Trends in Microbiology

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Review

New technologies for developing phage-based tools to manipulate the human microbiome

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Gut bacteria play an essential role in the human body by regulating multiple functions, producing essential metabolites, protecting against pathogen invasion, and much more. Conversely, changes in their community structure are linked to several gastrointestinal (GI) and non-GI conditions. Fortunately, these bacteria are amenable to external perturbations, but we need specific tools for their safe manipulation as nonspecific changes can cause unpredicted long-term consequences. Here, we mainly discuss recent advances in cultivation-independent technologies and argue their relevance to different key steps, that is, identifying the modulation targets and developing phage-based tools to precisely modulate gut bacteria and restore a sustainable microbiome in humans. We finally suggest multiple modulating strategies for different dysbiosis-associated diseases.

Highlights

Microbiome manipulation should be precise to avoid unwanted consequences – phages may provide a solution due to their host-specific nature.

It is essential to shift from classical isolation and characterisation methods to novel culture-independent techniques to discover the full potential of phages as microbiome modulators.

New culturing technologies enable mechanistic studies of yet-to-be-cultured gut bacteria, providing insight into their physiology and network interactions.

Multi-omics are more accurate than mono-omics in identifying bacterial taxa and functional traits related to human health and disease.

Introduction

The human gut contains trillions of microbes, including bacteria, viruses, fungi, and protozoa [1]. Bacteria and their viruses, called bacteriophages or phages, are the most abundant microbes in the gut [1,2]. The total number of gut bacteria is estimated to be 3.8×10^{13} [3], and the phage-to-bacteria ratio is 1:1 or lower [4]. Bacteria in the gut are not passive bystanders but regulate multiple host functions, including circadian rhythms, metabolism, and immunity [5–7]. By contrast, **dysbiosis** (see Glossary) is associated with several human diseases or conditions, including inflammatory bowel disease (IBD), colorectal cancer (CRC), cardiovascular disease (CVD), type 2 diabetes, recurrent *Clostridium difficile* infections, and more [8–12]. This dysbiosis is not just associated with imbalances in the diversity of gut bacteria but also their functions. For instance, multiple gut species belonging to the phyla Firmicutes and Proteobacteria produce trimethylamine N-oxide (TMAO) as a by-product of their metabolism, which, in turn, is associated with CVD [13]. *Enterococcus faecalis*, a commensal gut bacterium, also generates cytolysin, a toxin that causes liver injury [14]. Therefore, accurate manipulation of the gut bacteria to eliminate pathogens or restore a balanced community in diseases associated with dysbiosis is of great interest.

Phages are suggested to play a major role in maintaining gut homeostasis through regulating bacterial abundance, diversity, and metabolism; they are also key drivers of horizontal gene transfer and bacterial evolution in most ecosystems [4,14,15]. Unique characteristics of phages as natural bacterial modulators make them promising tools for **microbiome** manipulation. Here, we describe three questions that are key to the precise modulation of the human microbiome using phages. We also summarise our current knowledge of the technological advances that can help to address these questions. Finally, we suggest multiple phage-based strategies for modulating gut bacteria and their relevance for restoring a **sustainable microbiome**.

Which gut bacteria could potentially contribute to human diseases?

Contrary to the two well-established diseases, *Helicobacter pylori*-associated gastric cancer and *C. difficile*-associated diarrhoea, the aetiology of most dysbiosis-associated disorders, as well as

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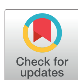

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their link to the gut microbiome, is mostly unknown [16]. Identifying the causative bacteria – pathogenic or nonpathogenic – that specifically contribute to a disease is the first step for precise microbiome manipulation. Establishing a comprehensive catalogue of gut bacterial isolates is necessary to get insights into their disease-causing capacity. However, many gut bacteria are still undetermined as they are recalcitrant to cultivation. Recent cultivation-independent studies have provided some insight into the disease-associated **community structure** of the gut microbiome by profiling and comparing the human microbiome in health and disease. Yet, the full diversity remains uncharacterised, and 40–50% of human gut species lack a reference genome [17]. This is partially due to the current computational shortcomings and existing public databases not representing the complete microbial diversity [17,18] – data mining may provide a solution (Box 1).

What disease-related functions do these bacteria code for?

Specifying whether the observed shifts in community composition contribute to disease pathogenesis requires an understanding of what is there and what they do. This is due to the fact that our microbiome is highly individualised [19] and often context-specific – for example, drastic changes occur in the microbiome during pregnancy so that it becomes identical to the microbiome in people with metabolic syndrome [20]. In addition, the gut bacteria may have a collective rather than individual impact, as suggested for CVD [10,21]. To address the microbiota's biomolecular activity rather than community structure, **multi-omics** analysis can be used.

The power of multi-omics

It has been suggested that studying both the community structure and functional features of the gut microbiota leads to more accurate identification of their roles in health and disease rather than using structure alone [10,22]. The gut bacteria show higher sensitivity to external intervention when studied for changes in gene expression compared with community structure [23]. Microbiome assemblages from different individuals with similar health status are also more alike in terms of their functional profiles than their composition [24,25]. The functional capacity encoded by the microbiome can be described by functional metagenomics, yet it is necessary to determine whether these genes are expressed and translated into proteins. Multi-omics analyses (Box 2) can validate metagenomic findings by describing mechanisms that link gut microbial metabolism to host physiology [10]. Using this approach, positive correlations between the gut bacterial species, including *Prevotella copri* and *Bacteroides vulgatus,* and insulin resistance have been identified for type 2 diabetes; further *in vivo* analysis has also confirmed the role of *P. copri* in increasing the serum levels of branched-chain amino acids (BCAAs), insulin resistance, and aggravation of glucose intolerance [6]. Identifying one specific bacterial target for possible modulation is more complicated for some dysbiosis-associated diseases such as CVD and

Box 1. Data mining

Exploring metagenomic databases using reference-free approaches can help to recover previously undetected members of the human microbiome, paving the way towards revealing their absolute composition [66,67]. Reference-free approaches have already proved to be powerful strategies for species discovery and characterisation [17,68]. These methods mainly make use of sequence information without any prior knowledge and group sequences into unlabelled bins. More specifically, shotgun metagenomic reads are assembled to contigs using *de novo* assembly. The produced contigs are then clustered into different bins based on sequence coverage and tetranucleotide frequency [68]. These approaches allow the recovery of potential genomes, termed metagenome-assembled genomes (MAGs), discovering new species, expanding phylogenetic diversity of microbes, and enriching the public databases that are important to future metagenome analysis [18,68]. Two recent independent studies alone reconstructed over 200 000 microbial genomes, representing several thousands of previously unknown species in public databases, using large-scale metagenomic assembly and binning [1,17]. This is not limited to the bacterial communities as mining viral databases has also changed our view of their diversity in the human body [66,67,69].

