Review

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Phage therapy: Targeting intestinal bacterial microbiota for the treatment of liver diseases

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Summary

Phage therapy has been overshadowed by antibiotics for decades. However, it is being revisited as a powerful approach against antimicrobial-resistant bacteria. As bacterial microbiota have been mechanistically linked to gastrointestinal and liver diseases, precise editing of the gut microbiota via the selective bactericidal action of phages has prompted renewed interest in phage therapy. In this review, we summarise the basic virological properties of phages and the latest findings on the composition of the intestinal phageome and the changes associated with liver diseases. We also review preclinical and clinical studies assessing phage therapy for the treatment of gastrointestinal and liver diseases, as well as future prospects and challenges.

Keywords: Microbiome; Virome; Phageome; Gut-liver-axis; NAFLD; NASH; PSC; Alcohol use disorder; Alcohol-associated hepatitis

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Introduction

Bacteriophages, or simply phages, are prokaryotic viruses that infect and kill bacteria. Although phages were already known antimicrobial agents by the early 1900s,[1,2] the development and spread of antimicrobial chemicals, beginning with the discovery of penicillin,[3] made phage therapy less important; however, the emergence of antimicrobial-resistant bacteria has brought phages back into focus for the treatment of infectious diseases.[4,5] Notably, since several specific bacteria in the gut are involved in the pathogenesis of gastrointestinal and liver diseases, restoring eubiosis by selectively eliminating these so-called pathobionts is an attractive approach.[6,7] Treatment with broad-spectrum antibacterial agents may promote further dysbiosis.[8,9] In this context, the host specificity of phages has the potential to precisely edit the intestinal bacterial microbiota. Taken together, "old and new" phages can target antimicrobial-resistant bacteria for the treatment of infectious diseases and precisely edit the bacterial microbiota for the treatment of diseases linked to pathobionts. From these perspectives, this review outlines the basic virological properties of phages and their current applications. We will summarise the role and composition, including disease-associated changes, of phages in the virome, and the applicability of phage therapy for the treatment of gastrointestinal and liver diseases. Finally, we will review challenges and future prospects for phage-based therapy.

Phage biology

Phages are ubiquitous, with an estimated population of more than 10^{31} particles, making them the most enriched biological entities in the biosphere.[10,11] Phages exist universally wherever living bacteria are present, including soil, lakes, oceans and the human body, and act as predators to regulate the bacterial microbiota.[12–15]

All phages possess a capsid enclosing their genome. The genomic nucleic acid of phages can be categorised as linear double-stranded DNA, linear single-stranded or double-stranded RNA, or circular single-stranded DNA. In addition, almost all phage capsids are connected to the tail, which is essential for attachment to host cells and injection of the genome from the capsid.[4,5,7] Previously, *Caudovirales* was a class of viruses known as tailed phages with double-stranded DNA that were divided into three groups based on their morphologies, namely *Myoviridae* (contractile tail), *Siphoviridae* (long non-contractile tail), and *Podoviridae* (short tail). Although phages have previously been classified by their morphology, the International Committee on Taxonomy of Viruses recently updated viral taxa based on the sequence information alone and abolished morphology-based taxa.[16] The renewed viral taxa encompass the class *Caudoviricetes*, comprised of the *Crassvirales*, *Kirjokansivirales*, *Thumleimavirales*, *Methanobavirales* and unspecified orders, giving rise to 22 newly identified bacterial virus families within this class.

The phage life cycle can be classified into lysogenic and lytic (Fig. 1). Phages that go through these two cycles are defined as temperate phages, whereas those that only go through lytic cycles are called virulent phages. During the lytic infection cycle, phages are primarily adsorbed to specific receptors on the bacterial surface, which allows them to inject their genome into the bacterium. After phage gene expression and genome replication,

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progeny virions are assembled, and fully packaged phage particles are released through lysis of the bacterium by endolysin,[4] while temperate phages can select whether to enter the lytic cycle or establish lysogeny. In the lysogenic cycle, viral genes required for bacterial lysis are completely shut off by repressors of temperate phages, and lysogenic phages integrate into the host chromosome or form a linear or circular plasmid, which can replicate by itself, called a prophage.[17] Once host cells are exposed to environmental changes or stressors, the lysogenic phage genome is excised and enters a lytic cycle.[18,19] Because of these characteristics, temperate phages have the potential to horizontally transmit virulent and antimicrobial-resistant genes among bacteria. In addition, a high proportion of bacteria survive as lysogens following temperate phage infection, making temperate phages less suitable for therapy.[5,20,21]

The host range of phages is specific and limited to a single bacterial genus or species.[5] The specificity is determined by bacterial host factors and the presence of anti-phage systems that counteract the various steps of phage infection,[22,23] such as restriction modification and CRISPR-Cas.[24] On the other hand, the bottleneck of the phage host range is primarily determined by the receptors on the surfaces of host bacteria. Structures exposed on bacterial surfaces such as outer membrane protein, lipopolysaccharide (LPS), pili, flagella and transporters can be bacterial receptors.[25–29]

Bringing phages back into focus

In 1927, d'Herelle and colleagues conducted a large clinical trial during a cholera epidemic in India.[30] In this trial, oral doses of *Vibrio cholera* phages reduced the mortality rate dramatically to ~6% (n = 74) in the treated group compared with a mortality rate of 63% (n = 124) in the group of patients who refused the phage treatment. With these early successes, many phage therapies were developed by scientists for the treatment of bacterial infections; however, the therapeutic outcomes were not always promising and consistent.[31,32] The heterogeneity of treatment results might be explained by i) the absence of adequate controls, ii) the dose and administration route of phages, iii) the narrow host range of phages, iv) the presence of bacteria-derived substances in phage products, and v) the effects of body fluids and immune responses.[33] Commercialisation of antibiotics largely replaced phage therapy, but the rise of antimicrobial-resistant bacteria has led to renewed interest.

