Gut bacteriophages and the pinball challenge

Luisa de Sordi

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Abstract

Five years ago, my first study on the mechanisms that govern the coexistence of intestinal bacteria and bacteriophages was published in Cell Host & Microbe. In this commentary, I use the following evolutionary steps of my career to discuss the larger frame of bacteriophage biology in gut health and disease.

Introduction: the microbiota, between -omics and -ology

Mankind has long sought the answer to what defines a human being through philosophy, social sciences, evolutionary biology, and multiple other disciplines. The Human Genome Project, conceived in the '80s as one of the most ambitious scientific projects of all time, opened the path to exploration of the genetic determinants of human physiology and launched a revolution in sequencing technology. It was at approximately the same time as its conclusion, in the early '00s, that a new awareness started to pervade the scientific community: man is also made of microbes, and this should not be ignored when considering who we are. In 2008, I started my Ph. D. studies and watched from afar as the Human Microbiome Project was launched and a worldwide effort made towards defining the composition of the microbiota and its importance for human health. Since then, progress in gene barcoding and metagenomics has unveiled an unprecedented diversity of microbes and additional "'omics" approaches have followed to complement the global genomic information with that of proteins, transcripts, and metabolites. Although this picture is far from being completed, it has started to outline the biological functions of the microbiota and correlate perturbations of such functions with genomic signatures of microbial abundance and diversity found in patients affected by several diseases and disorders. Most efforts have been towards defining the densest microbial niche, the intestinal tract, and its most numerous inhabitants, bacteria. More recently, relatively neglected microbes, such as fungi, archaea, and viruses, have also been acknowledged to be important components of intestinal and human health, which sparked my research interest in bacterial viruses, or bacteriophages (phages), the most abundant of intestinal viruses.

Certain phage biological features, such as the range of hosts, can be extrapolated by metagenomics data or combinational approaches of phage isolation and sequencing and comparison with metagenomic databases (Camarillo-Guerrero *et al.*, 2021; Hryckowian *et al.*, 2020). However, for the most part, our understanding of gut phages, and the microbiota in general, is still descriptive and the search for the biological mechanisms governing these communities cannot keep pace with the petabytes of sequence data deposited. As a microbiologist, I find it frustrating that most of these organisms never enter the laboratory as pure cultures for study. Here, I claim that there is still a place for biology in studying the microbiota, and that it is important to do so.

The microbiota as a phage pinball machine

Many metaphors have been used to describe the nature and functions of gut phages, the nano-organisms of the intestine. Russian dolls have repeatedly been used as a proxy for nested relationships between phage, bacteria, and the human host. In addition, “ripple” and “domino” effects describe well the chain of events that may start from the infection of bacteria by phages to regulation of the microbial community, ending with a potential effect on human health. Indeed, a strong case can be made for these points of view, but I find them somehow too linear and organised for our actual understanding of the topic. Here, I propose my personal vision, in which the microbiota is like a pinball machine, and I ask the reader to bear with me while I use this commentary to explain what I mean. In such a game of pinball, phages can be likened to little balls bouncing off various targets that are much bigger than they are in an apparently chaotic manner. The levels of interaction include, naturally, bacteria, but also possibly other viruses, the intestinal barrier, the immune system, and, more vaguely, other cellular compartments, with a resulting variable impact on human health, the pinball score (Figure 1). In the following sections, I will retrace some steps of my career during which I have attempted to obtain a better understanding of the biology of intestinal phages through these interactions and propose a number of future challenges faced by phage biologists, the pinball players.

Phage interactions with other microbes

Five years ago, while I was a postdoctoral researcher at the Institut Pasteur of Paris in the laboratory of Laurent Debarbieux, we published an article in Cell Host & Microbe showing that gut phages can perform successive passages of infection in multiple intestinal bacteria and select for structural mutations that allow adaptation to infect previously non permissive hosts (De Sordi *et al.*, 2017). These viral "host jumps" were not new to virologists and epidemiologists studying zoonoses and it was hard to imagine that, a few years later, viral mutations would become a common topic of conversation at a family dinner during a Coronavirus pandemic. However, at the time we started this project, few studies had experimentally examined these mechanisms in complex microbial communities, but a solid basis had already been established, starting from the 70s and 80s, on how individual phage-bacteria couples can coevolve with each other. In our article, we studied a simple community composed of one virulent phage that could infect an *E. coli* host strain but that was unable to bind to and lyse a second *E. coli* strain, even after weeks of patient coincubation *in vitro* and in the gut of axenic mice colonised with our three-partner community. All changed when this community was added to the microbiota of conventional mice, and it behaved differently. The phage was then able to infect both *E. coli* strains and selected genomic mutations that did not appear in the other experimental conditions. The microbiota changed what our phage could do, but how? We reasoned that the phage could have replicated on other bacterial hosts on the basis that *E. coli* is nearly omnipresent in the mammalian gut. Although this hypothesis appeared to be reasonable, proving it turned out to almost as hard as finding the proverbial needle in a haystack. With a mix of method, patience, resistance, and luck, the haystack of the mouse microbiota yielded what we were looking for: an *E. coli* strain that our phage was able to infect and that allowed the host-jump towards the non-infectable host. Changes in the viral host-range can be extrapolated from studies of phage-bacteria infection networks, but it was exciting to see them happen before our eyes.