Glossary

Community structure: indicates the composition of the microbial community in a given environment, the number of species in that community, and their relative abundance.
CRISPR: the clustered regularly interspaced short palindromic repeats and their associated proteins (Cas) cause immunity to phage infection.
Dysbiosis: an imbalance in community composition of microbes that live in the human body.
Faecal microbiota transplant (FMT): the process of transferring a faecal microbial community from a healthy donor to the gut of a recipient suffering from a dysbiosis-associated disease.
Functional diversity: the richness and distribution of different functions encoded by the microbial community.
Functional redundancy: when multiple different bacterial taxa share similar functional capacity in the population.
Microbiome: the genetic pool of microbial communities that live in and on the human body.
Multi-omics: an analytical approach that integrates multiple omics – such as genomics, proteomics, transcriptomics, and metabolomics.
Postbiotics: metabolites released by probiotics with a potential health benefit to the host.
Prebiotics: dietary compounds that can specifically induce the growth or activity of beneficial bacteria.
Probiotics: live microbes suggested to have a health benefit on the host when administered.
Sustainable microbiome: a robust and resilient microbial community that remains balanced in response to external interventions.
Temperate phages: bacterial viruses that can grow lytically or enter a more dormant stage either by integrating their genome into the bacterial chromosome or by staying as a plasmid without causing immediate cell lysis.
Virulent phages: bacterial viruses that can only go through a lytic grow cycle, that is, lyse the bacterial cells shortly after infection and production of their progeny.

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Box 2. Data integration

The transition from mono-omics studies to multi-omics analyses, integrating data from metagenomics, metatranscriptomics, metaproteomics, and metabolomics, can address the current shortcomings with microbiome analyses. Yet, the main challenge in analysing multi-omics data is the integration and interpretation of the extensive heterogeneous omics data in order to provide biological insights. New computational tools, such as Multi-Omics Factor Analysis (MOFA), have been developed to discover the principal sources of variation in omics data sets. MOFA is an unsupervised method that provides powerful group factor analyses armed with mean-field approximation-based Bayesian inference to account for multiple types of quantitation, with the ability to reveal factors shared across different omics data [70]. In contrast to the model-based methods for integrating multi-omics data that rely on mathematical deconvolutions to identify latent structures, network-based tools like iOmicsPASS include experimentally acquired biological network data in the analysis to integrate heterogeneous multi-omics data. iOmicsPASS uses a scoring system and the nearest shrunken centroid algorithm to identify subnetworks consisting of molecular interactions within and between related omics data [71]. gNOMO is a new multi-omics pipeline developed for integrating up to three omes, including metagenomics, metatranscriptomics, and metaproteomics data, from both host and microbiome data. gNOMO incorporates preprocessing, binning, assembly, taxonomic, and functional annotations to analyse host–microbiome interactions [72].

IBD, as several taxa might be involved. For example, about eight different TMAO-producing taxa, including *Anaerococcus hydrogenalis*, *Clostridium asparagiforme*, *Clostridium hathewayi*, *Clostridium sporogenes*, *Escherichia fergusonii*, *Proteus penneri*, *Providencia rettgeri*, and *Edwardsiella tarda*, have been identified, which directly contribute to platelet hyper-reactivity, and this enhances the risk of thrombosis [21,26]. The number of bacterial taxa that potentially contribute to IBD is even higher – ~10 – including five different *Bacteroides* species, *Eubacterium rectale*, *Roseburia intestinalis*, *Escherichia coli*, and *Faecalibacterium prausnitzii* [10]. Multiple metabolites are also involved, such as acylcarnitines, bile acids, and short-chain fatty acids – making any intervention more challenging [10].

Novel methods for culturing the uncultured

While recent sequencing-based studies have provided valuable insights into the community structure and function of gut bacteria they are semi-quantitative, provide relatively low resolution (it is hard to generate strain-specific data), and they are prone to experimental and computational biases. In addition, it is essential to isolate and culture bacteria in order to explore their physiological features, virulence properties, and their relation to the pathology of certain diseases. This is due to the fact that data generated by these approaches is difficult to understand without fundamental knowledge of the underlying mechanisms, which cannot be inferred from sequencing data alone. Thus, without cultivation, many questions will remain unanswered about bacterial functions in their natural habitats. For example, the role of *Clostridium butyricum* in necrotising enterocolitis, and the impact of the gut microbiota on cancer immunotherapy outcomes, have recently been revealed using culture-based methods [27–29]. In addition, unknown functional properties of bacteria or traits predicted solely based on their genomic information can be best studied in isolation or together with a small number of species that rely on each other to grow [29]. This approach has led to the discovery of a new enzymatic reaction and pathway – which would not have been possible by genomic methods alone [30]. Despite the importance of cultivating gut bacteria to fully understand their role in human health and diseases, numerous – up to 55% – human-associated bacteria and archaea have remained uncultured [31]. Recent culturomics studies that use multiple modified culture conditions for the growth of microbes from human specimens have enabled the recovery of 77% of the total 1525 bacteria and archaea present in the human gut [29]. As the remaining uncultured taxa might have central roles in health and disease, it is essential to try culturing them using recent innovations that benefit from advances in membrane diffusion, microfluidic systems, and fluorescence-activated cell sorting.

The main reason we currently cannot grow some gut bacteria under laboratory conditions is our inability to replicate critical aspects of their native environment. One alternative is to incubate them

while physically separated in their natural habitats using diffusion-based technologies that utilize filters or membranes, which allow access of growth-promoting factors from the environment while letting the growth-inhibitors produced by the replicating bacteria diffuse away [32]. One example of a diffusion-based method is the isolation chip (ichip) which consists of several hundred miniature diffusion chambers with pore sizes between 0.03 μm and 1 mm in diameter [32]. The smallest ichip variants are about 12 mm, which can fit into a capsule similar to what is used in endoscopy [33], enabling their application for cultivating gut bacteria *in situ*. It is also possible to use microfluidic systems for growing gut bacteria not yet cultured. The main advantages of microfluidic systems are the high scalability and low footprint of these technologies that enable the cultivability of numerous single-cell bacteria to be tested in multiple substrates and physicochemical conditions. For instance, a gene-targeted microfluidic system was recently used for culturing the first representative of a *Ruminococcaceae* genus [34]. Cell-sorting technologies are also used for isolating and culturing undetermined bacteria. Using this approach, millions of cells can be dispersed into separate wells, at the single-cell level, containing various growth media. This technology, combined with genome-informed antibody engineering (that uses antibodies against predicted cell surface proteins of bacteria) has recently led to the isolation and culturing of three oral Saccharibacteria species [35]. Similarly, a consortium of phylogenetically diverse bacteria linked to mucin degradation was identified using stable-isotope probing, and Raman-activated cell sorting (RACS), that provides label-free cell sorting, showed its potential for the isolation of bacteria by their functional properties [36].