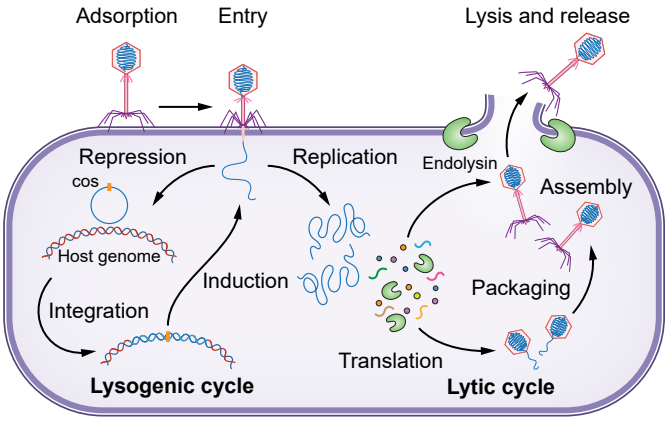

Fig. 1. Lytic and temperate phage infection cycle. A phage life cycle begins with attachment of the phage particle to the receptors on the bacterial cell surface by its tail structure, which allows the phage to inject its DNA into the cytoplasm. In the lytic cycle, after replication, transcription and translation of the phage DNA, the replicated phage genome is packaged into the structural proteins and the mature particle is assembled. Eventually, endolysins, peptidoglycan hydrolytic enzymes translated from the phage genome, cleave the cell wall peptidoglycan of targeting bacteria. Lysis of the bacteria leads to the release of progeny viruses. In the lysogenic cycle, the phage DNA, which forms a circle connected at the cohesive site (cos), integrates into the bacterial genome. The resulting lysogenic phage DNA replicates through host cell division. By certain stimulations, phage DNA can be excised from the bacterial genome and return to the lytic cycle.

Key points

- Although phages have been overshadowed for decades by antibiotics, they are being revisited as a powerful approach against antimicrobial-resistant bacteria.
- The bacterial microbiota have been mechanistically linked to gastrointestinal and liver diseases, prompting renewed interest in phage therapy.
- The gut virome in humans is dominated by phages and is altered in patients with liver diseases compared with healthy individuals.
- Pre-clinical studies have shown that selective elimination of a pathobiont by phages can lead to improvements in inflammatory bowel disease, primary sclerosing cholangitis, ethanol-induced liver disease and non-alcoholic fatty liver disease in mouse models. Appropriately designed clinical trials are required to strengthen our knowledge on the potential of phage therapy for the treatment of gastrointestinal and liver diseases.

Infectious diseases caused by antimicrobial-resistant bacteria have a considerable negative impact on public health. It has been estimated that 10 million people will die annually due to antimicrobial-resistant bacterial infections by 2050 unless a global programme to reduce antimicrobial resistance is implemented.[34] In this context, phage therapy has received significant attention for the treatment of antimicrobial-resistant infections because phages have a different bactericidal mechanism to existing antibiotics, making it easier to kill antimicrobial-resistant bacteria.[35–38] This is one aspect of the therapeutic applicability of phages (Fig. 2A). Notably, a 2017 case report on the use of phage therapy, following Emergency Investigational New Drug approval by the FDA, to treat necrotising pancreatitis caused by a multidrug-resistant strain of *Acinetobacter baumanii* was the first case of successful phage therapy reported in the US.[39] After that, there have been case reports on the use of phage therapy, under Emergency Investigational New Drug applications, against multidrug-resistant *P. aeruginosa, S. aureus* and *Mycobacterium abscessus*.[40] In addition, several clinical trials have tested phage therapies against *P. aeruginosa* otitis externa, burn infections (PhagoBurn trial) and diarrhoea caused by *E. coli* (Table 1).[41–43] Although clinical trials for *P. aeruginosa* otitis externa showed good results, others failed to demonstrate conclusive efficacy, and no phage products for therapies have yet been approved.

Phageome in the intestinal microbiome

Virus-like particles dominate the gut microbiota, with an estimated 10^9 to 10^{10} per gram of faeces.[44,45] In fact, the phageome accounts for 90% of the human intestinal virome, with eukaryotic viruses accounting for the other 10%.[46] The predominant families in the intestinal phageome are *Myoviridae*, *Podoviridae* and *Siphoviridae*, followed by the smaller isometric family *Microviridae*.[44,47] Although the role of the phageome is not fully

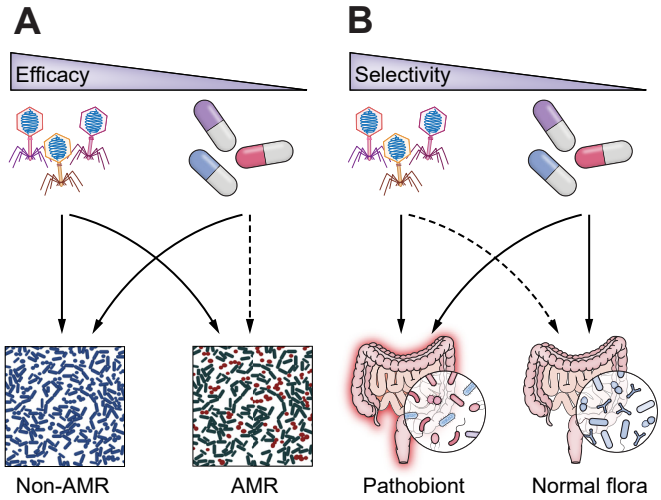

Fig. 2. Advantages of phages over antibiotics in the context of addressing bacterial infections and dysbiosis. (A) Antibiotics have been conventionally applied to bacterial infections; however, the use of antibiotics has led to the emergence of antimicrobial-resistant strains of bacteria, and there are cases in which antibiotics are not expected to be effective. On the other hand, killing of antimicrobial-resistant bacteria by phages is expected to be more effective because phages possess completely distinct bactericidal mechanisms compared to antibiotics. However, the narrow host range of phages may limit their application in the treatment of infectious diseases. (B) There is concern that the use of conventional antibiotics may kill even useful microbes in the human body inducing dysbiosis. On the other hand, phages have a very specific host range. Therefore, phages have the potential to selectively eliminate pathobionts in the dysbiotic bacterial microbiota of patients. AMR, antimicrobial resistance.

understood yet, progression of certain diseases including liver diseases is associated with changes in the intestinal phageome.[6,7,48] Therefore, phages are considered an important component in the microbiota, with impacts on human health.