Determining the microbiota as a promoting factor supported the idea that this ecosystem is continuously shaped and expanded by parasitic and predatory mechanisms driven by gut phages. Their study will bring us to the next challenge: how do these mechanisms interconnect with other

97 microbial ecological dynamics? Bacterial resistance, persistence, and abortive infections are also
98 among the factors expected to affect the community structure, together with phage-associated fitness
99 advantages driven by prophages carrying useful features, such as superinfection exclusion, immune-
100 evasion, or even the expression of virulence factors.

101 In addition, virus-virus interactions have been little studied in the intestinal environment but
102 cooperation between phages has been recently shown *in vitro* (Chevallereau *et al.*, 2020). As the
103 concept of sociovirology is firmly taking hold, it only seems predictable that it could be key to the
104 coexistence of densely associated mixed phage subpopulations in the intestinal tract. Improving our
105 understanding of their diversity may cast light on new roles for virus-virus interactions, similarly to
106 when we became aware of bacterial community behaviours a few decades ago.

107 ***Phage-bacteria interactions in the intestinal environment***

108 The microbiota lives and evolves in a defined environment, the intestinal tract, with mutual effects on
109 microbial and intestinal physiology. While in the Debarbieux lab at the Institut Pasteur, I had the luck
110 to also work with a then Ph. D. candidate, Marta Lourenço. Marta set up a model to study phage-
111 bacterial interactions in mice hosting a defined microbiota of 12 bacteria. We chose this model to
112 work in a controlled setting in which we could follow changes in the entire microbial community. We
113 separately challenged two *E. coli* strains with different phages, but the density of the colonising *E. coli*
114 was only slightly affected (Lourenço *et al.*, 2020). The phages, however, were actively replicating, as
115 shown by their high titres. This might seem puzzling, but is not surprising *per se*, as the gut is colonised
116 by a stable density of bacteria and their predators and parasites. But how do they coexist? Decades of
117 research have revealed several models of phage-bacteria coevolution based on the emergence of
118 bacterial resistance to phage predation and the corresponding mechanisms of phage counter-
119 resistance. The hypothesis that coevolution is key to maintaining stable bacterial and viral intestinal
120 populations is appealing. However, although we previously characterised coevolution in *in-vivo*
121 experimental settings (De Sordi *et al.*, 2019), in this study, hours of plaque assays did not reward us

with the isolation of resistant bacterial clones. We reasoned that we did not know how the spatial heterogeneity of the intestinal tract could affect phage-bacteria interactions and decided to investigate where in the intestine phage infections were more likely to take place. We found that the phage-to-bacteria ratio was much lower in the mucus layer than in the luminal compartment of different gut sections. We proposed that actively mobile bacteria can become better established in the micro compartment of the outer mucus, thus finding refuge from phage predation, more prominent in the gut lumen (Lourenço *et al.*, 2020). If trivially compared to the predator-prey dynamics of higher organisms, this may function like a rabbit-hole, providing a haven for rabbit reproduction and protection from a chasing falcon. We published this work in 2020 in Cell Host & Microbe and contributed to conveying the message that the spatial organisation of the microbiota may have an influence on the community structure and interactions with the host.

