Digestive and Liver Disease
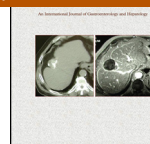
Progress Report

The human gut microbiota and virome: Potential therapeutic implications

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ABSTRACT

Human gut microbiota is a complex ecosystem with several functions integrated in the host organism (metabolic, immune, nutrients absorption, etc.). Human microbiota is composed by bacteria, yeasts, fungi and, last but not least, viruses, whose composition has not been completely described.

According to previous evidence on pathogenic viruses, the human gut harbours plant-derived viruses, giant viruses and, only recently, abundant bacteriophages. New metagenomic methods have allowed to reconstitute entire viral genomes from the genetic material spread in the human gut, opening new perspectives on the understanding of the gut virome composition, the importance of gut microbiome, and potential clinical applications.

This review reports the latest evidence on human gut "virome" composition and its function, possible future therapeutic applications in human health in the context of the gut microbiota, and attempts to clarify the role of the gut "virome" in the larger microbial ecosystem.

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1. Introduction

Before the surge of interest into neglected components of the "gut microbiota" (including fungi and viruses), studies on the "gut bacterial microflora" and its widespread and well known bacterial species have collected evidence on the microbiota's role in metabolic, gastrointestinal, immune diseases and, lately, in cancer development [1].

The Western lifestyle is associated with serious metabolic sequelae (diabetes, obesity, metabolic syndrome, increased cardiovascular risk, etc.) [2]; this has driven clinical and basic researchers' attention to the possible modulation of gut microflora through diet, antibiotics, and pre-/probiotics with encouraging results, however awaiting wider population-based studies [2].

Thus, the microbiologic environment has attracted attention and resources from the clinical and economic sectors of our society to achieve a better understanding of the microbiota ecosystem. These efforts have led to the discovery of other components of gut microbiota such as yeasts, fungi, archaea, and last but not least, viruses [1].

This last subset of findings has been mostly unexpected because of the common representation of the gut virome as a source of pathogens. Enteroviruses, Norwalk, Rotaviruses are well known in daily clinical practice and are known to be responsible for common infectious gastroenteritis [3].

However, because gut viruses not amenable to culture with common microbiological techniques, the development of non-culture based metagenomic methods have allowed to reconstitute viral particles from single genetic sequences from almost every environment. This has moved our idea of gut viruses from a mere source of pathogens to a physiological component of the healthy human microbiota [3].

Based on the new findings obtained through metagenomic methods, this review will focus on the composition of the human gut virome, its role in the gut microbiota ecosystem, and possible future clinical applications.

2. Human gut microbiota composition

After birth the human intestine is progressively colonized by several microbial strains that fluctuate and change during our lifespan according to anatomical, dietary and nutritional status

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changes (e.g. obese, anorexic, lean nutritional status), environmental (e.g. climate, familial composition, life-style, working place, etc.), pathological (gastro-intestinal and systemic infections), and pharmacological factors (e.g. use of antibiotics, prokinetics, laxatives, probiotics) [4].

The main components of gut microbiota are bacteria, fungi, yeasts, archaea and viruses [4]. While the Human Gut Microbiome project has shed new light on the entire human intestinal bacterial composition, the last three decades of microbiological/clinical research have helped to understand how food, pre-/probiotics, antibiotics can modulate the intestinal bacteria quali-/quantitative pattern resulting in different microbial-host functions [2–5].