In the healthy intestinal virome, a small core gut phageome composed of an estimated 20-25 phages exists and is shared by more than 20% of adults, co-existing with phages that are unique to each person.[49,50] In addition, intra-patient diversity of the intestinal phageome reveals relative stability over time, primarily due to the prevalence of specific phage groups.[49,50] On the other hand, there is an extremely high level of inter-patient diversity in the intestinal phageome.[51,52] No viruses are detected in the neonatal gut at birth.[53] The phageome evolves in the intestine within the first week of life, likely via the induction of prophages from gut bacteria.[53] Interestingly, bacterial diversity increases during early life whereas the diversity and richness of the intestinal phageome decreases slowly by age 2-3 years. Initial predominant colonisers of the gut phageome are *Caudovirales,* but the *Microviridae* family gradually becomes more predominant and most abundant by age 2, similar to gut viromes in adults.[54,55] The phageome is affected by environmental factors such as the diet and by diseases.[51,56] However, results of phageome studies are at times discrepant and contradictory, which is likely due to different methods of sample preparation, detection and bioinformatics analysis.[45,57,58] Recent comprehensive virome analyses support the presence of a stable and predominantly virulent core phageome in healthy individuals,[59,60] while others suggest a lysogeny-rich environment in the healthy phageome.[51,52,61] Therefore, standard and accurate methods for the evaluation of intestinal phages need to be developed.

Changes in phageome structure have been described in liver diseases, including in patients with alcohol use disorder, alcohol-associated hepatitis and non-alcoholic fatty liver disease (NAFLD), and are associated with disease severity. Although viral diversity from faecal samples increased in patients with alcohol use disorder and alcohol-associated hepatitis,[62] patients with NAFLD had a lower intestinal viral diversity compared with controls.[63] A similar phage diversity as controls was observed in patients with cirrhosis.[64] In addition, *Escherichia, Enterobacteria* and *Enterococcus* phages were more abundant in patients with alcohol-associated hepatitis.[62] Notably, an increasing abundance of *Staphylococcus* and *Citrobacter* phages was associated with more severe alcohol-associated hepatitis. An increased abundance of *Escherichia, Enterobacteria* and *Lactobacillus* phages was observed in patients with advanced NAFLD.[63] Furthermore, *Lactococcus* and *Leuconostoc* phage abundance was inversely correlated, whereas *Lactobacillus* phage abundance was positively correlated, with the severity of liver fibrosis.[64] In patients with alcohol use disorder, some of the changes in the faecal phageome associated with progression of the liver disease are

Table 1. Clinical trials of phage therapy for bacterial infections.

Disease	Target bacteria	Trial design	Phage and dose	Population and treatment method	Outcome and interpretation	Ref.
Otitis	*P. aeruginosa*	Placebo-controlled, double-blind	6 phages (10^9 PFU) Single dose	12 individuals received phages, 12 individuals received glycerol-PBS buffer. Intra-aural administration	*P. aeruginosa* counts and clinical indicators were significantly lower only in the phage treated group. No adverse effects.	41
Diarrhoea	*E. coli*	Placebo-controlled, double-blind	11 T4-like phages (3.6×10^8 PFU) or ColiProteus (1.4×10^9) Three times/day for 4 days (12 doses)	39 individuals received 11 T4-like phages and 40 individuals received ColiProteus, 41 individuals received rehydration solution. Oral administration	Safe but no significant difference between phage treatment group and placebo group.	42
Burn wound	*P. aeruginosa*	Placebo-controlled, blind	12 phages (2×10^7 PFU was expected but 200-2,000 PFU was actual) One time/day for 7 days (7 doses)	12 individuals received phages, 13 individuals received 1% sulfadiazine silver (standard of care). Topical administration	Phage treatment decreased bacterial burden in burn wounds slower than standard of care at significantly lower dose than expected and trial halted.	43

PFU, plaque-forming unit.

partially reversible after a short period of abstinence.[65] A recent study investigating the intestinal viromes of patients with alcohol use disorder and metabolic dysfunction-associated fatty liver disease (MAFLD) demonstrated that viral composition and diversity were significantly different in patients with MAFLD with low and moderate alcohol consumption compared with those with MAFLD with no alcohol consumption.[66] Notably, patients with alcohol use disorder and alcohol-consuming patients with MAFLD had a more similar abundance of *Lactococcus* phages than non-alcohol-consuming patients with MAFLD and controls, indicating that *Lactococcus* phages could better predict alcohol use in the MAFLD population. However, whether changes in the intestinal phageome contribute to liver disease has not been elucidated. In addition, how changes in the intestinal phageome modify the bacterial microbiota or vice versa is not fully understood.[6,7,48] Therefore, further studies are required to clarify the causative links between the intestinal phageome and disease onset and progression. In addition, a recent study using plasma samples of septic patients (n = 285) and control individuals (n = 177) reported the existence of a circulating phageome. There was an association between infection and overrepresentation of pathogen-specific phages, which may allow for the identification of pathogens in septic patients.[67] Although the systemic phageome has not been evaluated in patients with gastrointestinal and liver diseases, information about the systemic phageome may provide insight into specific pathobionts.