However, in our study, we only tested a handful of phages, and it would be presumptuous to believe that their interactions with their hosts can be generalisable to the billions of particles present in an intestine. Indeed, a few months after we showed that our phages were weak mucus colonisers, Green *et al.* published the characterisation of phages with a strong affinity for mucin and intestinal glycan and capable of targeting pathogenic *E. coli* embedded in the mucus layer (Green *et al.*, 2021). On the one hand, this is a reminder of how phage-specific these mechanisms can be and that an unprecedented diversity of such mechanisms could be hidden in the microbiota community. On the other hand, it should motivate the continuing isolation of more intestinal viruses if we are to characterise their biological role. This is easier said than done, as four years have elapsed from the first computational identification of the universally abundant Crass-like phages and the *in vitro* cultivation of their first representative, but definitively worth the effort (Shkoporov *et al.*, 2018)

Another aspect that was highlighted in our study of the intestinal spatial heterogeneity is that bacterial resistance to phages is not required for this model to work, and we thought that other resistance-independent mechanisms could be involved in the coexistence of intestinal phages and bacteria.

Indeed, in a recent study, we reported the intestinal-specific transcriptional profile of *E. coli*, showing downregulation of phage membrane receptors. The consequence is bacterial phenotypic resistance to phage infection that relies on the physiological response of the bacteria to the gut environment rather than irreversible genomic mutations, thus explaining the return to susceptibility of the bacteria *in vitro* (Lourenço et al., 2022).

Interestingly, resistance seems to minimally impact the bacterial ability to colonise different eukaryotic hosts under virulent phage predation, as shown by recent studies using *Pseudomonas syringae* infecting tomato leaves, and bobtail squid symbiont *Vibrio fischeri* (Hernandez and Koskella, 2019; Lynch *et al.*, 2022*)*. Yet, all these studies share the rapid evolution of bacterial resistance during free, planktonic, lifestyle. This confirms that protecting host factors favour bacterial colonisation in different host-microbe associations, leaving us with a lot to be done to understand the biology of phage-bacteria interactions *in vivo*.

The pinball challenge

After four years of postdoctoral studies on gut phage research, I started an academic position at Sorbonne University in Paris in 2018, bringing my phage experience with me and the determination to use it to understand gut diseases. This gave rise to a new group, ready to take on new challenges in the context of intestinal inflammation. Indications that phages may play a role in intestinal conditions have come from several viral metagenomics studies showing that the abundance and diversity of gut phages is altered in patients relative to that of healthy subjects. In Crohn's disease, an expansion of free temperate phages at the expense of virulent phages has been observed (Clooney *et al.*, 2019). What is the biological basis for this observation and what does it mean for the development of the disease? An elegant *in vivo* experimental study using a P2-like phage of *Salmonella spp.* showed that intestinal inflammation can act as a potent inducer of the lytic cycle of temperate phages (Diard *et al.*, 2017). The outcome of interactions between this new pool of phages and the intestinal environment is still largely unexplored. For example, whether phages activate the immune system is still debated.

However, certain evidence suggests a role for unbalanced phage communities in aggravating inflammatory symptoms, as shown by faecal phages from ulcerative colitis patients during flare-ups, specifically inducing IFN-γ by $CD4^+$ T (Gogokhia *et al.*, 2019). If more functional studies manage to complete the metagenomic characterisation of disease-associated phage communities, we may begin to appreciate their possible involvement in intestinal conditions.

Interesting biological questions to be tackled go back to the bouncing pinball analogy introduced before and include interactions with the unbalanced microbial community, the mucus, the epithelial barrier function, and the above-mentioned immune cells. This brings me to the next question: can we, as scientists and clinicians, control the flippers? That is, can we use our knowledge of phage biology to improve the health score linked to the microbiota by adding specific microbiota components? Attempts to engineer the microbiota have long been based on the use of pre-/probiotics or faecal material transplant (FMT) to modulate bacterial communities, with variable outcomes. Little has been done using phages. A traditional way of looking at the problem is the use of phages as therapeutic weapons against targeted bacteria (aka phage therapy). Although phage therapy has consistently proven its value in treating various bacterial infections, results are still largely missing for the selective targeting of intestinal pathogens or the outgrowth of intestinal commensals or pathobionts. While the pioneering treatment of *Shigella* infections by Felix d'Herelle launched the fame and diffusion of phage therapy worldwide in the early 20^{th} century, more recent data proved that therapeutic phages may fail to replicate in the desired host, leading to phage loss, like a pinball inexorably hitting the bottom of the machine without hitting the flippers or any target. On the one hand, this observation strengthens the argument for more detailed biological characterisation of phage replication in the intestinal tract. On the other, it suggests that we may need a broader approach towards gut engineering and that to hit a single bacterial microbiota member, we may need to act on the coexisting community associated with its expansion, and thus on multiple targets. This approach is routinely used to treat recurrent infections from *Clostridioides difficile* with FMT, and a number of results suggest a role for the gut virome in its therapeutic success. In addition, filtered, bacteria-depleted, viral

transplants (FVT) have been shown to be effective in several experimental *in vivo* models of disorders. As for its bacterial counterpart, active components in this mysterious mixture are unknown: do phages from healthy individuals act as master regulators of the community and, directly or indirectly, intestinal physiology or do a few key phages restore bacterial diversity and evenness by targeting the out growers?

