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What is (not) known about the dynamics of the human gut virome in health and disease

Leen Beller and Jelle Matthijnssens

The human gut virome has an important role in human health but its dynamics remain poorly understood. Few longitudinal studies in healthy adults showed a stable temporal gut virome, with high inter-individual diversity. In contrast, the infant virome shows a high temporal intra-individual diversity. Unfortunately, these virome studies ignore an enormous amount of unknown 'dark matter' sequences, leading to incomplete analyses and possibly incorrect conclusions. Also, the interactions between prokaryotes and bacteriophages in the gut seem to be too complex for currently available models. Therefore, there is a huge need of larger longitudinal cohort studies focusing on both the bacterial and viral component of the microbiome to be able to describe and understand this complex ecosystem.

Address
KU Leuven Department of Microbiology, Immunology and Transplantation, Laboratory of Viral Metagenomics, Leuven, Belgium, Belgium

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Introduction

In recent years, the relevance of the human gut microbiota in health as in disease has become increasingly apparent, resulting in an increased interest in this ecosystem. It includes a prokaryotic, eukaryotic and viral component, and the former has by far received the most attention. Population-wide studies have resulted in a better understanding of this prokaryotic component, its beneficial functions in healthy individuals, and the factors that influence their composition, such as diet, age, disease states, medication and geography [1–3]. Research focusing on the viral component in the gut microbiome, or simply the *gut virome*, is lagging behind and its relevance in health and disease has yet to be shown.

Current methods to investigate the gut virome composition, mostly start with a purification of viral particles and an elimination of all cells and free-floating nucleic acids [4]. This step often involves filtration, centrifugation and enzymatic reactions and neglects intracellular and very large viruses. Next, nucleic acids are extracted and amplified, but the lack of conserved regions in viral genomes (like the 16S rRNA gene in prokaryotes) for targeted amplification, as well as the difficulty to simultaneously amplify multiple types of viral genomes (circular or linear, RNA or DNA, single stranded or double stranded) make this step challenging. In addition, incomplete viral databases and bioinformatics challenges are just a few more factors explaining the limited research of the human gut virome [5].

The first paper ever mentioning the 'virome' was published in 2003 [6], recognizing the presence of a viral component in the human microbiome, and in the same year the first bacteriophages were sequenced from human feces and their huge novelty and diversity was recognized [7]. The observation that gut viruses can be found in all individuals led to the realization that, in contrast to pathogenic effects, there might actually be an important role of the gut virome in normal homeostasis. The gut virome refers to both eukaryotic viruses and bacteriophages. The former can infect the human host, the eukaryotic component of the gut microbiota, including fungi and parasites, or just pass through the gastrointestinal tract as part of our diet (e.g. plant viruses) [8]. The pathogenic effect of eukaryotic gut viruses, resulting in transient or latent infection and disease, is well established; however, evidence for a beneficial role of eukaryotic gut viruses in health is scarce. One study showed that a persistent norovirus infection can restore intestinal morphology and functions in a germ-free or antibiotic-treated mice suggesting that this eukaryotic virus can act as a commensal bacterium with beneficial functions in the gut [9]. Also, the frequent observation of members from the *Anelloviridae* and *Circoviridae* in human stool, without any clear pathology, indicates they may be commensals [10,11]. In contrast to the infant gut virome, in healthy human adults, low abundances of eukaryotic viruses are reported.

Bacteriophages are the most abundant entities on earth and, unsurprisingly, this observation also extends to the gut, where bacteriophages constitute the most abundant inhabitants. Most bacteriophage genomes sequenced from human gut or feces samples belong to the dsDNA families *Siphoviridae*, *Podoviridae*, and *Myoviridae* (order *Caudovirales*) and ssDNA family *Microviridae* [12–14]. Only few studies report archaeal viruses of the family *Lipothrixviridae*, probably because of the low abundances of their hosts [15]. Gut bacteriophages can influence the prokaryotic communities in terms of composition and function, by direct lysis of their hosts (lytic phages) or by genome integration, which can change the fitness and phenotype of the host. Such gene transfers can result in antibiotic resistance, production of toxins or increased energy harvest [16]. Moreover, some phages can change the surface structures of prokaryotes, such as lipopolysaccharides, and influence the interactions of the prokaryote with the human host [17]. Therefore, phages in the gut are more than only predators, in some cases helping their host by providing genes conferring a competitive advantage in the ecosystem they both reside in.