Potential pathobionts of liver disease as targets for phage therapy

The close relationship between the gut and liver stems from a bidirectional communication through the portal vein and biliary tract, establishing the liver as the primary recipient of venous blood from the intestine. Therefore, the gut microbiota is known to modulate the severity of different liver diseases, and several specific bacterial pathobionts have been associated with the development of liver diseases. In order to manipulate the gut microbiota, antibiotics and faecal microbiota transplantation are established as untargeted microbial therapies.[48] In addition, the use of prebiotics and probiotics, as well as dietary changes, are options for manipulating the gut microbiota.[48] On the other hand, as phages possess selective bactericidal activity, they could be used to precisely target and reduce the abundance of specific pathobionts (Fig. 2B).[6,7] Hence, we summarise data on faecal bacteria known to be increased in human liver disease that could be potential targets for phage therapy (Table 2).

Several intestinal bacterial genera are increased in patients with liver diseases, including *Bacteroides*,[68–70] *Blautia*,[70,71] *Dorea*,[71,72] *Lactobacillus*,[72,73] *Roseburia*[72] and *Ruminococcus*[70,71] in patients with NAFLD and *Blautia*,[74] *Dorea*,[74] *Prevotella*[75] and *Veillonella*[76,77] in patients with alcohol use disorder. Faecal proportions of *Lactobacillus*, *Streptococcus, Enterococcus* and *Veillonella* are increased in patients with primary sclerosing cholangitis (PSC),[78–80] and *Prevotella*[81,82] and *Streptococcus*[70,77,81,83] in patients with cirrhosis. Prior to using phages to edit the microbiota, it is very important to identify changes in the intestinal microbiota on the bacterial species level and to establish a mechanistic link between the pathobiont and progression of liver disease. So far, bacterial species that have been identified include *Bacteroides vulgatus*,[68] *E. coli* and *K. pneumoniae* in patients with NAFLD,[68,84,85] and *Enterococcus faecalis* in patients with alcohol-associated hepatitis.[86] In addition, *Enterococcus gallinarum*[87] and *Veillonella dispar*[88] are increased in patients with autoimmune hepatitis, and *E. faecalis*,[77] *Veillonella atypica* and *Veillonella parvula*[83] are increased in patients with cirrhosis. A recent study reported that the faecal proportions of *K. pneumoniae* and *E. gallinarum* are increased in patients with PSC.[89,90] Notably, it is desirable to use virulent phages instead of temperate phages to edit the intestinal bacterial microbiota; however, virulent phages against pathobiontss have been isolated and cultured only from *E. coli*,[25,38,91] *K. pneumoniae*[85,89,92] and *E. faecalis*.[86,93,94] Although only a temperate phage against *E. gallinarum* has been identified,[95] a novel virulent phage was reported in 2023.[96] On the other hand, although temperate phages or potential temperate phages have been identified for *B. vulgatus*[97] and *Veillonella spp*,[98] no specific virulent phages have been discovered. Therefore, in addition to the identification of pathobionts, further phage isolation against candidate bacteria and their virological characterisation, such as host range and genomic characteristics, are required to expand the utility of phage therapy for the treatment of liver diseases. Additionally, similar to the success of *Lactobacillus salivarius* in treating acute liver injury by restoring balance to the microbial environment in mice,[48] a better understanding of the relationship between specific bacterial changes and dysbiosis in general could drive the applicability of phages to these targets.

Precise editing of the intestinal bacterial microbiota by phages

To ameliorate dysbiosis and pathologies caused by pathobionts, precise editing of the intestinal bacterial microbiota by phages has been attempted for gastrointestinal and liver diseases. In particular, research on phage therapy for inflammatory bowel disease (IBD) has been progressing, and useful preclinical studies and clinical trials have been reported (Table 3). Adherent-invasive *E. coli* strains are known to contribute to the pathogenesis of Crohn's disease[99,100] by stimulating antigen-presenting cells and eliciting a T helper 17 (Th17) cell response, which causes chronic intestinal inflammation.[101] The application of phages specifically targeting adherent-invasive *E. coli* has been tested as a treatment against Crohn's disease. In a preclinical study using conventional mice, oral administration of the three phages reduced *E. coli* colonisation and dextran sodium sulphate-induced colitis.[102] Prior to conducting a clinical trial for Crohn's disease, the *in vitro* killing activity and specificity of a cocktail of seven phages (EcoActive) was assessed against 210 clinical strains of adherent-invasive *E. coli*, with the cocktail demonstrating killing activity against 95% of tested strains. In addition, in mice colonised with adherent-invasive *E. coli*, inflammation was attenuated in those receiving the cocktail twice a day for 15 days.[91] Furthermore, a phase I/IIa randomised, double-blind, placebo-controlled trial is ongoing to investigate the safety and efficacy of EcoActive against intestinal adherent-invasive *E. coli* in 30 patients with Crohn's disease (ClinicalTrials.gov: NCT03808103). In addition, a recent report by Federici *et al.* on the analysis of IBD-associated microbiota in four different regional cohorts (United States, France, Germany, and Israel, n = 537) showed that *K. pneumoniae* was strongly associated with IBD exacerbation and severity.[103] By using an optimised phage dosing protocol (10^9 plaque-forming units [PFU]/ml of five different phage types administered three times a week), they determined that *K. pneumoniae* was suppressed in colitis-prone mice, reducing inflammation and disease severity. The authors eventually assessed the viability of orally co-administered phages in human volunteers in a phase I randomised, single-blind,

Table 2. Potential bacterial targets for treatment of liver diseases.