Finally, external intervention may include bacterial lysogens carrying prophages of clinical interest. With Laurent Debarbieux, we were recently involved in a study from the group of Laurence Zitwogel in which it was shown that specific microbiota components can improve the efficacy of cancer immunotherapy. In particular, one epitope of a prophage protein from *Enterococcus hirae* triggered cytotoxic T-cell responses that cross-reacted with tumour-associated antigens. As a result, the immunogenic reaction was restricted to bacterial strains carrying the prophage, whereas the non-lysogenic form of the same species failed to show therapeutic efficacy (Fluckiger *et al.*, 2020). In this perspective, pools of temperate gut phages could represent an arsenal of molecular mimics with immunogenic potential against tumour antigens and could be used as personalised microbiota modulators via the administration of bacterial lysogens.

Conclusions

The network of biological interactions between phages, the microbiota, and their human host can shed light on the fundamentals of gut health and disease, represented in this commentary as a pinball machine. As phage biologists, we, the pinball players, can diversely contribute to resolving this network via structural, molecular, genomic, evolutionary, and mathematical studies. A fundamental understanding of the microbial alterations highlighted by metagenomic studies in clinical settings well support the hypothesis of translational engineering of intestinal ecosystems using various features of the versatile nature of phages. This spans the spectrum from their therapeutic applications against pathogenic bacteria to being regulators of microbial populations via FVT through the modulation of innate and adaptive immunity, among other possible mechanisms (Figure 1). The outcome is targeted

intervention for acute and chronic conditions aimed at improving the clinical symptoms, the disease score.

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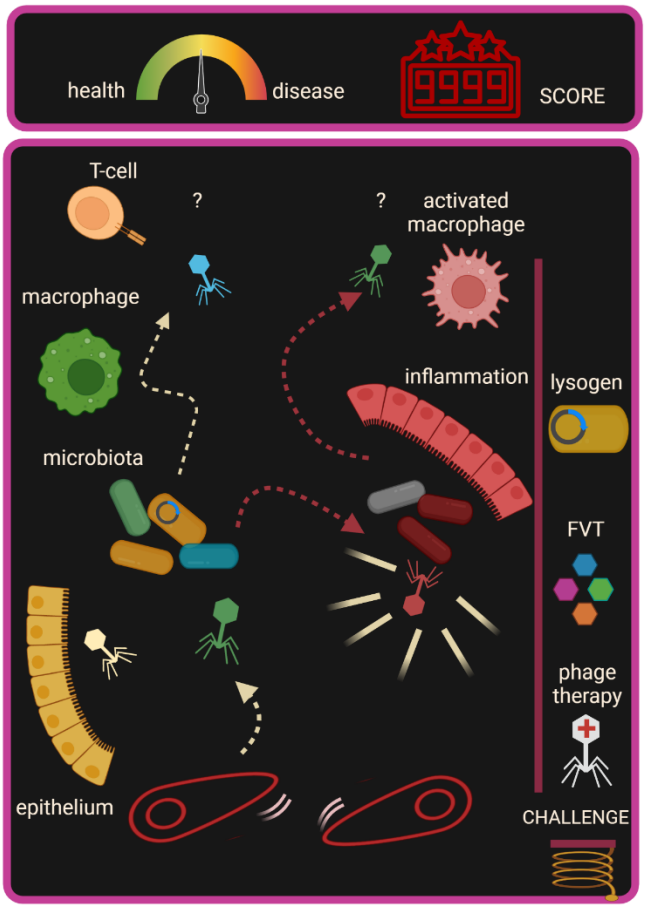
Figure 1: The pinball challenge of gut phages

Intestinal phages are pinballs bouncing off different targets in the pinball machine, represented by bacteria of the microbiota and possibly other compartments, like epithelial layers and immune cells, influencing a gut health score. The challenge, represented by ready-to-be-launched phages (phage therapy, FVT and prophages, described in the main text) is to change the score towards a healthy state.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CHALLENGE

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