Specifically starting from the observations of an obese/lean gut microbiota associated with overweight or lean status it became clear how microbiota manipulation by diet was possible and how microbiota could be responsible not only for overweight but also for the chronic inflammatory state typical of the metabolic syndrome (MetS) [6]. However diet and the gut microbiota's role in obesity pathogenesis is not simply causative as was initially expected. In fact, a recent observation by Ridaura et al. has showed how co-housing mice with an obese twin's microbiota with mice containing the lean co-twin's microbiota prevented the development of increased body mass and obesity-associated metabolic phenotypes (greater polysaccharides metabolism and proteins degradation) in obese cage mates. More interestingly, an obesogenic diet (high in saturated fats and low in fruits and vegetables) counteracted the protective effect of the lean gut microbiota observed during lean and obese mice co-housing [7]. The role of diet in gut microbiota modulation is strengthened by the recent metagenome-wide association study by Qin et al. in type 2 diabetic patients, with a mainly diet-associated insulin resistance status; the Authors showed that these patients have a peculiar decrease in some butyrate-producing bacteria, an increase in various opportunistic pathogens and an enrichment of other microbial functions conferring sulphate reduction and oxidative stress resistance [8].

Starting from these observations, the possible functions of gut microbiota were quickly related to other organs/apparata. The previous association between spontaneous bacterial peritonitis and small bowel bacterial overgrowth in liver cirrhosis [9] has led to the understanding of the microbial molecular patterns triggering inflammation and fibrosis in liver diseases such as non-alcoholic liver disease (NAFLD) and its complications, i.e., non-alcoholic steatohepatitis (NASH), liver cirrhosis and hepatocellular carcinoma (HCC) [10]. Moreover, as in a vicious cycle, the role of microbial pathogen molecular patterns (PAMPs, e.g. gram-negative polysaccharide, LPS) in NAFLD pathophysiology has been linked to those of MetS, which is frequently concomitant [10].

More recently intestinal bacteria have been implicated in the pathophysiology of psychiatric diseases such autism [11]. In fact, gut bacteria seem to interact with the central nervous system (CNS) via the enteric nervous system through the endocannabinoid system. Thus gut microbiota can affect the neuro-psychiatric state of the host and, conversely, the CNS is able to affect its composition through food intake regulation [11,12].

Bacteria reach more than 1 kg of weight and account for more than 1100 species; Bacteroidetes and Firmicutes are the predominant phyla in adults, followed by Actinobacteri and Proteobacteria [13].

Indeed, the human microbiota also contains other more neglected components such as archaea, viruses, fungi, yeasts and other Eukarya (such as Blastocystis and Amoebozoa) [4,13].

Little is still known about commensal fungi, archaea and protozoa [4,13].

However, some emerging microbiological data on yeast composition and functions have clarified their subsequent clinical use in the modulation of gut microflora. In fact, *Saccharomyces boulardii* is currently used with significant efficacy over placebo in the treatment of post-infectious and post-antibiotic diarrhoea [14].

Knowledge on the composition of the gut virome has evolved from a niche of the gut microbiome populated by pathogens only (e.g. Norwalk, Rotavirus, Enterovirus, etc.), responsible for gastroenteritis by direct damage of enterocytes or through alteration of ion and water secretion in the colon, to an enlarged list of undetectable giant viruses (derived mainly from protozoa and parasites), and more recently to plant-derived viruses and bacteriophages, thanks to new metagenomic methods [3,13].

3. Human gut virome composition

As mentioned above the concept of the existence of a "gut virome" is, paradoxically, very recent [3] although the presence of viruses as pathogenic organisms in human intestine has been known and documented for more than a century [13].

Thus, the description of the gut virome composition can begin with these pathogenic viruses (Table 1), whose viral particles were discovered by microbiologists mainly because they could be cultured [13,15]. Norwalk, Rotavirus and Enterovirus are the well-known agents of gastroenteritis in man [15]. The reason we consider linking these pathogens with the gut virome is that the infection of the gut is responsible for enterocyte and bacterial microflora changes. These can affect the host not only in the acute phase of the infection with gastrointestinal complaints such as nausea, vomiting, diarrhoea and weight loss, but also in the long-term with persistence of symptoms and the possible eliciting of functional gastrointestinal disorders such as functional dyspepsia and post-infectious irritable bowel syndrome [16] (Table 1).