Target bacteria Genus	Target bacteria species	NAFLD	AUD	Autoimmune Hepatitis	PSC	Cirrhosis	Ref.	Isolation of virulent phages	Ref.
Bacteroides							68–70		
	B. vulgatus	↑					68	No	
Blautia		↑	↑				70, 71, 74		
Dorea		↑	↑				71, 72, 74		
Escherichia									
	E. coli	↑					68, 84, 85	Isolated	25,38,91
Enterococcus					↑		78–80		
	E. faecalis		↑			↑	77, 86	Isolated	86,93,94,
	E. gallinarum			↑	↑		87, 89, 90	Isolated	96
Klebsiella									
	K. pneumoniae	↑			↑		68, 84, 85, 89, 90	Isolated	85,89,92
Lactobacillus		↑			↑		72, 73, 78–80		
Prevotella			↑			↑	75, 81, 82		
Roseburia		↑					72		
Ruminococcus		↑					70, 71		
Stereptococcus					↑	↑	70, 77–81, 83		
Veillonella			↑		↑		76–80		
	V. atypica					↑	83	No	
	V. dispar			↑			88	No	
	V. parvula					↑	83	No	

AUD, alcohol use disorder; NAFLD, non-alcoholic fatty liver disease; PSC, primary sclerosing cholangitis.

placebo-controlled trial (ClinicalTrials.gov: NCT04737876).[103] In this clinical trial, two *K. pneumoniae*-targeting orally administered phages were safe and well tolerated after passage through the gastrointestinal tract of healthy individuals without off-target dysbiosis. These results, showing the suppression of *K. pneumoniae* and limited off-target effects of phage therapy, are promising; clinical trials in patients with IBD-associated clade *K. pneumoniae* strains are being awaited. While two clinical trials using the commercial bacteriophage product PreforPro, containing a mix of phages targeting *E. coli,* revealed that the phages were safe and well tolerated, the evidence for efficacy was not clear.[104,105] One randomised, placebo-controlled crossover trial (ClinicalTrials.gov: NCT03269617) evaluated the effect of PreforPro and another randomised, double-blind, placebo-controlled trial (ClinicalTrials.gov: NCT04511221) assessed the additive effect of PreforPro on probiotics *Bifidobacterium animalis subsp. lactis* BL04. Both trials included healthy individuals with self-assessed mild-to-moderate gastrointestinal distress but who had not been diagnosed with gastrointestinal disorders. The lack of efficacy using PreforPro in humans is not surprising, as the causative impact of *E. coli* on abdominal symptoms has not been established. Therefore, the connection between intestinal bacteria targeted by phages and symptoms should be addressed carefully prior to trials using phage therapy.

Regarding phage therapy for treatment of liver diseases, there are four preclinical trials (Table 3), but no clinical trials. High alcohol-producing strains of *K. pneumoniae* (HiAlc Kpn) are present in the human gut, and their analysis using 43 patients with NAFLD (non-alcoholic fatty liver [n = 11] and non-alcoholic steatohepatitis [n = 32]) showed that 61% of patients with NAFLD carried HiAlc and medium alcohol-producing (MedAlc) Kpn, whereas this value was only 6.25% in healthy individuals. The alcohol-producing ability of *K. pneumoniae* was significantly stronger in the faeces of patients with NAFLD.[85] Inoculation of mice with HiAlc Kpn elicited steatohepatitis by inducing Th17 cells. Intestinal microbiota containing HiAlc Kpn was transplanted from a patient with non-alcoholic steatohepatitis into germ-free mice, which resulted in hepatic steatosis. On the other hand, faecal microbiota transplantation after selective elimination of HiAlc Kpn using two *Klebsiella* phages *ex vivo* prior to faecal microbiota transplantation showed amelioration of liver disease.[85] Moreover, oral treatment with a single phage led to the alleviation of HiAlc Kpn-induced steatohepatitis in mice.[106] In a similar preclinical study targeting *K. pneumoniae*, Ichikawa *et al.* found that Th17 cell responses were induced by PSC-derived *K. pneumoniae*, which accelerated liver injury in hepatobiliary injury-prone mice. Oral and intravenous phage cocktail administration decreased *K. pneumoniae* and improved liver inflammation and disease severity without off-target dysbiosis.[89] Furthermore, in 2019, Duan *et al.* reported that 5.59% of faecal bacteria in the gut bacterial microbiota of patients with alcohol-associated hepatitis (n = 75) were *Enterococcus* spp., and faecal *E. faecalis* was significantly more abundant than in healthy individuals and patients with alcohol use disorder.[86] Cytolysin-, which is a secreted exotoxin, positive *E. faecalis* correlated with the severity of alcohol-associated hepatitis, and importantly 89% of cytolysin-positive patients with alcohol-associated hepatitis died within 180 days of admission compared with only 3.8% of cytolysin-negative patients. Mice gavaged with cytolysin-positive *E. faecalis* showed more severe ethanol-induced liver damage and hepatic steatosis compared with control mice. Germ-free mice transplanted with faecal samples from cytolysin-positive patients with alcohol-associated hepatitis also exhibited more ethanol-induced liver disease. Oral gavage of multiple phages specifically targeting cytolysin-positive *E. faecalis* abolished ethanol-induced liver injury and steatosis without affecting the overall composition of the gut microbiota. Improvement in liver disease was not observed in gnotobiotic mice treated with phages targeting *Caulobacter crescentus* (control phages). In a similar experiment using phages against cytolysin-negative *E. faecalis*, features of ethanol-induced liver disease were not attenuated compared with control phages, suggesting that improvement in liver disease by phages is due to the specific elimination of cytolysin-positive *E. faecalis* rather than a reduction in total *E. faecalis*. These reports strongly indicate that selective elimination of a pathobiont by phages can improve liver diseases in mouse models.

Table 3. Preclinical and clinical trials of phage therapy for gastrointestinal and liver diseases.

