477724.2019.1632062

Shahin, K., and Bouzari, M. (2018). Bacteriophage application for biocontrolling *Shigella flexneri* in contaminated foods. *J. Food Sci. Technol.* 55, 550–559. doi: 10.1007/s13197-017-2964-2

Shahin, K., Bouzari, M., and Wang, R. (2018). Isolation, characterization and genomic analysis of a novel lytic bacteriophage vB_SsoS-ISF002 infecting *Shigella sonnei* and *Shigella flexneri*. *J. Med. Microbiol.* 67, 376–386. doi: 10.1099/jmm.0.000683

Shahin, K., Bouzari, M., and Wang, R. (2019a). Complete genome sequence analysis of a lytic *Shigella flexneri* vB(-)SflS-ISF001 bacteriophage. *Turk. J. Biol.* 43, 99–112. doi: 10.3906/biy-1808-97

Shahin, K., Bouzari, M., Wang, R., and Khorasgani, M. R. (2019b). Distribution of antimicrobial resistance genes and integrons among *Shigella* spp. isolated from water sources. *J. Glob. Antimicrob. Resist.* 19, 122–128. doi: 10.1016/j.jgar.2019.04.020

Shahin, K., Zhang, L., Bao, H., Hedayatkhah, A., Soleimani-Delfan, A., Komijani, M., et al. (2021). An *in-vitro* study on a novel six-phage cocktail against multi-drug resistant-ESBL *Shigella* in aquatic environment. *Lett. Appl. Microbiol.* 72, 231–237. doi: 10.1111/lam.13418

Sivapalasingam, S., Nelson, J. M., Joyce, K., Hoekstra, M., Angulo, F. J., Mintz, E. D., et al. (2006). High prevalence of antimicrobial resistance among *Shigella* isolates in the United States tested by the National Antimicrobial Resistance Monitoring System from 1999 to 2002. *Antimicrob. Agents Chemother.* 50, 49–54. doi: 10.1128/AAC.50.1.49-54.2006

Sorensen, M. C., van Alphen, L. B., Harboe, A., Li, J., Christensen, B. B., Szymanski, C. M., et al. (2011). Bacteriophage F336 recognizes the capsular phosphoramidate modification of *Campylobacter jejuni* NCTC11168. *J. Bacteriol.* 193, 6742–6749. doi: 10.1128/JB.05276-11

Stone, E., Campbell, K., Grant, I., and McAuliffe, O. (2019). Understanding and exploiting phage-host interactions. *Viruses* 11, 567. doi: 10.3390/v11060567

Sur, D., Ramamurthy, T., Deen, J., and Bhattacharya, S. K. (2004). Shigellosis : challenges and management issues. *Indian J. Med. Res.* 120, 454–462.

Taneja, N., and Mewara, A. (2016). Shigellosis: epidemiology in India. *Indian J. Med. Res.* 143, 565–576. doi: 10.4103/0971-5916.187104

Tariq, A., Haque, A., Ali, A., Bashir, S., Habeeb, M. A., Salman, M., et al. (2012). Molecular profiling of antimicrobial resistance and integron association of multidrug-resistant clinical isolates of Shigella species from Faisalabad, Pakistan. *Can. J. Microbiol.* 58, 1047–1054. doi: 10.1139/w2012-085

von Seidlein, L., Kim, D. R., Ali, M., Lee, H., Wang, X., Thiem, V. D., et al. (2006). A multicentre study of Shigella diarrhoea in six Asian countries: disease burden, clinical manifestations, and microbiology. *PLoS Med.* 3, e353. doi: 10.1371/journal.pmed.0030353

Wang, Z., Zheng, P., Ji, W., Fu, Q., Wang, H., Yan, Y., et al. (2016). SLPW: a virulent bacteriophage targeting methicillin-resistant *Staphylococcus aureus in vitro* and *in vivo*. *Front. Microbiol.* 7, 934. doi: 10.3389/fmicb.2016.00934

Wei, B., Cong, C., Zhang, L., Zheng, L., Chen, L., Yu, W., et al. (2021). Complete genome analysis of the newly isolated *Shigella sonnei* phage vB_SsoM_Z31. *Arch. Virol.* 166, 2597–2602. doi: 10.1007/s00705-021-05121-y

WHO (2013). *World Health Statistics*. Geneva: World Health Organization.

Yamaki, S., Omachi, T., Kawai, Y., and Yamazaki, K. (2014). Characterization of a novel Morganella morganii bacteriophage FSP1 isolated from river water. *FEMS Microbiol. Lett.* 359, 166–172. doi: 10.1111/1574-6968.12560

Yang, C., Wang, H., Ma, H., Bao, R., Liu, H., Yang, L., et al. (2018). Characterization and genomic analysis of SFPH2, a Novel T7virus Infecting Shigella. *Front. Microbiol.* 9, 3027. doi: 10.3389/fmicb.2018.03027

Zhang, H., Wang, R., and Bao, H. (2013). Phage inactivation of foodborne *Shigella* on ready-to-eat spiced chicken. *Poult. Sci.* 92, 211–217. doi: 10.3382/ps.2011-02037

Ziedaite, G., Daugelavicius, R., Bamford, J. K., and Bamford, D. H. (2005). The Holin protein of bacteriophage PRD1 forms a pore for small-molecule and endolysin translocation. *J. Bacteriol.* 187, 5397–5405. doi: 10.1128/JB.187.15.5397-5405.2005

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