A recent paper by Li et al. offers a clear and advanced example of how metagenomics has changed the professional perspectives of microbiologists and translational researchers in the study of gut microbiome. Using first national, then intercontinental catalogues of reference genes of the human gut microbiome, in the last two decades, researchers used sequence reads and relative gene content to map the profile of the microbial species in biological samples. This is the fundamental principle of metagenomics which, instead of deep genome sequencing after sample culture, collects different sequences from various genetic materials (e.g. from human gut) and is able to use them to mark families, taxa and genera for both microbiome and virome. The knowledge of gene abundance levels can be also associated to different diseases in the attempt to setup a specific genetic marker. Another advantage of metagenomic analysis arises from the possibility to extract genetic material directly from faecal samples without any changes and/or contamination, which may arise during culture [17].

Very recently, thanks to these metagenomic methods, novel enteric eukaryotic viruses were found to be responsible for acute diarrhoea in children's small bowel enteropathy in developing areas of Australia. Interestingly these new data, confirmed by quantitative real time polymerase chain reaction, have shown that diarrhoea in children contains a higher abundance of viruses, many of them not previously known to be pathogenic, such as the Adenoviridae, Picornaviridae, Reoviridae families. Within the Picornaviridae family, Enterovirus were the most represented [18].

In the past decades microbiological investigations have further discovered viruses infecting human intestinal parasites such as amebae (e.g. Mimiviridae, Mamaviridae, Marseilleviridae) from cooling towers, rivers, lakes, and seawater. These viruses are defined "giant" because of their dimensions. They are DNA viruses and their existence has been frequently doubted because they are undetectable by small-pore filtration (Table 1). Some of the Mimiviruses have been associated with pneumonitis and diarrhoea in humans although evidence is controversial [19].

Table 1
Known virotypes according to culturomics and metagenomics.

Virus type	Genome type	Environment	Associated disease
Eukaryotic virus			
Rotavirus, Astrovirus, Calicivirus, Norovirus, Hepatitis E virus, Coronavirus and Torovirus, Adenovirus (serotypes 40 and 41)	All RNA except Adenovirus (DNA)	Human small bowel and colon	Gastroenteritis (small bowel epithelium and the absorptive villi disruption, with consequent malabsorption of water and an electrolyte imbalance) (all the mentioned eukaryotic viruses)
Adenoviridae, Picornaviridae and Reoviridae (genus enterovirus)	RNA	Human intestine	Unknown (all the mentioned viruses)
Plant derived virus			
Pepper mild mottle virus (PMMV), oat blue dwarf virus, Grapevine asteroid mosaic-associated virus, Maize chlorotic mottle virus, Oat chlorotic stunt virus, Panicum mosaic virus, Tobacco mosaic virus	RNA	Plants and human faeces	Pathogenic for plants Non pathogenic for humans (all the mentioned plant derived viruses)
Giant virus (>300 kb)			
Mimiviridae, Mamaviridae, Marseilleviridae, Poxviridae, Iridoviridae, Ascoviridae, Phycodnaviridae, Asfaviridae	DNA	Human faecal protists, amoebae in lake, river and seawater	Pneumonitis, Children diarrhoea (Mimiviridae only)
Prophages			
Myoviridae, Siphoviridae, Podoviridae, Tectiviridae, Leviviridae, Inoviridae	dsDNA	Human faeces specimens	Unknown (all the mentioned prophages)
Virus (<145 kb)			
Microviridae family (Microvirus, Gokushovirinae, Alpavirinae, Pichovirinae)	ssDNA	Seawater, human gut bacteria	Unknown (all the mentioned Microviridae viruses)

dsDNA: double stranded DNA; ssDNA: single stranded DNA.

More recently plant viruses such as pepper mild mottle virus (PMMV), oat blue dwarf virus, grapevine asteroid mosaic-associated virus, maize chlorotic mottle virus, oat chlorotic stunt virus, panicum mosaic virus, and tobacco mosaic virus have been described [20] (Table 1).