Unfortunately, the dynamics in the human gut virome remain poorly understood. The scope of this work is to highlight recent advances regarding these human gut virome dynamics by specifically focusing on longitudinal studies. These studies aid in understanding the composition of the gut virome and its changes over time in healthy conditions, disease states and following antibiotics treatment. Furthermore, studies focusing on both the gut prokaryotic and virome composition reveal the interaction between those two populations.

Interpersonal variation in the human gut virome and the responsible factors

Almost a decade ago, Reyes *et al.* were the first to recognize the high interpersonal variation of the gut virome in adults: every individual had a unique virome composition. Monozygotic (MZ) adult twins and their mothers shared similar bacterial communities, whereas their viral gut communities were not shared [14]. In contrast, Lim *et al.* found that infant twins harboured a more similar virome compared to unrelated children [15], which might be explained by their shared environment and diet. A more recent study, showed that the gut virome of adult MZ twins, in contrast to observations made by Reyes *et al.*, is more similar between twins compared to unrelated individuals, even though their bacterial microbiomes did not share this similarity [18••].

This major knowledge cavity was exemplified in 2014, when a previously unidentified bacteriophage, named 'CrAssphage' (Cross-Assembled phage), was detected in the majority of all available fecal viromes [19]. Today, a whole new family of CrAss-like phages are described containing mostly temperate phages with Podoviridae-like tails that likely infect members of the Bacteroidetes [20•,21]. Although it is still a matter of debate, the observation of a specific set of shared gut viruses in the human population was confirmed in other studies as well and led to hypothesis of a 'core virome'. CrAss-like phages, other *Caudovirales* phages and members of the *Microviridae* were identified as a part of this 'core virome' [18••]. Further research is needed to validate the existence and relevance of this 'core virome' both in health and disease.

In addition to relatedness, multiple factors have been correlated to the inter-individual virome variation, including diet, birth mode and disease.

Diet

Minot *et al.* investigated the effect of a dietary intervention on the gut virome of six healthy adults to the same diet [12]. In agreement with previously mentioned studies, the main source of variance still was interpersonal variation, although the virome of individuals exposed to the same diet converged. This shows that diet can influence gut phage populations, either directly or indirectly. Of note, the fact that diet can indeed affect the virome composition could explain the contradicting result of the adult MZ twin studies, which both did not correct for the fact that co-twins could be on a different diet.

Birth mode

In a recent study on the infant virome at 1 year of age, a strong correlation between the virome diversity and birth mode (naturally versus caesarian section) was found while the bacterial 16S composition, representing the prokaryotic fraction of the microbiome, did not show significant differences [22•].

Disease

During the last decades, intense research has been carried out to unravel the role of the gut microbiome in disease. The prokaryotic component of the gut microbiota has since then been associated with a plethora of diseases going from gastro-intestinal and auto-immune diseases to liver diseases and even diseases of the central nervous system [23]. The role of the gut virome in disease has yet to be shown but some associations with diseases are known. In case of inflammatory bowel disease (IBD), associations with both the eukaryotic virome and bacteriophages have been found [24–27]. Furthermore, the gut phageome composition has been linked to type I diabetes and colorectal cancer [28,29], while the eukaryotic virome had been associated with diarrhea in infants and AIDS [30,31]. In a longitudinal study on malnutrition in twin pairs, the malnourished twin could be distinguished from the healthy twin solely based on both phage populations as well as the increased prevalence of eukaryotic viruses classified to the *Anelloviridae* and *Circoviridae* families [10]. Whether or not the altered gut virome compositions observed between patients with these diseases and controls is the cause or the consequence of the disease, still remains a question.