How to specifically target unwanted gut bacteria?

The current strategies used for microbiome manipulation are limited to a few options that have mostly led to inconclusive results, with **faecal microbiota transplant** (**FMT**) being the most prominent approach. FMT is an ancient method that is still in use without significant changes. Despite its high rate of success for the treatment of recurrent *C. difficile* infection, and its use in certain experimental studies on IBD, there are serious safety concerns with FMT, including the potential to transfer harmful multiresistant pathogens from the donor to the receiver [37]. Moreover, the impact of recent strategies, such as **probiotics**, **prebiotics**, and **postbiotics**, on the gut bacteria is often transient, individualised, and affected by differences in genetic background and microbiome structure of the host [38]. The biggest drawback with most of these methods is their lack of specificity. The human gut is home to thousands of bacterial and archaeal species with tremendous functional capacities. Thus, careless modulation of these communities can lead to severe long-term consequences [38].

Phages as microbiota modulators

Phages have the potential to serve as precise tools for modulating the human microbiome due to the following unique features: (i) high specificity – phages are often species-specific with regard to their host, and most of them infect only a single bacterial species, or multiple strains within a species, leaving the nonhost bacteria unharmed [39,40]; (ii) ubiquity – phages outnumber their bacterial host by roughly tenfold in most ecosystems but the human gut. This suggests that there can be numerous phages against every target bacterium in the environment, making the isolation of desirable phages straightforward [4]; (iii) evolvability – phages have high genomic plasticity and multiplication rates; they are also locked in constant coevolution with their bacterial host, thus, they have developed diverse strategies to infect their hosts and survive their defence mechanisms; (iv) diversity in infection strategy – phages infect their host through two major strategies, lytic and lysogenic, which makes them suitable to be used for different modulation approaches, for example, the use of **virulent phages** for eliminating unwanted taxa and temperates for gene delivery; and (v) self-replication – a unique characteristic of phages as modulators is their ability to reproduce at the site of infection in the presence of the target bacteria. Thus, low doses can potentially be used in therapy [39,40].

Furthermore, there is some evidence that phages are highly efficient in clearing infections by pathogenic bacteria, including one successful placebo-controlled double-blind Phase I/II clinical trial against chronic otitis, caused by drug-resistant *Pseudomonas aeruginosa*, which showed improvement in the treated group compared with the control group [41], and several personalised case studies [42]. They have also shown promising regulatory effects on gut bacteria: the phage community from stunted children changed bacterial abundance and diversity from healthy children in an age-specific manner *in vitro* [15]; the transfer of viral communities from lean to obese mice reduced symptoms of type 2 diabetes and obesity [43]; a cocktail of four phages that were used to target cytolytic *E. faecalis* could attenuate alcoholic liver disease in humanised mice [14]; and transferring sterile-filtered stool samples rich in phages from healthy donors to a small number of patients with recurrent *C. difficile* infection could sufficiently relieve the symptoms associated with the infection [44]. This potential has also been recognised by biotech companies, and a platform technology called PhageBiotix, which uses virulent phages for targeting unwanted bacterial species in the GI tract, has been recently developed (http://www.intralytix.com).

The limitations of phages and the help of emerging technologies

There are several unknowns about phages as modulators – mainly we lack full knowledge of phage diversity, the phage–bacteria infection networks, and their interactions with human cells (Box 3), which are largely limited by the inability to culture many bacteria and their phages. Of the estimated 10^{31} phages on earth, only a few thousand have been isolated and sequenced. This is not limited to their quantity as their diversity is also under-represented – phages characterised until now infect only a few of >1000 predicted bacterial phyla [45]. The vast majority of these phages infect common pathogenic bacteria that can be easily cultured in the laboratory. By contrast, no virulent phages have yet been isolated against many bacterial taxa, particularly those that are fastidious or anaerobic, such as *C. difficile*, despite all the attempts that have been made [46]. This is mainly due to the limitations of the classical approaches commonly used for phage isolation. These methods are usually culture-dependent, which restricts the isolation of phages to those that can form plaques. To address this issue, we should move towards using emerging cultivation-independent strategies for their isolation and characterisation.

Novel methods for characterising uncultured phages

Fortunately, multiple cultivation-independent experimental methods have been recently developed to physically link phages to their bacterial hosts (Figure 1). First, the proximity ligation

Box 3. Novel preclinical models for studying phage kinetics

There are multiple unknowns when studying phage kinetics, such as diffusivity through the body, as well as their interactions with the immune system and human cells, which may have important implications for phage-based modulations. To shed light on these, preclinical models with high accuracy are sought after [39,73]. 2D cultures in a microfluidic system that enable live-cell imaging under conditions similar to the human body have recently been used for studying phage kinetics. They showed that phages are rapidly internalised by the endothelial cells through micropinocytosis – the uptake rate was significantly affected by the size and morphology of the phage as well as by the cell types tested [73]. Yet, despite their popularity, 2D cultures do not truly represent how cells live and function *in situ*, suggesting an inevitable shift from 2D to 3D cell cultures. 3D cultures, such as patient-derived organoids, show a high degree of phenotypic and genotypic similarity to the original patient tissues, indicating how patients respond to the treatments *in situ* [74]. *In vivo* studies have already provided significant insight into phage kinetics [40] but still can benefit from forthcoming technological advances in the field. One example is the development of transparent mouse models that enable imaging of the subcellular details in the animal through bones, skin, and tissues without the need for further dissection. These models are highly beneficial for studying phage kinetics as they allow unbiased and comprehensive studies of the interactions between phages and the whole mammalian body [75]. In addition, a new technique has recently been developed that allows real-time observation of cellular processes in the colon of live animals using high-resolution, chronic fluorescent imaging of the organs, tissues, and more for weeks with no pain, discomfort, or intestinal obstruction [76]. This technique can be helpful to study the phage-based modulation in diseases more relevant to the colon, such as CRC.