Target bacteria	Disease	Type	Design	Phage and dose	Population and treatment method	Outcome and interpretation	Ref.
E. coli	Gastrointestinal distress	Clinical trial - Complete	Placebo-controlled, double-blind	PreforPro (4 phages) Daily for 28 days	32 healthy individuals with mild to moderate gastrointestinal distress Oral administration	Safe but no therapeutic effects compared with placebo	104
E. coli	Gastrointestinal distress	Clinical trial - Complete	Placebo-controlled, double-blind	PreforPro (4 phages) with probiotics (*Bifidobacterium animalis subsp. lactis* strain BL04) Daily for 28 days	68 healthy individuals with mild to moderate gastrointestinal distress Oral administration	Safe but no evidence of therapeutic effects	105
E. coli	Crohn's disease	Preclinical	Adherent-invasive *E. coli* colonised mouse model	EcoActive (7 phages) Twice a day for 15 days	Colitis-prone mice colonised with adherent-invasive *E. coli* strain Oral administration	EcoActive was safe and did not induce dysbiosis, and protected mice from clinical and histological manifestations of inflammation	91
E. coli	Crohn's disease	Clinical trial - Active	Placebo-controlled, double-blind	EcoActive (7 phages) Twice a day for 15 days	30 individuals with inactive Crohn's disease in clinical and objective remission Oral administration	Estimated primary completion date: September 30, 2023	ClinicalTrials.gov: NCT03808103
K. pneumoniae	IBD	Preclinical	*K. pneumoniae* from IBD patient - colonised mouse model	5 phages (10^9 PFU/ml) Three times per week	Colitis-prone mice colonised with Kp2 strain Oral administration	*K. pneumoniae* was suppressed in mice, reducing inflammation and disease severity	103
K. pneumoniae	PSC	Preclinical	*K. pneumoniae* from PSC patient - colonised mouse model	4 phages (10^9 PFU/ml) Every 3 days for 2 or 3 weeks	SPF, germ-free and hepatobiliary injury-prone SPF mice with clinical *K. pneumoniae* isolate Oral or intravenous administration	Levels of *K. pneumoniae* were suppressed by phages and liver inflammation and disease severity were attenuated	89
K. pneumoniae	Healthy (safety trial)	Clinical trial - Complete	Placebo-controlled, double-blind	2 phages (2.8×10^{10} PFU) Twice a day for 3 days	18 healthy individuals received oral esomeprazole (40 mg once a day) to increase gastric pH Oral administration	Phages were safe and well tolerated after passage through the gastrointestinal tract	103
K. pneumoniae	NAFLD	Preclinical - *ex vivo*	Stool samples from patients with NAFLD – colonised mouse model	2 phages Pretreat before faecal microbiota transplantation	Germ-free mice transplanted with faecal microbiota of patients with NAFLD	Faecal microbiota transplantation with phage pretreatment attenuated steatohepatitis development	85
K. pneumoniae	NAFLD	Preclinical	Stool samples from patients with NAFLD – colonised mouse model	1 phage (10^6 PFU maximum) Once a day for 1, 4 or 7 days	Germ-free mice transplanted with faecal microbiota of patients with NAFLD Oral administration	Phages targeting alcohol-producing *K. pneumoniae* alleviated steatohepatitis without obvious side effects	106
E. faecalis	Alcohol-associated liver disease	Preclinical	Stool samples from patients with alcohol-associated hepatitis – colonised mouse model	3 or 4 phages (10^9 PFU) 1 day before ethanol binge	Germ-free mice transplanted with faecal microbiota of patients with alcohol-associated hepatitis Oral administration	Phages targeting cytolytic *E. faecalis* precisely edited the intestinal microbiota and abolished ethanol-induced liver disease in microbiota humanised mice	86

IBD, inflammatory bowel disease; NAFLD, non-alcoholic fatty liver disease; PFU, plaque-forming unit; PSC, primary sclerosing cholangitis; SPF, specific pathogen free.

