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## Gut bacteriophages and the pinball challenge

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1 **Title: Gut bacteriophages and the pinball challenge**

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21 **Abstract**

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

26 **Introduction: the microbiota, between -omics and -ology**

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

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

#### 54 **The microbiota as a phage pinball machine**

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

#### 70 ***Phage interactions with other microbes***

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

94 Determining the microbiota as a promoting factor supported the idea that this ecosystem is  
95 continuously shaped and expanded by parasitic and predatory mechanisms driven by gut phages.  
96 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

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

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

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

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

152 Interestingly, resistance seems to minimally impact the bacterial ability to colonise different  
153 eukaryotic hosts under virulent phage predation, as shown by recent studies using *Pseudomonas*  
154 *syringae* infecting tomato leaves, and bobtail squid symbiont *Vibrio fischeri* (Hernandez and Koskella,  
155 2019; Lynch *et al.*, 2022). Yet, all these studies share the rapid evolution of bacterial resistance during  
156 free, planktonic, lifestyle. This confirms that protecting host factors favour bacterial colonisation in  
157 different host-microbe associations, leaving us with a lot to be done to understand the biology of  
158 phage-bacteria interactions *in vivo*.

### 159 **The pinball challenge**

160 After four years of postdoctoral studies on gut phage research, I started an academic position at  
161 Sorbonne University in Paris in 2018, bringing my phage experience with me and the determination  
162 to use it to understand gut diseases. This gave rise to a new group, ready to take on new challenges in  
163 the context of intestinal inflammation. Indications that phages may play a role in intestinal conditions  
164 have come from several viral metagenomics studies showing that the abundance and diversity of gut  
165 phages is altered in patients relative to that of healthy subjects. In Crohn's disease, an expansion of  
166 free temperate phages at the expense of virulent phages has been observed (Clooney *et al.*, 2019).  
167 What is the biological basis for this observation and what does it mean for the development of the  
168 disease? An elegant *in vivo* experimental study using a P2-like phage of *Salmonella spp.* showed that  
169 intestinal inflammation can act as a potent inducer of the lytic cycle of temperate phages (Diard *et al.*,  
170 2017). The outcome of interactions between this new pool of phages and the intestinal environment  
171 is still largely unexplored. For example, whether phages activate the immune system is still debated.



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

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

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

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

### 213 **Conclusions**

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

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

225

## 226 **Acknowledgements**

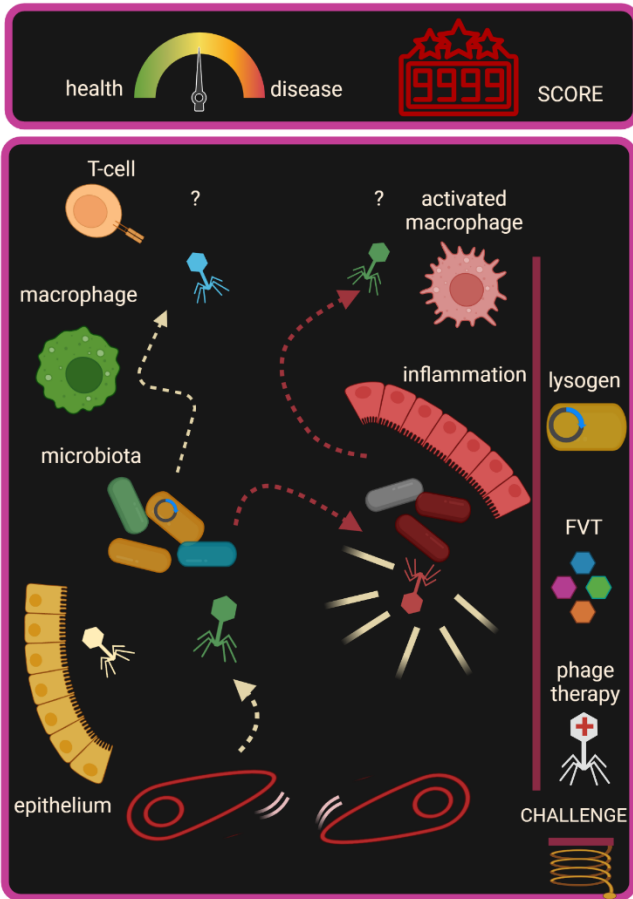
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232

## 233 **Figure 1: The pinball challenge of gut phages**

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

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