Intrapersonal variation: stability over time

Dynamic development of the infant gut virome towards a more stable adult-like gut virome

Directly after birth, the human gut is exposed to and colonized by an enormous number of viruses, prokaryotes and eukaryotes. In the following weeks and months, a stable community of these organisms is formed. Multiple environmental factors influence the development of the infant gut microbiota and are suggested to be confounding factors in virome studies. Whether or not the gut of newborns is completely sterile, is still a matter of debate. Viral-like particles (VLPs) could not be detected via microscopic examination of the meconium; however, they were identified in a stool samples of the same infant at week 1, arguing in favor of a 'nearly sterile' gut at birth [32]. Bacteriophages, mainly *Siphoviridae*, dominate early colonization of the gut after birth and are derived from the environment rather than the mother or the diet. The virome changes drastically in the first weeks of life, as more than half of the viral genotypes that were present in the samples of week 1 were not detectable anymore in the sample of week 2 [32]. In 2015, Lim *et al.* characterized the gut virome and bacteriome of four twin pairs in samples collected at six time points between birth and the second year of life. The phageome was composed of members from the order *Caudovirales* and no RNA phages were detected. In contrast to the increasing bacteriome diversity and richness over time, the highest richness, diversity and abundance of phages was observed in the first months of life and decreased significantly over time (graphical abstract). The sole exception being the bacteriophage family *Microviridae*, which increased in abundance and diversity over time in the first years of life. The latter observation was confirmed by Reyes and colleagues [10,15].

Eukaryotic viruses from the families *Adenoviridae*, *Anelloviridae*, *Astroviridae*, *Parvoviridae*, *Picornaviridae*, *Picobirnaviridae* and *Reoviridae* have been detected in infant stool samples, even in the absence of clinical symptoms [15,33]. Their richness increased with age, suggesting that they are derived from environmental exposure [15]. In the study of Lim *et al.*, members of the *Anelloviridae* were present from three months of age and most abundant between 6 and 12 months. The same anelloviruses were detected in two samples from one infant, taken 12 months apart, indicating that they cause persistent infections [15]. The high prevalence of anelloviruses in early life, was confirmed in another study where Malawian twins were investigated [10]. It is speculated that the presence of anelloviruses may be correlated with the lowered immune state associated with the decrease in maternal IgGs [15].

The healthy adult gut virome is temporally stable

In contrast to the drastic changes observed in the virome of infant guts, the adult gut virome seems temporally stable (graphical abstract) [12]. Reyes *et al.* identified that 95% of the viral genotypes identified in the same individual sustained over a period of one year [14], while Minot *et al.* identified that 80% persisted over a period of two and a half year [13]. However, in the latter study the individual contigs exhibited high evolution rates, which were family-dependent. Bacteriophages of the *Microviridae*, a family of lytic phages, showed the highest mutation rates, up to 10^{-5} substitutions per day, whereas temperate phages showed the lowest substitution rate [13]. Temperate phage DNA replication is driven by the more reliable bacterial polymerases in the prophage state and the replication rate is also slower compared to lytic phages, possibly explaining the latter observation [13]. Remarkably, the substitution rate for one member of the *Microviridae* was so high that within the two and a half year study period it evolved into a new species. Knowing that gut phages can evolve so rapidly within one individual, the enormous inter-individual variability of the gut virome comes as no surprise.

Bacteriome-phageome dynamics are dominated by a temperate lifestyle

No kill-the-winner dynamics in the healthy human gut

Microscopic investigation of fecal samples measured an abundance of the order 10^8 to 10^9 VLPs per gram of stool compared to a bacterial abundance of around 10^9 cells per gram, resulting in a phage-to-bacteria ratio of approximately 1:1 or even as low as 0.1:1 [34,35]. This number is much lower than the 10:1 ratio estimated in, for example, marine samples, even taken into account the recent discovery that this ratio can vary from 2.6:1 to 160:1 [36,37]. The cyclic change in abundance, where an outgrow in bacteria is followed by an increase in abundances of their infecting bacteriophages leading to a decrease of bacterial loads ('kill-the-winner' dynamics), as is seen in ocean environments, does not seem to apply in the gut microbiome [38]. The relatively high abundance of lysogenic compared to lytic phages in the gut might explain the low phage-to-bacteria ratio [39]. Indeed, so far, no clear observation of oscillating predator pray relationships between bacteria and phages have been observed in the human gut [12,14]. The inverse correlation between bacteria and phages in early life, detected by Lim *et al.*, is thought to be a typical predator-prey relationship; however, only one cycle is observed [15]. The temperate lifestyle of the gut virome is further confirmed by the observation that most bacteriophage genomes derived from human fecal samples encode integrase genes and other genes typical for a temperate lifestyle [14]. The models that describe phage-bacteria dynamics in other ecological environments are clearly not sufficient to explain the richness association and diversity association of gut prokaryotes and viruses, so new models are needed to understand these dynamics [18••].

