Phages to shape the gut microbiota?

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The concept of (bacterio)phage therapy is simple; target the phage to the bacterial pathogen causing disease. As phages are natural killers of bacteria, one could expect this to be an easy task. However, when it comes to phage therapy within the gut, it might not be quite that simple. Already without exogenous intervention, a multitude of phage–bacterial interactions occur within the human gut, some of which might play a direct role in disease progression. In this perspective, we aim to summarise the current understanding of phages within our gut, moving from infancy, adulthood, and then into disease progression. We then highlight recent advances in phage-based interventions, both conventional phage therapy and the progressing field of whole virome transplant.

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Introduction

The human gut is home to a number of microorganisms, most prominently bacteria and the viruses that infect them; the bacteriophages. Phage colonisation of our guts begins in early infancy, after which they stably coexist with bacterial commensals. Recent studies have shown the phage community to be altered during disease, suggesting a potential role in disease progression. In contrast, traditional use of phage therapy within the gut has been challenging, raising the question as to whether phages truly modulate the gut, or simply reflect the changes in bacterial community composition. Deeper understanding of the novel phage biology occurring within the gut ecosystem is ultimately required in order to progress the field of intestinal phage therapy. Herein, we summarise the current understanding of phage biology within the gut and highlight recent advancements in phage-based interventions.

The human gut virome: in sickness and in health

### The infant gut is rapidly colonised by phages

At birth, the human gut is considered to be largely devoid of microorganisms. Yet in the following hours and days postpartum, the gut is rapidly colonised; housing the greatest microbial diversity within the body. This early colonisation includes phages, which have been found in infant stool samples as early as a few days after birth (Figure 1a) [1,2]. For the first few years of life, the viral gut composition appears to be dynamic, with an ongoing turnover in viral species [1,2]. This is thought to be the result of a stepwise colonisation by phages of different lifestyles [3]. When bacterial biomass is low and potential hosts for invading phages are scarce, the virome is predominantly composed of temperate phages. These phages likely piggybacked on pioneering bacteria as integrated prophages, to later be induced in the gut [4]. Whether the lysogenisation of bacterial hosts provides a competitive advantage during colonisation of the infant gut is currently unknown, but mouse models have shown a competitive advantage provided by phage induction to ward off closely related (but non-lysogenised) strains [5]. Overtime, the expansion of bacterial species throughout the gut facilitates the colonisation of strictly virulent phages. Consistent with this is the increased abundance of the virulent phage families, Microviridae and crAss-like, observed in later infancy [2,3].

The adult gut is stable and has an individualised signature

In contrast to the infant gut, the adult virome is considerably stable over time [6,7,8]. An in-depth analysis of ten healthy adult viromes attributed this stability to a set of highly abundant and persistent phages [8]. These persistent phages seem to mainly consist of the crAss-like and Microviridae phage families, both of which are generally considered to be virulent (Figure 1b). The crAss-like phage family is a particularly high abundance member of the human gut, with some viromes having as high as 90% of viral reads accredited to this family [9–11]. Further, the persistent virome is highly individualised, with few if any phages shared across individuals [8]. So far, two longitudinal studies have observed a slow, but significant, genetic drift within the virome over time [6,8]. This genetic drift, together with stable integration of high abundance virome members, might provide an explanation for the uniqueness of our viromes.

Alongside the set of persistent phages, each virome also contains a number of transiently detected viral clusters [8]. These clusters generally outnumber the persistent