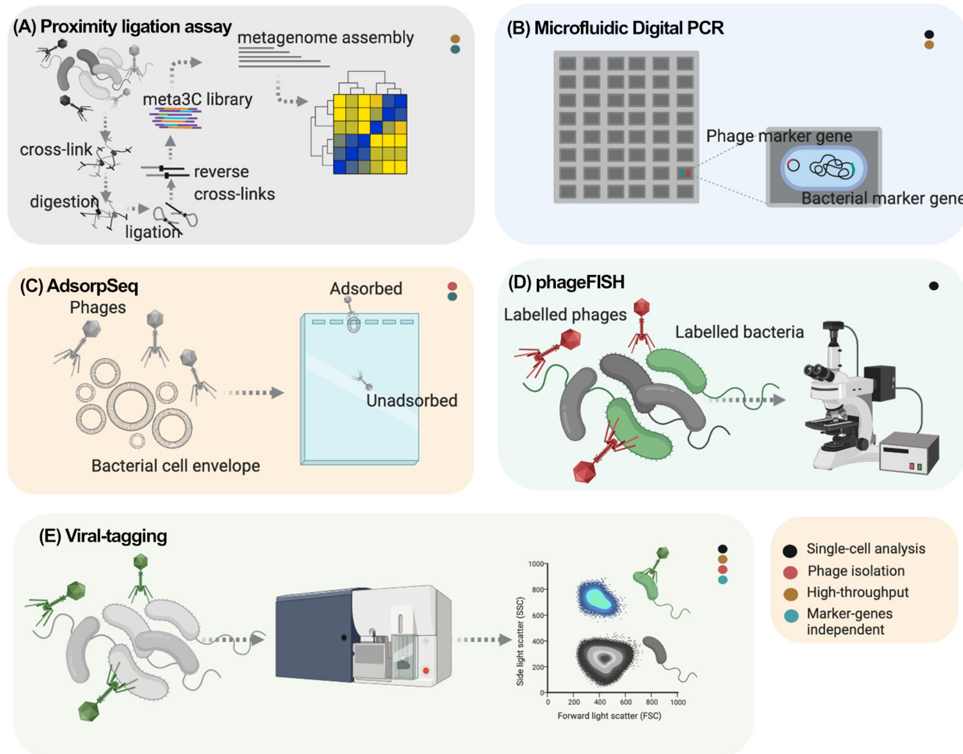

Figure 1. Cultivation-independent methods for linking phages to their bacterial host. (A) the proximity ligation assay combines experimental and computational methods to determine physical contacts between DNA molecules of phages and bacteria to identify their proximity. (B) Microfluidic digital PCR uses marker genes to codetect internalised phages and their bacterial host using digital PCR. (C) AdsorpSeq applies gel electrophoresis for identifying phage–host interactions based on the affinity of the phage for the surface receptors on the envelope of target bacteria. (D) phageFISH (fluorescence *in situ* hybridization) uses sequence-based, fluorescently labelled probes for simultaneous identification of infecting phages and their hosts using epifluorescence microscopy. (E) Viral-tagging (VT) identifies unknown phage–bacteria pairs using fluorescence-activated cell sorting (FACS) without requiring the use of marker genes. Panel (A) is adapted from [77]; panel (B) is adopted from [48]; and panel (D) is adapted from [49] with permission from the relevant publishers.

assay (Figure 1A) uses metagenomic chromosome conformation capture (meta3C), which combines experimental and computational methods to explore the physical contacts between DNA molecules to identify their proximity [47]. Specifically, sequences present in complex microbial communities are crosslinked and digested to produce a meta3C library, which is assembled and assigned to bacterial sequences based on their physical contact frequencies [47]. Second, microfluidic digital PCR (Figure 1B) allows simultaneous identification of phages and their host bacteria using marker genes at a single-cell level. Specifically, microbial cells are collected from the environment, diluted, and loaded into a multichannel microfluidic digital PCR, which codetects internalised phages and host bacteria using digital PCR [48]. Third, phageFISH (fluorescence *in situ* hybridization) (Figure 1C), an enhanced geneFISH variant that increases gene detection efficiency by more than 50%, has been developed to identify and visualise intra- and extracellular phage DNA. This method is based on epifluorescence microscopy for visualisation and uses sequence-based fluorescently labelled probes for identification [49]. Fourth, the polymerase colony called polony is a solid-phase PCR amplification method that uses highly degenerate primers to probe for phage signature genes. Specifically, phages are distributed in a thin polyacrylamide gel containing a 5′-acrydite-modified primer, and individual PCR colonies are produced from template phages. The colonies can then be assessed to see whether they

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contain phage marker genes using fluorescently labelled probes. This method can distinguish and quantify DNA/RNA phages from different genera [50]. Fifth, adsorption sequencing (AdsorpSeq) (Figure 1D) uses gel electrophoresis to detect phage–host paring. Specifically, purified virus-like particles from the environment are mixed with the cell envelope of target bacteria and applied on agarose gels. The adsorbed phages are separated via gel-electrophoresis and then collected for metagenomic analysis. Using AdsorpSeq, 26 unknown phage–bacteria parings were detected in hospital sewage [51].

Sixth, viral-tagging (VT) is the last method we discuss here (Figure 1E). It separates the phage-infected bacterium with its infecting phage(s) from noninfected bacteria using fluorescence-activated cell sorting (FACS) without requiring the use of marker genes. VT enables experimental linkage of any phages to known/unknown bacteria at both the population and single-cell levels. It also allows downstream analysis of phage sequences infecting a given host when combined with metagenomics. In a single experiment, VT revealed over 363 new phage–host parings in the faecal samples of 11 healthy volunteers, which could not be detected by other methods [52].

Ultimately, the identified phages should be isolated for further processing before being used for microbiota modulation. However, cultivation-independent methods that allow for phage isolation are scarce. The best example is VT as it enables the sorting of phage–bacterium pairs at both population and single-cell levels. The isolated unculturable phages can be reproduced in large numbers using multiple technological advances, including new cultivation techniques (mentioned earlier in this work) and synthetic platforms such as cell-free systems to construct phage particles from isolated phage genomes [53]. The cell-free system allows the production of infectious phage particles directly from purified phage DNA by maintaining cellular processes without an intact cell membrane [53]. Alternatively, phage genomes can be synthesised based on VT data and electroporated into the host bacteria to reproduce phage particles [54]. Moreover, VT combined with multi-omics enables the study of the interactions between not-yet-cultivated phages and their bacterial hosts, leading to the identification of previously unknown phage regulatory strategies that can be used to target unwanted bacterial taxa as stand-alone antibacterials – a suggested approach is illustrated in Figure 2.