Their biological importance depends on their concomitant presence in plants as pathogens and as "commensals" in human faeces, explaining how food intake has conditioned and continues to condition human gut virome composition and enterocyte life-cycle. These data also suggest that these small plant-derived viruses can affect the intestinal bacterial quali-/quantitative composition and its functioning with consequences for the human host. There is no data on the presence of these viruses in patients with diarrhoea [20].

Finally yet importantly, intestinal bacteriophages have been discovered as the main component of the gut virome, accounting for about 90% of its composition [3].

Bacteriophages were one of the first microbiological entities characterized in the literature. Our knowledge about their life cycle is quite extensive; bacteriophages can be quite literally defined as "viruses of bacteria". Bacteriophages are commonly known as bacterial "parasites", who inject their genome in their host, integrating with its genetic material (prophage state) and inducing other phage particle synthesis with bacterial cell lysis (lytic state) [3,21].

Bacteriophages are viruses with double-stranded DNA (dsDNA) [22,23]. Single-stranded DNA (ssDNA) bacteriophages are mainly found among the Microviridae family and were initially considered secondary players in the environmental viral community because of their modest genome size [3,22,24].

Microviridae are small icosahedral viruses with circular single-stranded DNA genomes [22] and their members are divided into microviruses (genus Microvirus) and gokushoviruses (subfamily Gokushovirinae) [24]; more recently, a new sub-family, the Alpavirinae, was described (Table 1). These viruses have been retrieved in bacteria belonging to two genera of the phylum Bacteroidetes: Prevotella and Bacteroides, with possible implications for human microbiota metabolism [22].

As described above, the study of gut virome composition has benefited from new non-culture based metagenomic methods. Thus, starting from viral genetic sequences, it has been possible to progressively classify the gut virome composition into families. These phage sequences had not been taxonomically classified beyond the family level, therefore microbiologists could not associate and/or correlate this composition to those of intestinal bacterial species. Very recently, Waller et al. used metagenomic viral sequences with a newer sampling procedure for microbial communities characterization at the taxa (about 15 taxa) and genera level. Moreover, these marker genes were found to be absent from 'non-prophage' regions of bacterial chromosomes. The marker genes are used to detect phage sequences in metagenomic samples containing both prokaryotes and phage DNA. Interestingly, Picovirinae and Spounavirinae genomes could be isolated from human-associated bacteria (e.g. Staphlococcus, Streptococcus, Clostridium, Mycoplasma, Listeria, Enterococcus, Bacillus). In particular, among the seven identified viral genera associated to bacterial genera, P22-like genus was found to be connected to genera of the Enterobacteraceae family (Escherichia, Salmonella, Klebsiella), or Spounavirinae with those of the Bacteroidales order. However, over 60% of the metagenomically marked genes could not be used to identify phage genera possibly associated with the respective bacteria, because of the methodological bias of not including lytic genomes [25].

4. Virome functions within the human gut microbiome

Currently there are few clear data regarding gut virome functions within the gut microbiota ecosystem. However, the life cycle of viruses provides indirect information about their possible roles.

The great part of the phages found in the human gut show a typical "temperate" behaviour, thus justifying the hypothesis that their composition is quite stable during the host's life, although the viruses are able to mutate spontaneously. In fact, several authors have used the terms "stability" and "variability" to define phage behaviour. It is interesting to note that these two kinds of

characteristics belonging to the bacteriophages in the intestine are linked as in a "virtuous" cycle. In fact, the stability of the viral genome is responsible for that of other microorganisms, such as the bacteria of the gut microbiota. This is proven by the fact that gut virome composition mimics the evolution of the infant bacterial microbiota, perhaps remaining stable in adult life [3,26,27], and by 51 hypervariable loci found in virus-like particles (VLPs) purified from human faecal samples. These are common between phages and bacteria, and are implicated in bacterial wall adhesion, immunoglobulin receptor synthesis, contributing to maintaining viral–bacterial immune tolerance in the gut. This allows the persistence of bacterial and viral species in the gut, exerting their effects on enterocytes and, more in general, on the host [3,28,29].

