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1 **The selection of antibiotic- and bacteriophage-resistant *Pseudomonas aeruginosa* is**
2 **prevented by their combination**

3

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16 # Abstract

17 Objectives: Bacteria developing resistance compromise the efficacy of antibiotics or
18 bacteriophages (phages). We tested the association of these two antibacterials to circumvent
19 resistance.

20 Methods: With the Hollow Fiber Infection Model (HFIM), we mimicked the concentration
21 profile of ciprofloxacin in the lungs of patients treated orally for *Pseudomonas aeruginosa*
22 infections and independently, mimicked a single inhaled administration of phages (one or two
23 phages).

24 Results: Each treatment selects for antibiotic- or phage-resistant clones in less than 30 h. By
25 contrast, no bacteria were recovered from the HFIM at 72 h when ciprofloxacin was started 4
26 h post-phage administration, even when increasing the initial bacterial concentration by a 1000
27 fold.

28 Conclusion: The combination of phages with antibiotics used according to clinical regimens
29 prevents the growth of resistant clones, providing opportunities to downscale the use of multiple
30 antibiotics.

31

32

33 **Keywords:** hollow fiber infection model, antimicrobial resistance, phage therapy,
34 pharmacokinetics, drug combination

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35 # Introduction

36 *Pseudomonas aeruginosa* is an opportunistic pathogen, naturally resistant to many antibiotics.
37 Moreover, repeated antibiotics treatments administered to patients with chronic airways
38 infections, such as cystic fibrosis (CF) patients, have led this bacterium to acquire additional
39 drug-resistance [1,2]. Acute exacerbations are treated by either intravenous (beta-lactams and
40 aminoglycosides), oral (ciprofloxacin), or inhaled (tobramycin and colistimethate sodium)
41 administrations of antibiotics [2,3]. The recent TORPEDO-CF study concluded that IV or oral
42 antibiotics administration could be equivalent [2].

43 In *P. aeruginosa* resistance to fluoroquinolones, such as ciprofloxacin, involves mutations in
44 *gyrA* or in genes regulating the expression of the MexEF-OprN efflux pump [4,5]. The increase
45 of the MIC is often modest for the first-step mutants, qualified as "less-susceptible" [5], but is
46 sufficient to favor their growth within the Mutant Selection Window (MSW) [6]. Next, multiple
47 mutations lead to a higher MIC, clinical resistance [4], and ultimately require ciprofloxacin to
48 be associated with other antibiotics, upscaling drug use [7,8]. Interestingly, the gradual increase
49 of MIC is reproduced *in vitro* by mimicking the clinical regimens of ciprofloxacin, providing a
50 mean to study the efficacy of combined treatments [9–11].

51 Bacteriophages (phages) are antibacterial viruses. Recently, an increasing number of successful
52 compassionate treatments in both Europe and the USA have confirmed the therapeutic potential
53 of phages, which has a long history of human use [12,13]. Phages have the peculiar capacity to
54 self-amplify at the site of infection, increasing their density locally, at the expense of bacteria
55 [14]. Nevertheless, as for antibiotics, bacteria have developed several ways to resist to phages
56 [15]. However, since the molecular mechanisms involved in drug and phage resistance do not
57 overlap, their association in cocktails or with antibiotics has been proposed to improve the
58 efficacy of treatments [16,17].

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59 By using the Hollow Fiber Infection Model (HFIM, Fig S1, S2) inoculated with *P. aeruginosa,*
60 we simulated the pharmacokinetics of ciprofloxacin in the human lungs for 72 h and evaluated
61 the antibacterial efficacy of its combination with phages administered to mimic a single local
62 inhaled treatment. We show that the combination of phages with a simultaneous or delayed
63 administration of ciprofloxacin leads to a stronger reduction of *P. aeruginosa* in the HFIM than
64 individual treatments, preventing the selection of both phage and ciprofloxacin resistant clones.
65 This reduction reached the limit of detection with the delayed combinations, suggesting that
66 coupling phages and antibiotics could downscale antibiotics consumption in clinics.

67

68 # Material and methods

69 ## Bacterial strain

70 The *Pseudomonas aeruginosa* strain K (PAK) with a MIC of ciprofloxacin of 0.064 µg/mL was
71 used for all the experiments.