Figure 1
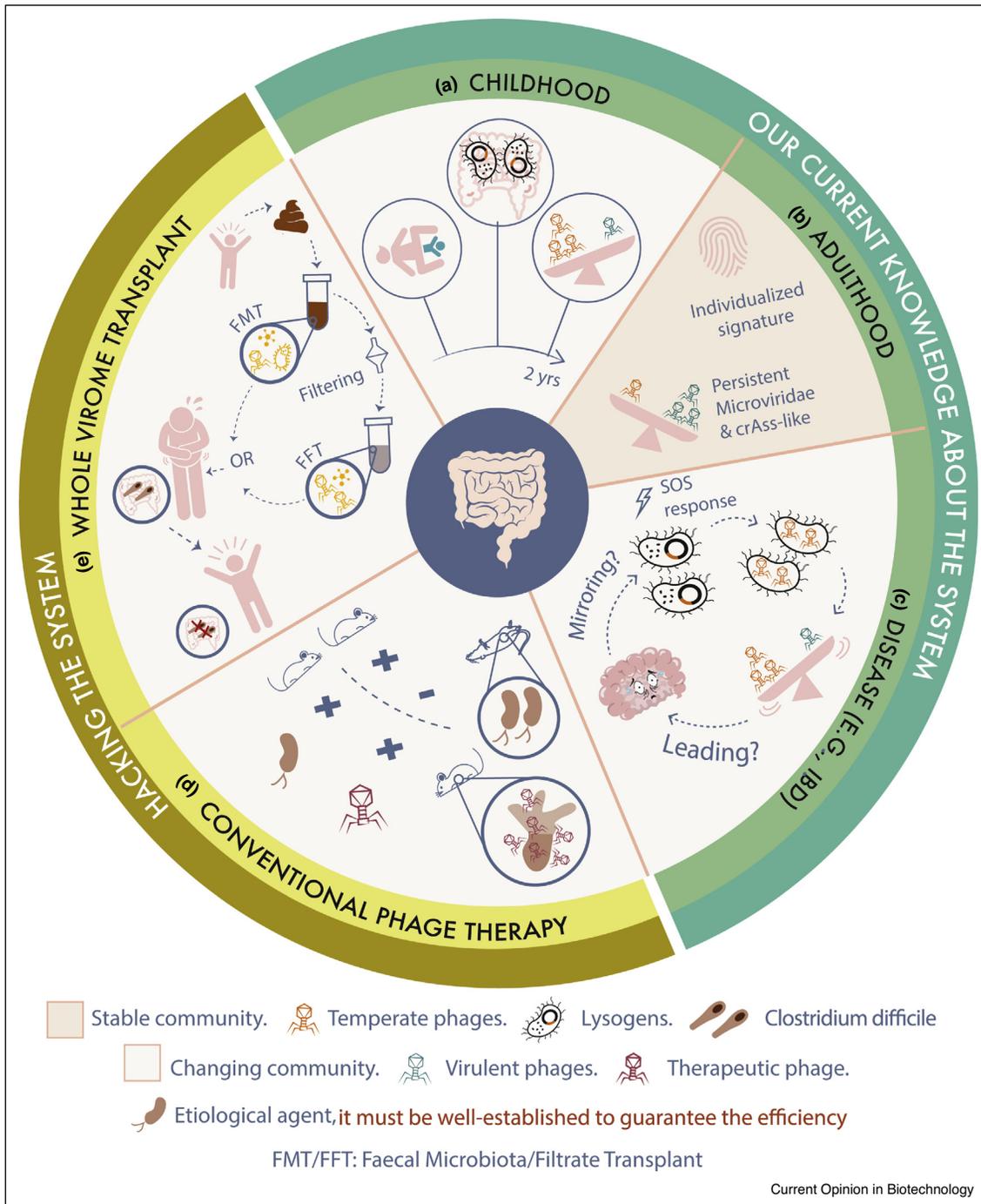
The phage community as the potential hub of human gut health: Understanding the system to hack it. **(a)** During childhood phages colonized the gut with a high predominance of temperate phages, which likely piggybacked on pioneering bacteria. **(b)** Two years after birth, the gut phage community matures and keeps stable during adulthood. **(c)** Disruptions of the phage composition, involving an increase of temperate phages, are linked with diseases like IBD. Whether phages can modulate the gut or simply react to changes within it is still unknown. **(d)** However, phage therapy interventions have been successful in mice. **(e)** Interestingly, the use of faecal filtrate transplant, which is enriched in viruses but lacks bacteria, has shown equal efficiency as faecal microbial transplants for treating recurrent *Clostridium difficile* infections.