Using cultivation-independent methods, we can significantly expand the diversity of phage-based modulators serving different manipulation strategies (Figure 3). (i) Targeting the bacteria by killing unwanted taxa while allowing beneficial taxa to grow using natural virulent phages [14] and phage-derived enzymes [55]. This strategy is most relevant in cases where only one bacterial taxon is responsible for the disease, for example, *C. difficile*-associated diarrhoea. (ii) Targeting the function by using phages as vectors to deliver a variety of modulatory interventions – such as specific genes to introduce a new function to the community (which is beneficial to the host), such as genes which encode essential metabolites; the **CRISPR**/Cas nuclease system, which can selectively eliminate bacterial taxa based on their genetic content [56], which is useful when there is **functional redundancy** in the population, as is the case with TMAO-producing bacteria; or programmable nuclease deactivated Cas9 to repress virulence genes, for example, suppressing shiga toxin expression in *E. coli* [57]. This is an interesting approach as its lower impact on bacterial fitness might protect against selection for resistance. (iii) Targeting the pathogenicity by silencing the virulence factors, such as bacterial surface structures. These structures are involved in bacterial pathogenicity, such as immune evasion and cell invasion, which are often used by phages to attack bacteria [58]. Upon phage infection, bacteria lose these structures through an evolutionary trade-off between phage resistance and virulence [58]. For example, *Listeria monocytogenes* loses the surface-associated Internalin B (InlB) upon phage infection, which prevents InlB-mediated cell invasion and lowers its pathogenicity [58]. Moreover, phages

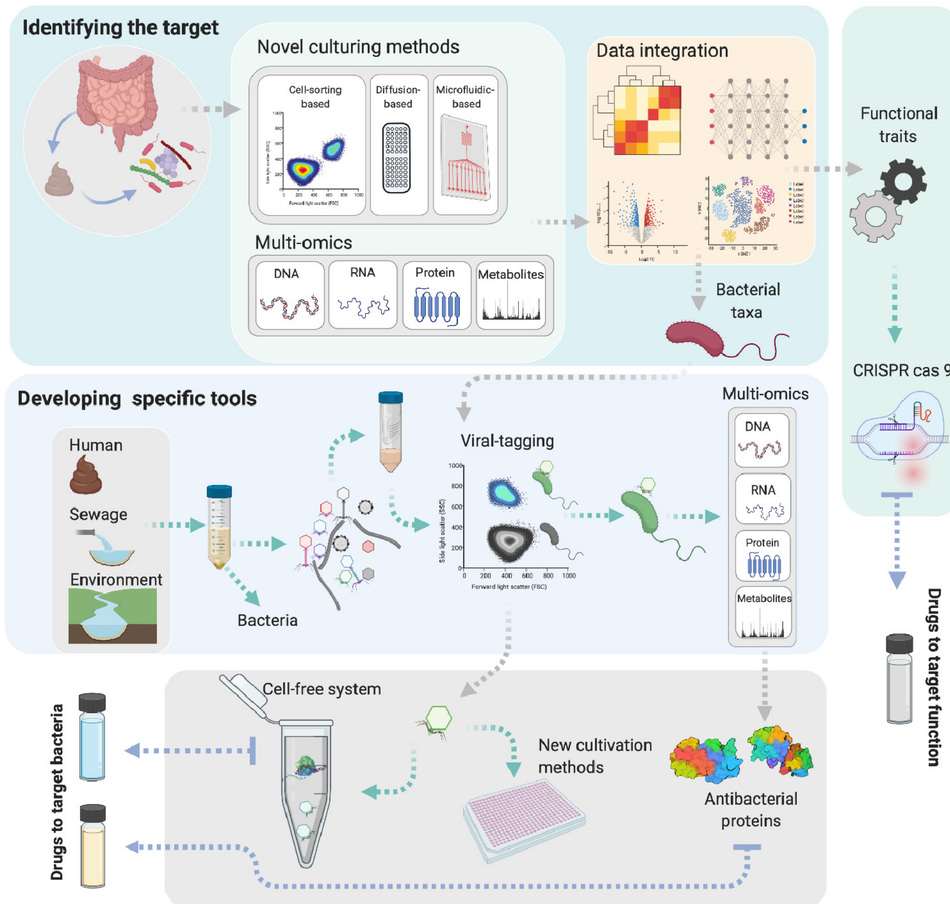

Figure 2. A suggested approach to develop phage-based strategies for precise manipulation of the human microbiome. First, bacterial and functional targets will be identified using advances in culturing methods and multi-omics. Second, phages will be isolated from different environmental sources through cultivation-independent technologies and reproduced using cell-free systems or novel cultivation approaches. Finally, the isolated phages and phage enzymes are developed for eliminating the undesirable taxa, disarming pathogenic bacteria by targeting their virulence, and for delivering specific genes with various functions to modify target bacteria. CRISPR/cas9 will be delivered to the target bacteria using modified phage vectors.

may also have a preventive effect through controlling the causative bacteria in dysbiosis-associated diseases, such as IBD, when administered early to people with a family medical history.

The power of synthetic biology

In addition to the unknowns mentioned earlier, there are multiple obstacles when using phages as modulators that can be partially addressed by the advances in synthetic biology and drug delivery, discussed later. (i) It is relatively hard to find phages that target wide ranges of bacteria due to their high specificity. However, the host range of phages can be altered by modifying receptor-binding proteins (RBPs) on their tail fibres which are used to recognise the cognate bacterial surface receptors [59]. To this end, these regions have been genetically engineered to generate **functional diversity** in a T3 phage's RBPs through site-directed mutagenesis, which changed its host range. The synthetic phage constructed could also prevent the appearance of resistant phenotypes [59]. In addition, hybrid phage particles that display different phage tails