72 ## Phages and ciprofloxacin

73 Phage PAK_P1, a virulent *Myoviridae*, was isolated using strain PAK[18]. Phage LUZ19v is a
74 variant of phage LUZ19, a virulent *Podoviridae* isolated on strain PAO1 [21], isolated
75 following serial passages on strain PAK. The efficiency of plating (EOP) of LUZ19 and
76 LUZ19v on strain PAK is 0.2 and 1, respectively, compared to the EOP on strain PAO1

77 Both phages were amplified in liquid lysogeny broth. Lysates were filtered-sterilized at 0.2µm,
78 and stored at 4°C until use. Phage titrations (serial dilutions) were spotted on tryptic soy agar
79 (TSA) supplemented with magnesium sulfate (10 g/L) and activated charcoal (10 g/L) covered
80 by a lawn of strain PAK made with 10^6 CFU.

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81 Stocks of ciprofloxacin (Sigma Aldrich) were stored at -20°C for less than 1 month and thawed
82 only once.

83 Minimal Inhibitory Concentration (MIC) determination

84 The MIC of ciprofloxacin for the strain PAK was determined in triplicate by broth microdilution
85 in cation-adjusted Mueller Hinton broth (MHB), according to the CLSI reference methods.
86 Frozen samples of bacteria collected at the end (72 h) of each HFIM experiment were thawed
87 and plated on MH agar overnight. Several colonies were sampled and the bacterial density was
88 adjusted to $5x10^5$ CFU/mL before MIC determination.

89 Hollow Fiber Infection Model (HFIM)

90 The HFIM includes a cartridge (C2011 polysulfone cartridge, FiberCell Systems, Inc.,
91 Frederick, MD, USA) with capillaries composed of a semipermeable polysulfone membrane.
92 The pore size of the capillaries (42 kDa) allows equilibration of ciprofloxacin, which can freely
93 circulate between the intracapillary and extracapillary spaces while the bacteria and the phages
94 are trapped in the extracapillary space of the cartridge (Fig. S1 and S2).

95 In this study, 20 mL of a suspension containing 5.5 log_{10} CFU/mL (standard inoculum) or 8.5
96 CFU/mL (high inoculum) of *P. aeruginosa* were inoculated into the extracapillary space of
97 each cartridge and incubated at 37°C in MHB for 1 h. Ciprofloxacin was added to the central
98 compartment to obtain the desired maximum concentration (C_{max}) of 1.5 µg/mL and was
99 continuously diluted with MHB to mimic an elimination half-life of 4 h [19]. A mean inoculum
100 of 7.5 log_{10} PFU/mL (8.8 log_{10} PFU *in toto*) of either one or two phages with equal amounts of
101 each phage was added once into the extracapillary space. Treatments with ciprofloxacin or
102 phages or both simultaneously were started 1 h after the inoculation of bacteria in the HFIM.
103 When testing the delayed combination, ciprofloxacin was added 4 h after the phages. All the

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104 experiments (except the untreated control and single phage combined to ciprofloxacin) were
105 performed in duplicate.

106 Bacteria and phages quantification

107 Samples of 1 mL were collected from the extracapillary space to count the bacteria and phages
108 at 0 (before the addition of phages or ciprofloxacin), 0.25, 0.5, 1, 2, 4, 6, 8, 24, 26, 29, 32, 48,
109 50, 53, 56, and 72 h. After centrifugation at 3000 g for 10 min, supernatants were recovered to
110 count phages and pellets were resuspended in 1 mL of NaCl 0.9% to count bacteria. The bacteria
111 and phage suspensions were serially diluted (10X), and spotted (10 µL) in triplicate on either
112 TSA (to count bacteria) or on TSA covered with strain PAK (to count phages) and incubated
113 overnight at 37°C. The limit of detection (LOD) was 1.5 log_{10} CFU/mL for bacteria and 1.5
114 log_{10} PFU/mL for phages.

115 Monitoring of ciprofloxacin and phage resistance

116 Twice a day the bacteria sampled from the HFIM were counted on agar plates containing 0.5
117 µg/mL of ciprofloxacin, corresponding to 8-fold the MIC of t strain PAK. The proportion of
118 less-susceptible bacteria was calculated as the ratio of colonies on drug-supplemented agar
119 (MIC 8X) divided by colonies on drug-free agar.