set, but exist in much lower abundance and tend to include temperate phages as well as eukaryotic viruses. Lysogens, bacteria with integrated prophage genomes, are known to be common within the gut [12]. Considering this, a temperate lifestyle has often been used to explain the seemingly peaceful coexistence (i.e. the temporal stability) of phages and bacteria. However, our current understanding suggests that virulent phages are abundant in the healthy adult gut [8••,13]. Explanations for this stable coexistence of virulent phages and their prey include; (i) arms race dynamics, whereby phages and bacteria constantly evolve to avoid extinction, (ii) microbiota facilitated host jump, in which phages can avoid extinction by jumping to a new host after depleting their previous host, and (iii) spatial refuges, in which bacteria can proliferate without being targeted by phages [14]. So far, there is limited evidence of extended arms race dynamics within the gut, although some instances of emerging phage resistance have been reported [15•,16]. High microbial diversity is known to promote host switching and the broad host range it entails has been suggested to aid the survival of virulent coliphages in the dynamic infant gut [17,18]. However, the involvement of this mechanism in the stable adult gut remains to be seen. Instead, recent evidence suggests that the spatial heterogeneity within the gut (i.e. the lumen versus mucosal surface) could be the main driver of coexistence [14]. However, considering our limited knowledge of phage biology within the gut, it is likely that other, yet to be characterised, mechanisms are also at play. For example, crAss-like phages have the ability to sustain productive infection without negatively affecting the growth of their bacterial hosts, as was demonstrated in broth cultures where spatial refuges are absent [19•].

Disease alters the gut virome, but does the gut virome alter disease?

The gut microbiota, usually referring to the bacterial fraction of the gut, is well recognised as a major player in human health [20]. Recently, the role of the virome, and in particular phages, is being investigated as a potential missing piece of the gut dysbiosis puzzle. For example, the viromes associated with colorectal cancer and type 2 diabetes have unique viral signatures compared to healthy controls [21–23]. Likewise, studies have identified a shift in the virome of patients suffering from inflammatory bowel disease (IBD) [24,25•,26]. In these patients, an overall increase in phage numbers, measured as increased virus-like-particle (VLP) counts [25•], as well as a shift from virulent towards temperate phages was observed (Figure 1c) [25•]. Consistent with this, inflammation (a hallmark of IBD) is known to activate the bacterial SOS system, leading to prophage activation and induction [27].

However, the role, if any, of the altered virome leading to disease progression is yet to be deciphered. As such, changes observed in phage compositions might merely mirror any changes seen in the bacterial host population. Indeed, increased phage abundances in a mouse colitis model have been linked to expansion of their pathogenic host [28]. In contrast, phage-mediated lysis could facilitate gut dysbiosis and depletion of beneficial symbionts [28–30]. For example, the reduction of *Faecalibacterium prausnitzii*, an important anti-inflammatory gut symbiont, concomitant with elevated levels of its temperate phages has been linked to IBD [29]. Interestingly, prophage induction caused by western dietary products, such as fructose and stevia, has been observed [31,32]. Similarly, increased levels of temperate phages were observed in a mouse model after switching to a high-fat, high-sucrose diet, providing a possible link between western diets, prophage induction, and gut disease [33]. Although intriguing considering our rapidly increasing consumption of refined sugars, this is still hypothetical and a causative role of phage in disease has yet to be established. In fact, even though an altered microbial composition has been observed in several human gastrointestinal diseases, the causative role of these alterations is far from being demonstrated [34]. In line with this uncertainty, contradictory results were recently reported in which a switch to a high-fat diet shifted the viromes of mice towards virulent Microviridae phages [35]. Clearly, the field of intestinal phage biology is still in its infancy and we are far from fully understanding the complex dynamics that governs gut health.

The guardians of the gut: can we harvest the power of phages?

Phages against gut disease, a fictional hero or a future reality?

Simply mentioning the word 'bacteriophage' evokes notions of bacterial predation, cell lysis, and anti-microbial interventions. Yet clinical evidence of their successful application in human gut diseases is so far limited. For example, the historical records of their use against *Vibrio cholera*, dysentery and Salmonella gut infections in the early 20th century, although impressive, lack standardisation and the use of proper controls that would be demanded today [36]. Similarly, in countries with long standing tradition of phage therapy, such as Poland, Russia and Georgia, the introduction of phage cocktails as a standard treatment preceded the development of double-blinded clinical trials. Further, the phage content within these cocktails are seldom characterised, which, together with limited documentation in English literature, has hindered their transfer to western medical practices [36]. In an attempt to progress intestinal phage therapy into a standardised clinical setting, Sarker *et al*. conducted a placebo controlled, double-blinded trial using phages to combat diarrhea in Bangladeshi children [37]. Sadly, neither clinical benefit, nor evidence of phage replication, was observed. Although unsuccessful, the trial unveiled some key hurdles for the development of

effective intestinal phage therapy [38]. For example, retrospective analysis of the clinical stool samples revealed either complete absence or low abundance of the targeted bacteria, implying their limited involvement in disease progression. As such, the study highlighted the need for a well-established disease etiology, and recognised the potential limitations associated with targeting low abundance or otherwise inaccessible pathogens, such as slow growing or biofilm associated strains [38].