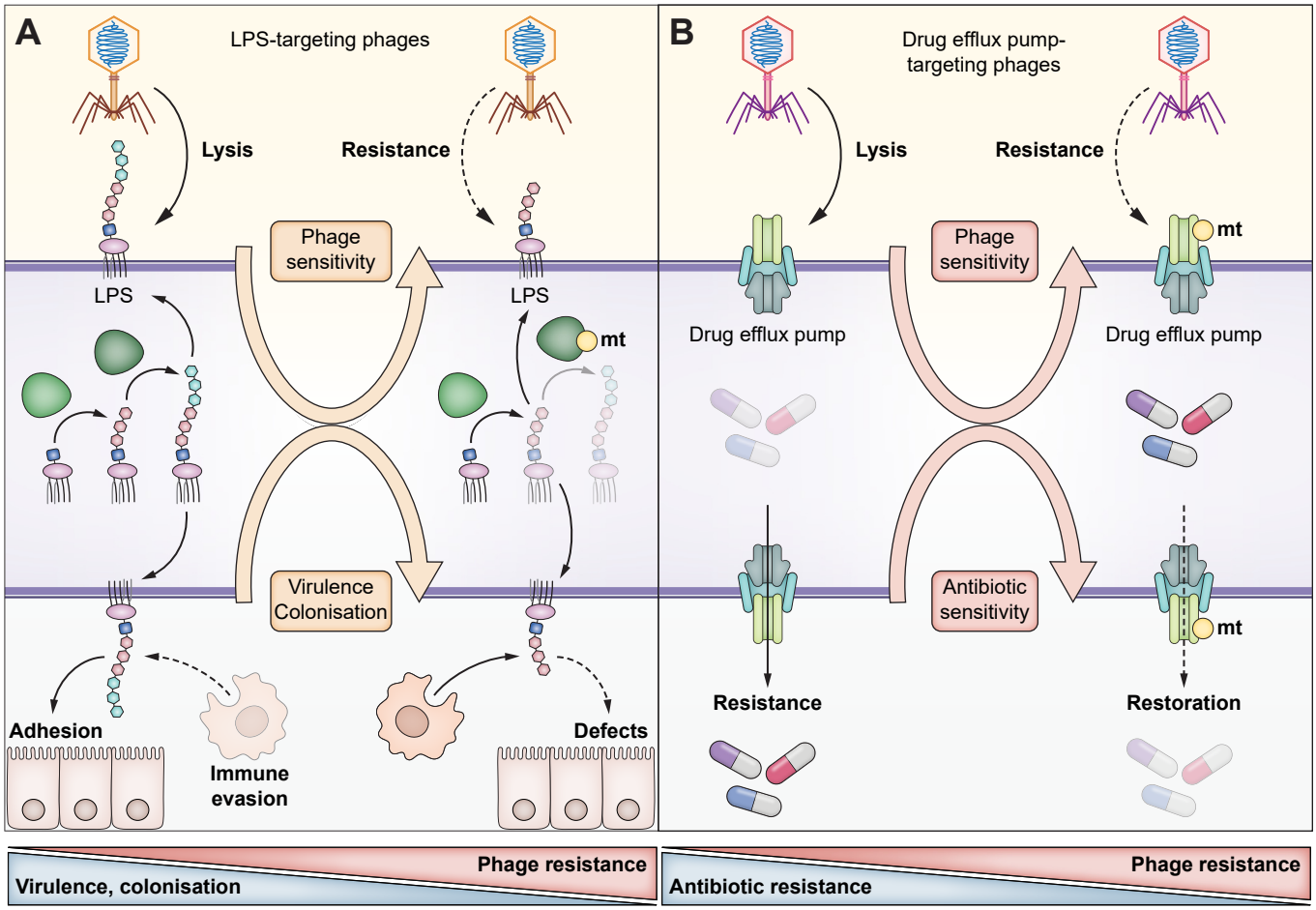

Fig. 3. Fitness trade-offs between phage infection, bacterial virulence and antibiotic sensitivity. Phage receptor mutations produce fitness trade-offs between phage bacterial virulence and antibiotic sensitivity. (A) In the case of LPS serving as the phage receptor, mutations can be accumulated in the coding sequences of LPS biosynthesis molecules, which will shorten the length of LPS and be inversely correlated with bacterial virulence. Alterations in the structure of LPS that result in phage resistance can potentially enhance clearance through reduced adhesion and host colonisation. (B) In the case that drug efflux pumps serve as phage receptors, mutations can be accumulated in drug efflux pump-encoding sequences. Non-synonymous mutations in the proteins can produce phage resistance and disrupt their original function of drug efflux, which will induce restoration of antibiotic sensitivity. LPS, lipopolysaccharide; mt, mutation.

Preparation and administration of phages for liver diseases

Regarding the preparation of phages against pathobionts, sewage obtained from treatment plants and clinical samples containing the targeted bacteria are promising sources for phage isolation.[10,86,89,103] Creating a phage library against specific bacteria will further contribute to their ready availability for future clinical use. Phages typically exhibit specificity towards bacterial genera, minimising their impact on non-targeted gut bacteria and the host. However, in cases where both pathobionts and beneficial bacteria belong to the same genus, it becomes crucial to either validate the phages' host range beforehand during the screening step or to design the bactericidal spectrum more precisely, potentially through synthetic phages. Additionally, it is essential to remove bacterial cell debris, such as endotoxins and peptidoglycans, from the purified phage solutions. This can be achieved using methods like filtration, chromatography, ultracentrifugation, and octanol treatment.[39,103,107] Administering purified phages in mice and clinical trials has shown no adverse effects, *e.g.* inflammatory reactions.[35,41–43,91,103–105] Regarding concerns about LPS release by bacterial cell lysis, it has been reported that the lysis of *E. coli* by phages releases a lower amount of endotoxin compared to β-lactams.[108] When administering phages clinically to target pathobionts in the gut, it is preferable to provide a phage cocktail orally, at doses ranging from 10^9 to 10^{10} PFU, over the course of several days. This range is based on prior clinical trials conducted on conditions such as IBD (Table 3). Although phages can spread systemically, phages accumulate in the liver after intravenous and intrapleural administration,[35,109] indicating that systemic phage administration may be effective against bacteria that have translocated to the liver or are causing infection in the liver. We previously reported that phages even spread systemically when administered orally in mice subjected to chronic ethanol feeding.[110] From this perspective, besides editing the gut microbiota, systemic or intraperitoneal administration of phages may also be effective for the treatment of spontaneous bacterial peritonitis and liver abscesses. These would be similar applications to the case report of a patient with necrotising pancreatitis and an infected pancreatic pseudocyst. Phages were administered intravenously and directly into the pseudocyst with good therapeutic outcomes.[39]

Challenges and prospects for phage therapy

Similar to the case with antibiotics, bacterial resistance to phages can emerge through a variety of molecular mechanisms, which are associated with bacterial changes that prevent phage infection.[22–24] In fact, several clinical studies of phage therapy against antimicrobial-resistant bacteria showed the occurrence of phage-resistant variants,[39,111–113] which represents a significant obstacle to the development of successful phage therapies. Thus, we need to carefully consider how to address phage resistance in advance.

