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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- γ by CD4⁺ 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 20th 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.

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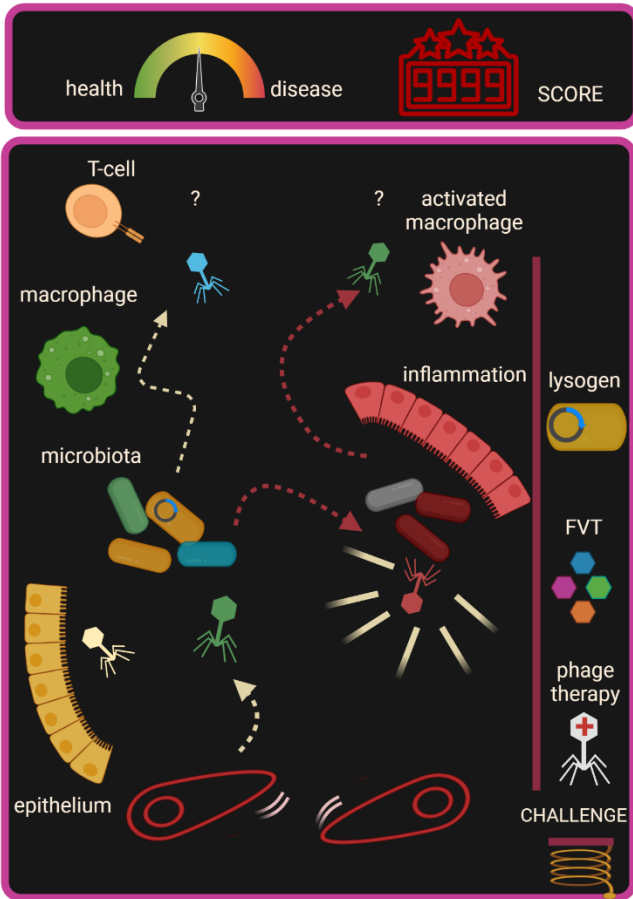
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232

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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