On the other hand the presence of one intrinsic variability, typical of the few lytic phages found in the intestine, not triggered by drugs or other environmental factors, including bacterial pressure, is an interesting characteristic of these viruses, allowing the generation of new species in a short time frame and allowing them to escape extinction [26].

Indeed, among the genes stably conserved during intestinal viral evolution discovered by metagenomics, there are also those involved in energy harvesting such as for carbohydrate transport and degradation [30–32]. These properties are common to diet-derived viruses of plants that can modulate human bacterial microbiota/host metabolism (e.g. carbohydrate synthesis/degradation, protein synthesis) [15]. This finding merits some interpretation according to those observed in intestinal bacterial species.

Cani et al. have discovered the association between lean and obese gut bacterial microbiota and their respective metabolic patterns [33] in man. A similar viral–metabolic association is conceivable also according to the evidence that twin pairs and their mothers have phage and bacterial community similarities in parallel with the same dietetic regimen assumed over time [30,34–36].

These data on the possible role of gut virome on metabolism regulation should be related to those derived from the intercontinental study by Li et al. on enrichment of gene catalogues. The authors showed how the phage intercontinental virome catalogue abundance is deeply influenced by a small group of individuals sampled, mainly genes involved in metabolism regulation and/or antibiotic resistance. On the other hand, similarly to the "Darwin postulate" on animal species survival, the most common genes mapped by the largest part of sampled individuals were those responsible for DNA replication and repair, namely a feature of "adaptation for survival" [25]. Thus gut virome has a deep influence on our gut microbiome and, perhaps, human genome maintenance over the generations.

Among the genes encoded in cryptic prophages of *Escherichia coli*, those for resistance to antibiotics and other stress factors have been found [37]. These findings explain the strict interaction between viral and bacterial particles in the intestine, a peculiar biological relationship that overcomes, only in this environment, the classical concept of bacteriophages as "predators" of bacteria. In fact, the transmission of genes between virus and the infected bacteria help the host to resist oxidative stress and antibiotic use, another proof of the "temperate" lifestyle of the gut virome.

Very recent evidence from the literature shows how the interaction of eukaryotic viruses and bacteriophages in the microbial gut ecosystem is more complicated than expected, especially considering animal models of inflammatory bowel disease (IBD). Kernbauer et al. have shown how persistent infection by the murine RNA norovirus strain CR6 is able to induce intestinal pathology in mice who are deficient in the IBD gene Atg16l1; however, in germ-free or antibiotic-treated animals, it is also able to re-establish the normal mucosal immunity and trophism, resembling the functions of commensal bacteria [38]. In this context, these findings are supported by the very recent work by Norman et al. showing how bacteriophages also play a role in the maintenance of mucosal inflammation in IBD with specific ulcerative colitis (UC) and Crohn disease (CD)-associated phage expansion. In particular, Caudovirales and Microviridae are more abundant in IBD in comparison with household members and controls but with a different Caudovirales genetic sequence distribution. These different bacteriophage increases, in UC and CD, were associated with a lower bacterial diversity, in general, and, more interestingly, with a peculiar disease-specific bacterial strain abundance. This implies a function of bacteriophages in the maintenance of mucosal inflammation in IBD, with a possible function as viral and bacterial antigen "generators" (namely PAMPs production) via their lytic state [39].

Altogether, these data show how viruses could be the target or even a means to face the pressing issue of antibiotic resistance, immune modulation in immune-related diseases, and human metabolism regulation.

5. Human virome therapeutic implications and future directions

The brief description of the evidence arising from metagenomic studies on gut virome is the basis of the discussion on the possible future therapeutic use of gut virome modulation in human health.