While the clinical evidence in favour of phage therapy is slim, some studies have demonstrated the ability of phages to modulate the gut microbiome [15•,39]. For example, the use of simplified gut communities within mouse models have revealed dynamic microbial and metabolic changes within the gut following phage administration [15•,39]. These changes were not limited to the targeted bacteria, but correlated to alterations in the abundance of other bacterial species, raising the question of whether careful targeting of less favourable communities can be paired with downstream increase of beneficial symbionts. Indeed, signs of this have recently been observed in humans [38]. In a placebo-controlled, cross-over trial, the use of an *Escherichia coli* phage cocktail in healthy adults was linked to increase in *Bifidobacterium bifidum* species, a common gut commensal [40].

Additionally, administration of phages has led to reduced pathogenicity in a number of mouse models (Figure 1d). For example, targeting of a colorectal cancer-associated bacterial species with virulent phages showed significant reduction in the targeted bacterium, followed by a subsequent reduction in cancer volume and increased survival [30]. Importantly, continuous phage administration was needed for sustained reduction in bacterial load. In an innovative approach, Dong *et al*. achieved clearance of a targeted bacterium and remodelling of the tumour microenvironment by combining the specificity of phages with antimicrobial agents [41]. Using the multifaceted phage display system, M13 phages capable of binding to the targeted bacteria were identified, after which antimicrobial nanoparticles were assembled on the phage surface. This use of phages as drug carriers, rather than agents of lysis, bypasses the need for infection and expands the versatility of the system. Engineered phages have also been used to deliver specific genetic material to their hosts. For example, a proof of principle study recently demonstrated the ability of M13 phagemids, being plasmids packaged into phage particles, to deliver CRISPR-Cas genes to bacteria within the mouse gut [42]. In another study, the efficacy of phage therapy against *Clostridium difficile* was increased by exploiting the bacteria's own CRISPR-Cas system [43]. By adding a chromosome-targeting CRISPR array to the phage, *in vivo* redirection of the endogenous CRISPR-Cas system was achieved leading to lethal DNA breaks on the bacterial genome. As isolation of strictly virulent phages against

Clostridium difficile has proved challenging, temperate phage mutants engineered to lack key lysogeny genes were used. This use of phage engineering to render temperate phages non-lysogenic has recently been highlighted as a valuable tool for rapid 'on-demand' production of phage cocktails [44]. Phage-based gene delivery has also been used to reduce the expression of virulence factors in the gut. For example, a temperate phage targeting Shiga toxin-producing *Escherichia coli* was recently engineered to encode a non-degradable repressor protein [45]. Stable integration of this temperate phage into the bacterial genome was achieved *in vivo* and served as a continuous inhibitor of toxin production. This targeted remodelling of bacterial function through *in situ* engineering using phages has recently been proposed as a promising future area for microbiota-based therapeutics [46]. Aside from phage engineering, beneficial phage traits can also be selected for during isolation of natural phages. For example, a recent study demonstrated the enhanced ability of a mucin-binding phage to clear the mouse intestine of pathogenic *Escherichia coli* [47]. Noteworthy, the phage, even though superior in the intestine, showed moderate efficacy in *in vitro* cultures. This highlights the need for life-like culture conditions when choosing candidate phages and raises the question of whether mucin-binding should be integrated to the development pipeline of future intestinal phage cocktails [48].

Despite these successes, a common drawback of animal models is the use of a reduced microbiota complexity, such as gnotobiotic or axenic mouse models. Although this reduced complexity is often needed to decipher complex inter-microbial interactions, it can hinder translation of findings to higher complexity environments, such as the human gut. To this end, a recent study used a humanized mouse model colonised with faecal bacterial communities from patients suffering from alcoholic liver disease [49••]. Targeting the pathogenic bacterial strain with phages successfully alleviated the disease without negative effects on the overall microbial composition. These recent successes of phages to attenuate disease by reducing bacterial load will spark a renewed interest of phage-based interventions in the clinical practice.