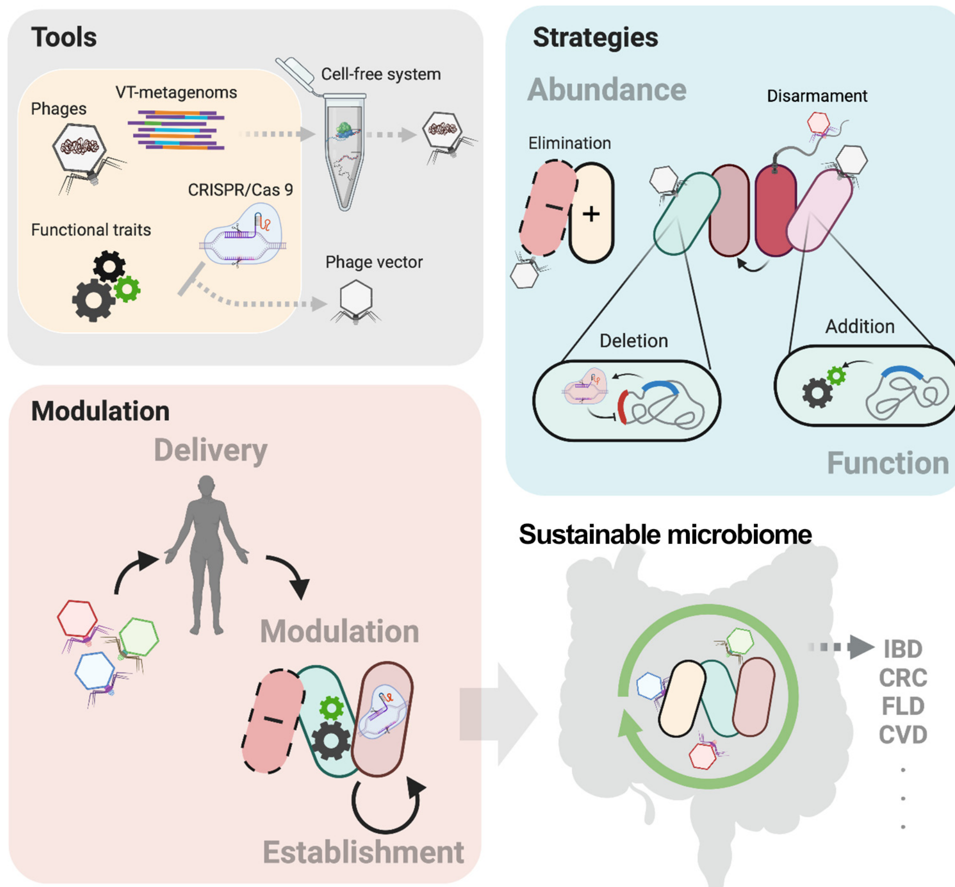

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Figure 3. An overview of phage-based tools and strategies for modulating the human microbiome. Virulent phages, phage-based antibacterial, and phage vectors will be developed and used for modulating the human microbiota through three different strategies: (i) targeting bacteria; (ii) targeting function; and (iii) targeting pathogenicity. The ultimate goal is to restore a sustainable microbiome in dysbiosis-associated diseases such as inflammatory bowel disease (IBD), colorectal cancer (CRC), fatty liver diseases (FLD), cardiovascular diseases (CVDs), etc. Abbreviation: VT, viral-tagging.

or tail-fibre proteins can be constructed to infect various unrelated bacterial taxa – a hybrid T7 phage produced using this approach could infect bacteria of several genera other than *Escherichia* [60]. (ii) Not all phages are equally efficient against their bacterial host. For example, **temperate phages** are usually not considered for therapeutic applications due to their low lysing efficiency and the risk of transferring virulent genes between bacteria [61]. However, their efficacy can be improved by removing lysogeny-related genes, such as the repressor gene, from their genome, hindering their integration into the host genome [61]. This is especially helpful when isolating virulent phages against a target bacterium is challenging [61]. For example, a cystic fibrosis patient with *Mycobacterium abscessus* infection was successfully treated by engineered temperate phages following bilateral lung transplantation [61].

Moreover, phages that are known to be highly efficient against bacteria – that is, those that display a large burst size, a short latent period, and a high adsorption rate, such as T1-like phages – can potentially be isolated from the environment using a gene-targeted approach [34]. (iii) Bacteria can prevent phage infection through an array of antiphage mechanisms, targeting different steps of the phage replication cycle [39]. These mechanisms include the

alteration – through mutation or phase variation – of receptors to inhibit phage adsorption [4,62], degradation of the phage genome through restriction–modification (R–M) systems, CRISPR immunity to phage infection, and more [4,39]. Phages can be engineered to overcome these mechanisms by changing their RBPs [59], arming them with anti-CRISPR proteins [63], and biofilm-degrading enzymes to enhance their efficiency against biofilm-forming bacteria [64], etc. (iv) Phages are sensitive to low pH – thus, their oral delivery is challenging. To protect phages against gastric acid and proteases in the GI tract they can be encapsulated using a layer-by-layer assembly approach that triggers phage release upon entry into the large intestine – that part of the GI tract which contains the highest number of bacteria [57]. (v) The host immune system can neutralise phages. To avoid this, the immunogenic phage proteins, such as head surface proteins, can potentially be altered to lower their immunogenicity and prevent their clearance by the immune system [65]. Yet, the current legislations limit the application of engineered phages. We expect that this will change in the near future, facilitating their application as therapeutics.

Concluding remarks

The importance of gut bacteria to human health, and their flexibility to external perturbations, provides an exciting therapeutic opportunity through editing their structure or function (Figure 3). Yet, this is not a simple task as thoughtless changes in this community can cause long-term unpredicted consequences. To avoid this, we believe that modulations should be precise, with an ultimate goal of restoring a sustainable microbiome – one which includes all essential functions and excludes or unarms undesirable bacterial taxa while remaining balanced in response to external perturbations (see Outstanding questions). To achieve this goal it is essential to shift from our current classical approaches for isolating and characterising phages towards using advances in cultivation-independent technologies and synthetic biology.

Outstanding questions

Can the gut bacteria and their functions be precisely edited by phages, phage enzymes, and phage vehicles? Will these changes be stable or transient?

What are the impacts of uncultivable bacteria and genes with unknown functions they encode in the human gut? How common is the functional redundancy in gut bacteria?

Can we finally reveal the full diversity of gut bacteria and their viruses using emerging culture-independent technologies? How about cultivating them – can we culture the remaining uncultured taxa in the near future?

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Declaration of interests

There are no interests to declare.