Considering the discovery of plant-derived virus [20] one can postulate that diet could favourably modulate gut virome composition and, in turn, the resident bacterial microflora. Moreover, although the genetic engineering of new probiotic strains is rapidly growing, and may produce patient-specific gut microflora modulation in a few years' time, the engineered modulation of gut virome through more "natural" pathways seems to be safer. In fact, not only diet, but also the use of probiotics and prebiotics can potentially affect gut virome composition. Finally, if metagenomic knowledge continues to progress, the engineering of viral genomes, and of bacteriophages in particular, could represent a patient-specific treatment "final destination". In particular, the ability to create viruses able to affect intestinal bacteria and impact on human metabolism seems to be one of the most important targets; a more direct application such as the prevention of viral gastroenteritis through a competitive competition for the gut microbiome ecosystem harbouring is also a possible therapeutic target [3].

Another potential use for bacteriophages is the regulation of immune response. In CD patients the evidence of a critical prevalence of sDNA Caudovirales and non-tailed ssDNA Microviridae, which alone and combined with related bacterial species condition the inflammatory response in IBD, is the basis for future phage-specific treatments. In fact, blocking bacteriophage infection of their bacterial hosts could alter the natural history of IBD. Moreover, since phage genes are more stable than those of bacteria, their catalogues can be used to set up an IBD marker (e.g. disease-specific Caudovirales could differentiate CD from UC when the clinical phenotype is indistinguishable) [39].

As mentioned above, an emerging problem in human health is the alarming prevalence of antibiotic-resistant strains of pathogenic bacteria, due to the horizontal and vertical transmission of resistance (e.g. multi-resistant *Staphylococcus aureus*, and more recently New Delhi metallo-beta-lactamase enterobacteriaceae) [37]. Indeed although huge efforts have been made by pharmaceutical industries, only a few novel antibiotics against these multi-resistant agents have been produced over the past decades [40].

In addition, the rationale for the use of bacteriophages against antibiotic resistance is the fact that these agents can actively down-regulate the proliferation of pathogenic multi-resistant bacteria and be used, after genetic manipulation, for the introduction of genes against pathogens [41].
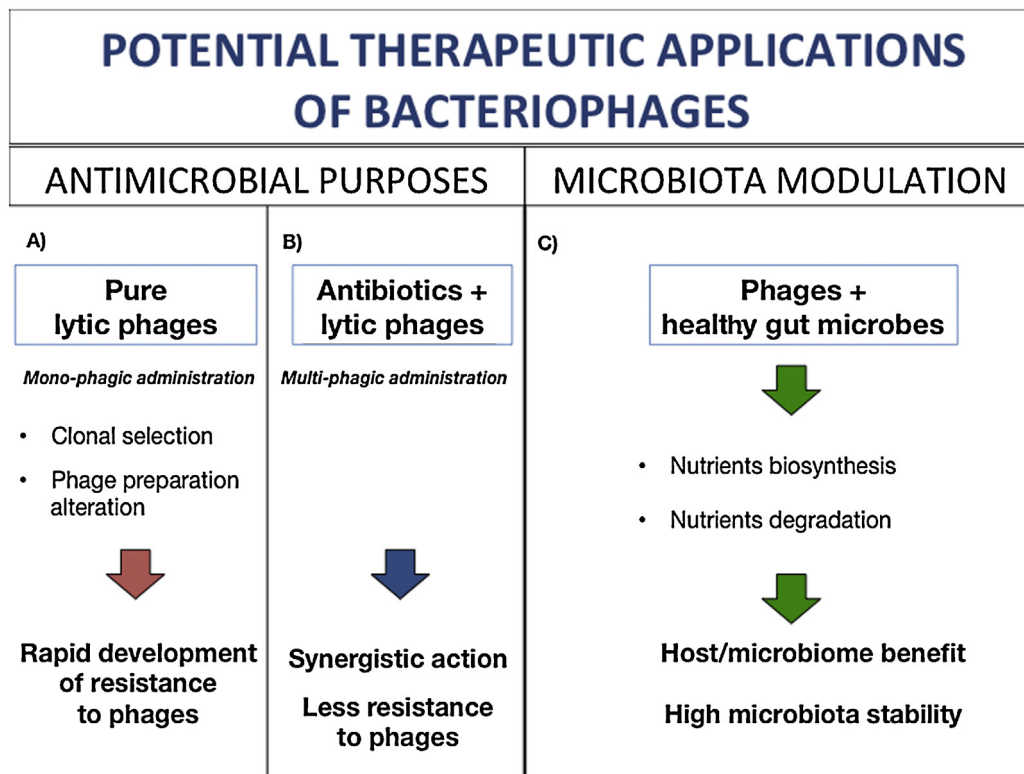
POTENTIAL THERAPEUTIC APPLICATIONS OF BACTERIOPHAGES