120 Bacteria sampled at 72 h were stored in 30% v/v glycerol at -80°C before phage resistance
121 analysis. Bacteria were thawed and immediately incubated with or without phages (10^8 PFU)
122 for one hour before plating on agar with or without a single pre-absorbed phage (10^8 PFU/plate).
123 The successive incubations in broth and on agar were made with the same phage (LUZ19v or
124 PAK_P1). The frequency of resistance to each phage was calculated by the ratio of colonies
125 growing in the presence of phages over those growing in the absence of phages.

126

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127 **Ciprofloxacin quantification**

128 Ciprofloxacin quantification was performed in samples withdrawn from the central
129 compartment and from the extracapillary space of the cartridge according to a standard protocol
130 (Supplementary file).

131

132 # Results

133 ## Pharmacokinetic analysis of ciprofloxacin in the HFIM

134 We simulated in the HFIM inoculated with *P. aeruginosa* strain PAK the concentration profile
135 of ciprofloxacin during 72 h corresponding to the administration of 500 mg twice daily in
136 patients, using a C_{max} at 1.5 µg/mL and a half-life of 4 h (Fig. S1 and methods). The predicted
137 *vs*. observed concentrations in the central and peripheral compartments of the HFIM fit well in
138 all experiments reported thereafter, including those with the combination of ciprofloxacin and
139 phages (Fig. 1). These data demonstrate the reproducibility of the disposition of ciprofloxacin
140 in the HFIM and reveal that the presence of phages in the peripheral compartment does not
141 affect it.

142

143 ## Clinically-relevant ciprofloxacin regimen selects rapidly for less susceptible
144 ## clones

145 Following the inoculation of *P. aeruginosa* in the extracapillary space of the HFIM cartridge
146 (Fig. S1 and S2), the bacterial concentration reached 5.7 $\log_{10}$ CFU/mL within 1 h, time point
147 at which different treatments were administered. In the absence of treatment, this bacterial
148 concentration increased to around 9.5 $\log_{10}$ CFU/mL over the first 24 h and remained roughly
149 stable during the next 48 h (Fig. 2A).

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150 Within 30 min after the start of the ciprofloxacin regimen, the density of *P. aeruginosa*
151 decreased by more than 3-logs and remained below the limit of detection (LOD) between 1 h
152 and 8 h (Fig. 2A). Subsequently, the bacterial density increased, despite the ciprofloxacin
153 regimen, reaching at 72 h a similar value (9.2±0.1 log_{10} CFU/mL) compared to the untreated
154 control.
155 Samples from the HFIM were plated twice a day on agar supplemented with 0.5 µg/mL
156 ciprofloxacin (8-fold MIC) to assess the selection of less-susceptible bacteria. No bacteria from
157 the initial inocula (n=17) grew on this selective medium. In samples exposed to ciprofloxacin,
158 the density of less-susceptible bacteria increased over the 72 h to reach 43-100% of the
159 population (Fig. 2A). The MIC of ciprofloxacin for the bacteria sampled at 72 h increased by
160 250 fold (16 µg/mL; Table 1), showing that within 24 h, the ciprofloxacin regimen administered
161 in the HFIM selected for less-susceptible bacteria.
162

163 ## Single or two-phage local administration selects for phage-resistance

164 We next assessed with the HFIM the susceptibility of *P. aeruginosa* to two phages, the
165 *Myoviridae* PAK_P1 and the *Podoviridae* LUZ19v, both positively evaluated previously for
166 the treatment of acute lung infections in mice [20,21]. The frequency of bacteria among the
167 naïve population that could grow in the presence of LUZ19v and PAK_P1 was $3x10^{-7}$ and $6x10^{-}$
168 ^{5}, respectively (Table 2).

169

170 Phages were administered once in the extracapillary space of the HFIM cartridge containing *P.*
171 *aeruginosa* to mimic a local administration (Fig. S1). Along these experiments, no phage was
172 detected in any of the samples taken from the central compartment, confirming that phages
173 were strictly maintained in the extracapillary space (Fig. S2). The single dose of phages was set