References

1. Pasolli, E. *et al.* (2019) Extensive unexplored human microbiome diversity revealed by over 150,000 genomes from metagenomes spanning age, geography, and lifestyle. *Cell* 176, 649–662 e20
2. Paez-Espino, D. *et al.* (2016) Uncovering Earth's virome. *Nature* 536, 425–430
3. Sender, R. *et al.* (2016) Revised estimates for the number of human and bacteria cells in the body. *PLoS Biol.* 14, e1002533
4. Shkoporov, A.N. and Hill, C. (2019) Bacteriophages of the human gut: the 'known unknown' of the microbiome. *Cell Host Microbe* 25, 195–209
5. Kuang, Z. *et al.* (2019) The intestinal microbiota programs diurnal rhythms in host metabolism through histone deacetylase 3. *Science* 365, 1428–1434
6. Pedersen, H.K. *et al.* (2016) Human gut microbes impact host serum metabolome and insulin sensitivity. *Nature* 535, 376–381
7. Zheng, D. *et al.* (2020) Interaction between microbiota and immunity in health and disease. *Cell Res.* 30, 492–506
8. David, L.A. *et al.* (2014) Diet rapidly and reproducibly alters the human gut microbiome. *Nature* 505, 559–563
9. Theriot, C.M. *et al.* (2014) Antibiotic-induced shifts in the mouse gut microbiome and metabolome increase susceptibility to *Clostridium difficile* infection. *Nat. Commun.* 5, 3114
10. Lloyd-Price, J. *et al.* (2019) Multi-omics of the gut microbial ecosystem in inflammatory bowel diseases. *Nature* 569, 655–662
11. Wong, S.H. *et al.* (2017) Gavage of fecal samples from patients with colorectal cancer promotes intestinal carcinogenesis in germ-free and conventional mice. *Gastroenterology* 153, 1621–1633 e6
12. Wu, H. *et al.* (2017) Metformin alters the gut microbiome of individuals with treatment-naive type 2 diabetes, contributing to the therapeutic effects of the drug. *Nat. Med.* 23, 850–858
13. Zhu, Y. *et al.* (2014) Carnitine metabolism to trimethylamine by an unusual Rieske-type oxygenase from human microbiota. *PNAS* 111, 4268–4273
14. Duan, Y. *et al.* (2019) Bacteriophage targeting of gut bacterium attenuates alcoholic liver disease. *Nature* 575, 505–511
15. Khan Mirzaei, M. *et al.* (2020) Bacteriophages isolated from stunted children can regulate gut bacterial communities in an age-specific manner. *Cell Host Microbe* 27, 199–212 e5
16. Walter, J. *et al.* (2020) Establishing or exaggerating causality for the gut microbiome: lessons from human microbiota-associated rodents. *Cell* 180, 221–232
17. Nayfach, S. *et al.* (2019) New insights from uncultivated genomes of the global human gut microbiome. *Nature* 568, 505–510