When fighting bad guys, it helps to bring your posse

Gut dysbiosis is often associated with a shift or loss of microbial diversity [20]. Faecal microbiota transplant (FMT), which is the infusion of healthy human donor faeces into the bowel, is aimed to restore this diversity. FMT is especially useful for treating recurrent *Clostridium difficile* infections (rCDI), a common opportunistic gut pathogen, with remission rates exceeding 90% [50]. During FMT, phages originating from the donor stool are also transferred to the recipient's gut. As such, a remodelling of the recipient virome towards that of the donor has been observed in a number of studies [30,51–55]. Interestingly,

engraftment of donor phages seems to correlate with positive-treatment outcomes. For example, a small study investigating the effects of FMT on patients suffering from ulcerative colitis (UC), a subtype of IBD, observed a reduced relative abundance of Caudovirales phages in patients responding to therapy compared to non-responders [29]. This is in line with previous observations of increased levels of Caudovirale phages in diseased patient versus donor stool [53,54]. Further, a recent study of FMT in rCDI found an association between treatment outcome and donor phage composition [55]. Specifically, high phage diversity but lower phage abundance in the donor samples seemed to correlate with remission rate.

The discovery of phage involvement in FMT raises the question of whether a 'whole virome transplant', being a faecal transplant devoid of bacteria, would generate similar effects? To this end, sterile faecal filtrate transfers (FFT) have been used to treat five patients suffering from rCDI, with clinical remission observed in all patients (Figure 1e) [56]. Likewise, a small pilot study comparing the use of FFT versus FMT in rCDI observed comparable remission rates (75% versus 80%, respectively), indicating 'whole virome transplants' as an effective alternative to FMT [57]. If the clinical outcomes are comparable, the use of bacterial-free filtrates would be preferred, as proven by a recent FDA recommendation highlighting the potential health risks associated with the transfer of live bacteria during FMT. This has also been demonstrated in a preterm piglet model of infant necrotising enterocolitis (NEC), in which the oral administration of FFT was both safer and more effective at preventing disease than FMT [58]. Further, beneficial effects of FFT in complex disorders other than NEC have been reported. For example, a recent study demonstrated the ability of FFT to reduce symptoms of type 2 diabetes in a diet induced obesity mouse model [59]. Another study used FFT to revert the gut microbiome of antibiotic perturbated mice to resemble the microbiome pre-antibiotic treatment [60]. Importantly, the active role of phages in FFT has been confirmed in two independent mouse models [39,60]. In both studies, a phage-free control, consisting of heat and nuclease treated faecal filtrates, were unable to elicit the same effects as the untreated FFT preparations. The ability of whole virome transplants to elicit a global modulation of the microbial composition may provide new avenues to treat complex gut disorders, even when the underlying disease etiology is unclear.

Concluding remarks
We are beginning to realise the power and potential of phages as natural modulators of our gut microbiota. Yet, we are barely scratching the proverbial viral surface. Attesting to this, metagenomic studies routinely finds 80–90% of viral reads within the gut to be novel or hypothetical, without sequence similarity to currently

characterised phages [61]. Further multifaceted studies into the vast viral unknown will uncover novel phage biology and provide a renewed understanding of how phages shape the gut. For example, metagenomic data have recently implicated elevated prophage induction in IBD, while mechanistic studies have provided deepened understanding of phage–bacterial interactions, leading to the discovery of spatial refuges wherein (potentially pathogenic) bacteria can escape phage predation [14,25•]. In tandem to this, further development of phage-based interventions for translation into clinical practice is on its way [41]. Lastly, the use of whole virome transplant provides a new and exciting resource in the fight against gut dysbiosis.

Conflict of interest statement
Nothing declared.

CRediT authorship contribution statement
Sofia Dahlman: Conceptualization, Investigation, Writing - original draft. **Laura Avellaneda-Franco:** Conceptualization, Investigation, Writing - review & editing, Visualization. **Jeremy J Barr:** Conceptualization, Investigation, Writing - review & editing, Supervision, Funding acquisition.

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- •• of outstanding interest

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