There are two effective ways to counter phage resistance: i) preventing the emergence of phage-resistant variants, and ii) inducing phage resistance in a way that is advantageous to phage therapy even if phage-resistant variants emerge. In order to prevent the emergence of phage-resistant variants, it has been reported that a combination of phages (phage cocktail) that recognises distinct receptors on the bacterial surface suppresses growth of target bacteria for longer and more efficiently than single phages by making it more difficult for bacteria to evolve resistance.[89,114,115] For instance, the cocktail of four *Salmonella* phages, which target the LPS O-antigen, outer core, inner core and the outer membrane proteins BtuB and TolC, could significantly suppress the emergence of phage-resistant variants compared to a single phage.[29] Li *et al.* suggested that the use of phage-resistant bacterial variants for phage isolation can increase the isolation frequency of ideal phages to enable more effective cocktail design *in vitro*.[116] This approach has been used in phage therapy following the appearance of phage-resistant variants, enabling the construction of a more potent and efficient phage cocktail.[39] In addition, in order to overcome phage resistance, Borin *et al.* demonstrated that co-evolutionary phage training could be a particularly powerful approach to obtain evolved phages which can re-infect phage-resistant variants because it employs the natural algorithm of evolution.[117] This approach can improve the therapeutic properties of phage therapy by using evolved phages, which possess broader host range and efficient infectivity towards phage-resistant variants.[118,119] On the other hand, even if phage resistance occurs during therapy, a fitness trade-off can be applied in which bacterial virulence is attenuated (Fig. 3A) or antimicrobial susceptibility is restored (Fig. 3B) at the cost of phage resistance.[120–122] Bacteria harbour saccharides and membrane proteins on their surface, which define bacterial virulence and many other characteristics. In *Escherichia, Shigella* and *Pseudomonas* that developed resistance against LPS-targeting phages, decreased virulence and increased clearance due to changes in LPS structure were observed.[123–125] In the case of enterococci, it is also known that the capsular polysaccharide or Enterococcal saccharide antigen (Epa) serves as a phage receptor.[93,126] Lack of Epa-encoding genes such as *epaS* and *epaR* reduced their ability to colonise the gut.[93,127] Furthermore, phage-resistant variants of *Salmonella,* which generated an incomplete LPS structure, resulted in host colonisation defects.[128] For gastrointestinal and liver diseases, this kind of phage resistance may result in a key fitness-cost contributing to the elimination of phage-resistant variants from the body more quickly during phage therapy (Fig. 3A). In addition, interestingly, drug efflux pumps exposed on the bacterial surface are also known to be phage receptors. When *Salmonella enterica* serovar *Enteritidis, E. coli,* and *P. aeruginosa* acquire phage resistance via mutations in drug efflux pumps, such as TolC or MexXY-oprM, accumulated mutations resulted in trade-offs including increased antibiotic sensitivity[29,129,130] (Fig. 3B). It is also known that bacteria become resistant to phages via acquisition of large-scale chromosomal deletions. Previous studies showed that loss of the fluctuating bacteriophage-induced *galU* deficiency region, caused by large-scale chromosomal deletions by DNA mismatch repair enzymes such as MutL,[124,131] is involved in trade-offs between the phage and fluoroquinolone sensitivity of *P. aeruginosa*.[132] In order to utilise these mechanisms to overcome phage resistance, it is necessary to uncover the details of phage receptors and the mechanisms of phage resistance at the molecular level.

Clinical observations of phage therapy indicated that administration of phages leads to the development of anti-phage antibodies. In the mouse immune model, induced antibodies recognised attachment-related proteins, such as long tail fibres, and reduced the plaque-forming activity of phages *in vitro*.[35,133] Nevertheless, it has been suggested that the impact of anti-phage antibodies would be negligible in the context of human phage therapy if the dosage of phages administered to humans (ranging from 3×10^7 to 6×10^{10} PFU/patient/day orally or topically) is notably lower than that employed in animal immunisation models, resulting in insufficient induction of anti-phage antibodies.[133,134] Conversely, a clinical case has been reported in which systemic administration via intravenous injection (10^9 PFU, twice daily, for 6 months) exhibited limited therapeutic effectiveness due to the potent production of phage antibodies.[135] This emphasises the importance of carefully considering the phage quantity and the chosen route and duration of administration.

Synthetic phages may help with the design of ideal phage cocktails and accelerate "super" precise editing of the intestinal microbiota, which are not easily achievable with natural phages. It has been suggested that synthetic phages, created by exchanging long tail fibres between phages, could broaden the host range, and cocktails composed of synthetic phages with a variety of long tail fibres could effectively suppress phage resistance.[136,137] In addition, employing engineered phages, such as Cas13a-carrying phages, which possess the ability to recognise genes unique to pathogens,[138] could facilitate the specific eradication of pathobionts without off-target effects on non-target bacteria. Furthermore, in cases where it is challenging to isolate virulent phages for pathogenic bacteria, synthetic biology platforms provide the capability to artificially synthesise virulent phages using prophage sequences found within pathogenic bacteria.[139] *E. faecalis* and *L. monocytogenes* phages which were converted to lysogeny-deficient variants, revealed efficient lytic activity against target strains.[140,141] In 2019, a 15-year-old patient with cystic fibrosis with a disseminated *M. abscessus* infection was treated with engineered phages synthesised from prophages.[142] This phage treatment brought clinical improvement and provided insight into advanced phage therapy.

Conclusion

Phages have once again come into the focus as a powerful approach for combating antimicrobial-resistant bacteria. In addition, as the relationship between the intestinal bacterial microbiota and diseases is unravelling, phages have been identified as a new approach for precisely editing the microbiota by selectively eliminating pathobionts. Thus, phages are an antimicrobial approach that can be applied not only for the treatment of bacterial infections, but also for chronic "non"-infectious diseases associated with changes in the bacterial microbiota. Future prospects for phage therapy, especially for liver diseases,

include therapeutic strategies that combine phages with either existing antibacterial compounds, such as antibiotics, or with conventional, non-microbe-targeting treatments. Engineering phages to broaden their host range and decrease resistance will maximise their efficiency, while preserving the beneficial intestinal microbiota. To this end, clinical trials will be needed to demonstrate the safety of using phage therapy to edit the gut microbiota in liver diseases. Phage-based therapy is expected to develop as a promising approach and give us powerful options in addition to classical antimicrobial strategies.

Abbreviations

Epa, Enterococcal saccharide antigen; HiAlc Kpn, high alcohol-producing strains of *K. pneumoniae*; IBD, inflammatory bowel disease; LPS, lipopolysaccharide; MAFLD, metabolic dysfunction-associated fatty liver disease; MedAlc Kpn, medium alcohol-producing strains of *K. pneumoniae*; NAFLD, non-alcoholic fatty liver disease; PFU, plaque-forming units; PSC, primary sclerosing cholangitis.

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Conflict of interest

B.S. has been consulting for Ambys Medicines, Ferring Research Institute, Gelesis, HOST Therabiomics, Intercept Pharmaceuticals, Mabwell Therapeutics, Patara Pharmaceuticals, Surrozen and Takeda. B.S. is founder of Nterica Bio. UC San Diego has filed several patents with J.F. and B.S. as inventor related to this work. B.S.'s institution UC San Diego has received research support from Axial Biotherapeutics, BiomX, ChromoLogic, CymaBay Therapeutics, Intercept Pharmaceuticals, NGM Biopharmaceuticals, Prodigy Biotech and Synlogic Operating Company.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Manuscript concept: JF and BS; drafting of the manuscript: JF and BS.

Supplementary data

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Author names in bold designate shared co-first authorship

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