Dynamics in disease states remain unclear

To date only one study, by Norman *et al.*, investigated both gut bacterial and phage populations in longitudinal samples of diseased patients (IBD) and healthy controls. They found that the two main types of IBD (Chron's Disease and Ulcerative Colitis) were associated with an expansion in phages of the order Caudovirales compared to healthy household controls, which was accompanied by a decrease in bacterial richness and diversity [25]. This observation leads to the hypothesis that in disease states, the bacteriophage population could drive the bacterial composition and might suggest a predator prey relationship between both. However, it is still debatable whether the changes in microbiota structure in IBD are the cause of consequence of inflammation [40]. If the former is true, a causal role for the gut virome in IBD and other disease states could be possible.

Bacteriophages provide non-host derived immunity

Interestingly, in 2013, Barr *et al.* discovered that the phage-to-bacteria ratio was not consistent throughout the gastrointestinal (GI) tract, and that in contrast this ratio was increased on the mucosal surfaces, not only in humans but across different animal species [41]. They proposed a 'Bacteriophage adherence to mucus' model explaining that phages are embedded in mucosa of the GI tract via interactions with mucin glycoprotein, driven by the Ig-like protein domains on phages, to protect the host mucosa from bacterial infections [41]. The adherence of phages to the mucus can also be advantageous for the microbiome itself as it increases the frequency of phages-bacterial host interactions, stimulating horizontal gene transfer.

Changes in the virome after antibiotic treatment

The gut phageome in healthy adults consists mostly of temperate phages. However, under stressful conditions such as antibiotic treatment, prophages are induced with potential effects on the bacterial gut community and the activation of kill-the-winner dynamics, as is seen in the gut viromes of swine [42]. In 2015, Abeles *et al.* investigated the influence of antibiotic therapy on different virome samples, longitudinally selected from four adults. Their main results showed that broad-spectrum intravenously administered antibiotics affected the oral viromes more than fecal viromes and that the observed effect is due to an effect on the prokaryotic hosts [43]. Willmann *et al.* investigated changes in gut bacteriome and phageome after antibiotic treatment in two adults. As expected, the bacterial abundances started to decrease from day one following treatment and was restored in the last sample taken 28 days after the start of the six days treatment [44]. Surprisingly, the overall phageome did not show a similar trend, instead, an increase in abundance of phage sequences containing antibiotic resistant genes was observed [45•].

Conclusion

In contrast to the increasing knowledge towards the bacterial microbiome and its role in health and disease, research focusing on the virome is only an emerging field of interest. For both, bacteriophages and eukaryotic viruses, potential beneficial roles have been suggested. Unfortunately, the dynamics in the human gut virome remain poorly understood. Longitudinal studies investigating the gut virome in healthy individuals showed a high interindividual diversity, meaning that everyone's viral gut composition is unique, in contrast to a low intraindividual diversity, indicating that the virome is stable over time. In infants however this intraindividual diversity is higher since the colonization process is very dynamic. The interactions between prokaryotes and bacteriophages in the human gut seem to be dominated by lysogeny but are too complex to be described using available models.

To be able to correctly describe the dynamics of the gut human virome, larger longitudinal cohorts are needed both in terms of numbers of individuals and number of time points sampled. Very importantly, all virome studies performed by now suffer from very incomplete databases and would benefit from improved bioinformatics analyses. It is estimated that 60–99% of the sequences obtained in viral metagenomics studies remain unidentified, unclassified or without functional annotation (graphical abstract) [46,47]. This results in incomplete analyses, which should be interpreted with great caution [48,49]. The fact that many recently published papers, contradict older ones, is proof of this [18••]. Finally, future research should include investigation of not only the bacterial part of the microbiome but should recognize the important role bacteriophages can have. Bacteriophages are able to change the composition and function of the bacterial microflora with important consequences for human health. *Why would one investigate a multidimensional ecosystem by only focusing on one dimension?*

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Conflict of interest statement

Nothing declared.

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Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

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