ANTIMICROBIAL PURPOSES		MICROBIOTA MODULATION

A)
Pure lytic phages
Mono-phagic administration

- Clonal selection
- Phage preparation alteration

Rapid development of resistance to phages

B)
Antibiotics + lytic phages
Multi-phagic administration

Synergistic action
Less resistance to phages

C)
Phages + healthy gut microbes

- Nutrients biosynthesis
- Nutrients degradation

Host/microbiome benefit
High microbiota stability

Fig. 1. Potential future therapeutic applications of bacteriophages in humans. Panel A: The "classic" lytic phage use as an antimicrobial, as a mono-phagic administration, has been stopped due to rapid bacterial resistance development from clonal selection and alterations in phage preparation. Panel B: Multiple phage administration can quickly overcome pathogenic bacteria resistance, in a synergistic action with antibiotics. Panel C: Phages could be used for gut microbiota modulation, in general, and for resident bacteria modulation, in particular. Genetically modified phages can be used, alone or in combination with pre-/probiotics, as "vectors" for nutrient biosynthesis and degradation, genetic modulation with the gut microbiome, and host beneficial effects (e.g. in obese, dysmetabolic patients) in a more "stable" microbiota environment.

Nonetheless, this therapeutic approach has been considered unreliable for decades although phages were discovered one century ago. A clearer metagenomic knowledge of the gut virome genome, together with more refined and specific genetic manipulation engineering techniques have made this option definitively feasible.

Specifically, bacteriophage use is reliable because they have low production costs, are easy to manipulate and have good target-host specificity with bacterial resistance that develops more slowly than for antibiotics [30,40]. Furthermore, the potential development of bacterial resistance to bacteriophages alone could be easily controlled by the combined use of antibiotics and bacteriophages in their lytic state (Fig. 1).

In a wider future perspective, the use of phages for gut microbiota modulation could have several therapeutic applications: lytic bacteriophages could modulate gut bacterial composition maintaining a "healthy" quali- and quantitative composition; bacteriophages alone or in combination with antibiotics could overcome bacterial antibiotic resistance; pro- and/or prebiotics could be used to modulate intestinal phages with yet unknown implications for the host physiology (Fig. 1).

The use of viruses, and of bacteriophages in particular, for gut microflora and microbiota modulation also raises the need to address safety concerns. Many safety issues, however, can be set aside due to the large body of evidence that no known detrimental effect in humans exposed to and living with millions of bacteriophages has been described [3].

One of the limitations of this review arises from source literature data: to date there are only association studies with human health and diseases of gut microbiota, in general, and gut virome, in particular. Thus, it is not possible to speculate or define a causative association, with solid pathophysiologic inferences, between gut microbiota and virome alterations and human diseases.

Only larger studies on gut virome composition with metagenomic methods may further enrich our knowledge on the physiological importance of phages in the gut microbiome; only robust animal and human interventional studies on phage modulation can be provide a solid basis for future therapeutic implications.

Conflict of interest

None declared.

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