18. Parks, D.H. *et al.* (2017) Recovery of nearly 8,000 metagenome-assembled genomes substantially expands the tree of life. *Nat. Microbiol.* 2, 1533–1542
19. He, Y. *et al.* (2018) Regional variation limits applications of healthy gut microbiome reference ranges and disease models. *Nat. Med.* 24, 1532–1535
20. Koren, O. *et al.* (2012) Host remodeling of the gut microbiome and metabolic changes during pregnancy. *Cell* 150, 470–480
21. Tang, W.H.W. *et al.* (2013) Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk. *N. Engl. J. Med.* 368, 1575–1584
22. Heintz-Buschart, A. *et al.* (2016) Integrated multi-omics of the human gut microbiome in a case study of familial type 1 diabetes. *Nat. Microbiol.* 2, 1–13
23. Maurice, C.F. *et al.* (2013) Xenobiotics Shape the Physiology and Gene Expression of the Active Human Gut Microbiome. *Cell* 152, 39–50
24. Tian, L. *et al.* (2020) Deciphering functional redundancy in the human microbiome. *Nat. Commun.* 11, 6217
25. Huttenhower, C. *et al.* (2012) Structure, function and diversity of the healthy human microbiome. *Nature* 486, 207–214
26. Zhu, W. *et al.* (2016) Gut microbial metabolite TMAO enhances platelet hyperreactivity and thrombosis risk. *Cell* 165, 111–124
27. Cassir, N. *et al.* (2015) *Clostridium butyricum* strains and dysbiosis linked to necrotizing enterocolitis in preterm neonates. *Clin. Infect. Dis.* 61, 1107–1115
28. Vétizou, M. *et al.* (2015) Anticancer immunotherapy by CTLA-4 blockade relies on the gut microbiota. *Science* 350, 1079–1084
29. Lagier, J.-C. *et al.* (2016) Culture of previously uncultured members of the human gut microbiota by culturomics. *Nat. Microbiol.* 1, 1–8
30. Nunoura, T. *et al.* (2018) A primordial and reversible TCA cycle in a facultatively chemolithoautotrophic thermophile. *Science* 359, 559–563
31. Lloyd, K.G. *et al.* (2018) Phylogenetically novel uncultured microbial cells dominate Earth microbiomes. *mSystems* 3, e00055-18
32. Berdy, B. *et al.* (2017) *In situ* cultivation of previously uncultivable microorganisms using the ichip. *Nat. Protoc.* 12, 2232–2242
33. Cummins, G. *et al.* (2019) Gastrointestinal diagnosis using non-white light imaging capsule endoscopy. *Nat. Rev. Gastroenterol. Hepatol.* 16, 429–447
34. Ma, L. *et al.* (2014) Gene-targeted microfluidic cultivation validated by isolation of a gut bacterium listed in Human Microbiome Project's most wanted taxa. *PNAS* 111, 9768–9773
35. Cross, K.L. *et al.* (2019) Targeted isolation and cultivation of uncultivated bacteria by reverse genomics. *Nat. Biotechnol.* 37, 1314–1321
36. Lee, K.S. *et al.* (2019) An automated Raman-based platform for the sorting of live cells by functional properties. *Nat. Microbiol.* 4, 1035–1048
37. DeFilipp, Z. *et al.* (2019) Drug-resistant *E. coli* bacteremia transmitted by fecal microbiota transplant. *N. Engl. J. Med.* 381, 2043–2050
38. Xiao, Y. *et al.* (2020) An ecological framework to understand the efficacy of fecal microbiota transplantation. *Nat. Commun.* 11, 3329
39. Mirzaei, M.K. and Maurice, C.F. (2017) Ménage à trois in the human gut: interactions between host, bacteria and phages. *Nat. Rev. Microbiol.* 15, 397–408
40. Dąbrowska, K. and Abedon, S.T. (2019) Pharmacologically aware phage therapy: pharmacodynamic and pharmacokinetic obstacles to phage antibacterial action in animal and human bodies. *Microbiol. Mol. Biol. Rev.* 83, e00012-19
41. Wright, A. *et al.* (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
42. Luong, T. *et al.* (2020) Phage therapy in the resistance era: where do we stand and where are we going? *Clin. Ther.* 42, 1659–1680
43. Rasmussen, T.S. *et al.* (2020) Faecal virome transplantation decreases symptoms of type 2 diabetes and obesity in a murine model. *Gut* 69, 2122–2130
44. Ott, S.J. *et al.* (2017) Efficacy of sterile fecal filtrate transfer for treating patients with *Clostridium difficile* infection. *Gastroenterol.* 152, 799–811 e7
45. Yarza, P. *et al.* (2014) Uniting the classification of cultured and uncultured bacteria and archaea using 16S rRNA gene sequences. *Nat. Rev. Microbiol.* 12, 635–645
46. Nale, J.Y. *et al.* (2016) Bacteriophage combinations significantly reduce *Clostridium difficile* growth *in vitro* and proliferation *in vivo*. *Antimicrob. Agents Chemother.* 60, 968–981
47. Marbouty, M. *et al.* (2017) Scaffolding bacterial genomes and probing host–virus interactions in gut microbiome by proximity ligation (chromosome capture) assay. *Sci. Adv.* 3, e1602105
48. Tadmor, A.D. *et al.* (2011) Probing individual environmental bacteria for viruses by using microfluidic digital PCR. *Science* 333, 58–62
49. Allers, E. *et al.* (2013) Single-cell and population level viral infection dynamics revealed by phageFISH, a method to visualize intracellular and free viruses. *Environ. Microbiol.* 15, 2306–2318
50. Baran, N. *et al.* (2018) Quantification of diverse virus populations in the environment using the polony method. *Nat. Microbiol.* 3, 62–72
51. de Jonge, P.A. *et al.* (2020) Adsorption sequencing as a rapid method to link environmental bacteriophages to hosts. *iScience* 23, 101439
52. Džunková, M. *et al.* (2019) Defining the human gut host–phage network through single-cell viral tagging. *Nat. Microbiol.* 4, 2192–2203
53. Yim, S.S. *et al.* (2019) Multiplex transcriptional characterizations across diverse bacterial species using cell-free systems. *Mol. Syst. Biol.* 15, e8875
54. Smith, H.O. *et al.* (2003) Generating a synthetic genome by whole genome assembly: phiX174 bacteriophage from synthetic oligonucleotides. *Proc. Natl. Acad. Sci. U. S. A.* 100, 15440–15445
55. Fujimoto, K. *et al.* (2020) Metagenome data on intestinal phage–bacteria associations aids the development of phage therapy against pathobionts. *Cell Host Microbe* 28, 380–389 e9
56. Bikard, D. *et al.* (2014) Exploiting CRISPR-Cas nucleases to produce sequence-specific antimicrobials. *Nat. Biotechnol.* 32, 1146–1150
57. Hsu, B.B. *et al.* (2020) *In situ* reprogramming of gut bacteria by oral delivery. *Nat. Commun.* 11, 5030
58. Sumrall, E.T. *et al.* (2019) Phage resistance at the cost of virulence: *Listeria monocytogenes* serovar 4b requires galactosylated teichoic acids for InlB-mediated invasion. *PLoS Pathog.* 15, e1008032
59. Yehl, K. *et al.* (2019) Engineering phage host-range and suppressing bacterial resistance through phage tail fiber mutagenesis. *Cell* 179, 459–469 e9
60. Yosef, I. *et al.* (2017) Extending the host range of bacteriophage particles for DNA transduction. *Mol. Cell* 66, 721–728 e3
61. Dedrick, R.M. *et al.* (2019) Engineered bacteriophages for treatment of a patient with a disseminated drug-resistant *Mycobacterium abscessus*. *Nat. Med.* 25, 730–733
62. Porter, N.T. *et al.* (2020) Phase-variable capsular polysaccharides and lipoproteins modify bacteriophage susceptibility in *Bacteroides thetaiotaomicron*. *Nat. Microbiol.* 5, 1170–1181
63. Landsberger, M. *et al.* (2018) Anti-CRISPR phages cooperate to overcome CRISPR-Cas immunity. *Cell* 174, 908–916 e12
64. Lu, T.K. and Collins, J.J. (2007) Dispersing biofilms with engineered enzymatic bacteriophage. *Proc. Natl. Acad. Sci. U. S. A.* 104, 11197–11202
65. Hodyra-Stefaniak, K. *et al.* (2019) Bacteriophages engineered to display foreign peptides may become short-circulating phages. *Microb. Biotechnol.* 12, 730–741
66. Gregory, A.C. *et al.* (2020) The gut virome database reveals age-dependent patterns of virome diversity in the human gut. *Cell Host Microbe* 28, 724–740 e8
67. Callanan, J. *et al.* (2020) Expansion of known ssRNA phage genomes: From tens to over a thousand. *Sci. Adv.* 6, eaay5981
68. Almeida, A. *et al.* (2019) A new genomic blueprint of the human gut microbiota. *Nature* 568, 499–504
69. Clooney, A.G. *et al.* (2019) Whole-virome analysis sheds light on viral dark matter in inflammatory bowel disease. *Cell Host Microbe* 26, 764–778 e5
70. Argelaguet, R. *et al.* (2018) Multi-omics factor analysis-a framework for unsupervised integration of multi-omics data sets. *Mol. Syst. Biol.* 14, e8124
71. Koh, H.W.L. *et al.* (2019) iOmicsPASS: network-based integration of multiomics data for predictive subnetwork discovery. *NPJ Syst. Biol. Applic.* 5, 1–10

72. Muñoz-Benavent, M. *et al.* (2020) gNOMO: a multi-omics pipeline for integrated host and microbiome analysis of non-model organisms. *NAR Genom. Bioinform.* 2, lqaa058
73. Bichet, M.C. *et al.* (2021) Bacteriophage uptake by mammalian cell layers represents a potential sink that may impact phage therapy. *iScience* 24, 102287
74. Vlachogiannis, G. *et al.* (2018) Patient-derived organoids model treatment response of metastatic gastrointestinal cancers. *Science* 359, 920–926
75. Cai, R. *et al.* (2019) Panoptic imaging of transparent mice reveals whole-body neuronal projections and skull-meninges connections. *Nat. Neurosci.* 22, 317–327
76. Rakhilin, N. *et al.* (2019) An intravital window to image the colon in real time. *Nat. Commun.* 10, 5647
77. Foutel-Rodier, T. *et al.* (2018) Chapter eight - generation of a metagenomics proximity ligation 3C library of a mammalian gut microbiota. *Methods Enzymol.* 612, 183–195