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174 to obtain 7.5 $\log_{10}$ PFU/mL in the HFIM (8.8 $\log_{10}$ PFU *in toto*), which corresponds
175 approximately to a phage:bacteria ratio of 100 at the time of administration.
176 Following the administration of phage LUZ19v or PAK_P1, the *P. aeruginosa* density dropped
177 to 1.9±1.3 or 2.8±0.9 $\log_{10}$ CFU/mL within 2 h, or 4 h, respectively, and then started to increase
178 continuously, reaching the density of the untreated control 24 h post-phage administration, and
179 remained stable for another 48 h (Fig. 2B and 2C). Corresponding to the drop of bacteria, the
180 density of LUZ19v or PAK_P1 increased during the first time points and reached a maximum
181 at 24 h or 48 h, respectively (Fig. S3). The susceptibility to phages of samples taken at 72 h
182 revealed that bacteria exposed to LUZ19v or PAK_P1 became nearly all resistant (20 to 50%
183 and 60 to 100%, respectively), while keeping a large susceptibility to the second phage ($<10^{-5}$
184 and 10^{-7}, respectively) (Table 2). Therefore, monophage treatments were as inefficient as
185 ciprofloxacin to control the growth of resistant bacteria in the HFIM during 72 h.
186 Since phages LUZ19v and PAK_P1 recognize two different bacterial receptors, the type IV
187 pilus [22] and the lipolysaccharide (LPS, [18]), respectively, we assessed if their combination
188 could lower the selection of resistant clones. The two-phage cocktail led to a similar reduction
189 of the bacterial density during the first time points compared to phage LUZ19v alone (Fig. 2D).
190 Then, the slope of the bacterial growth between 8 and 24 h was less steep compared to
191 monophage treatments. Bacterial counts reached similar levels to the untreated control at 48 h
192 and in the 72 h samples. The proportion of bacteria resistant to either phages was about one
193 order of magnitude lower compared to single treatments (Table 2). Therefore, the use of two
194 phages instead of one delays the selection of phage-resistant bacteria but does not prevent it.
195 The MIC of bacteria at 72 h following exposure to one or two phages was similar to the MIC
196 of the inoculated strain showing that the exposure to phages does not select for less susceptible
197 clones to ciprofloxacin (Table 1). Reciprocally, the bacteria from the HFIM exposed to
198 ciprofloxacin were as susceptible to either phages as the control (Table 2).

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199

200 **The combination of phages with ciprofloxacin prevents the growth resistant**
201 **bacteria**

202 To assess the impact of the combination of phages with ciprofloxacin, we tested two modalities
203 corresponding to a simultaneous or a delayed treatment (phages first and ciprofloxacin 4 h
204 later). The simultaneous treatment led to a rapid killing of bacteria, since their density dropped
205 below the LOD in 15 min (Fig. 3A). Impressively, we could not detect any colony on samples
206 taken during the next 72 h. During these experiments (n=2), the density of phages was stable,
207 suggesting that they did not amplify (Fig. S4). When adding ciprofloxacin 4 h after the two-
208 phage cocktail, the density of phages slightly increased and then remained stable up to 72 h.
209 Here, again, the combination rapidly killed the population of *P. aeruginosa*, and no viable
210 bacteria were recovered at any time after 1 h (Fig. 3B). Similar results were obtained when
211 either phage was simultaneously added with ciprofloxacin (Fig. S5).
212

213 **The antibacterial efficacy of the combination of phages with ciprofloxacin is**
214 **density dependent**

215 To assess the robustness of the combined treatment, the HFIM was inoculated with a 1000- fold
216 higher bacterial inoculum while the regimen of either ciprofloxacin and phages remained
217 unchanged. Following such inoculation, the bacterial density reached 8.6±0.3 $\log_{10}$ CFU/mL
218 within 1 h, which corresponds to a phage:bacteria ratio of 0.1. The simultaneous addition of
219 phages and ciprofloxacin rapidly killed bacteria with a density falling below the LOD between
220 30 min and 8 h (Fig. 3C). Subsequently, the bacterial density increased reaching 8.6±0.06 $\log_{10}$
221 CFU/mL at 72 h. The bacteria recovered had a reduced susceptibility to ciprofloxacin (16 to
222 32-fold higher MIC) and to phages compared to the naïve population (Tables 1 and 2). In the

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223 two independent assays, the proportion of bacteria resistant to each phage increased with
224 bacteria becoming fully resistant to LUZ19v and partially resistant to PAK_P1 in one replicate
225 and the other way around in the second.
226 By contrast, when adding ciprofloxacin 4 h after the two-phage cocktail, the initial reduction of
227 bacteria was slower compared to the simultaneous administration with bacterial density falling
228 below the LOD at 1 h for one replicate and 6 h for the other (Fig. 3D). However, after this
229 decline, no increase of the bacterial density was observed and again no colony could be
230 recovered on samples taken during the next 72 h. We concluded that when phages reduce first
231 the size of the bacterial population, the remaining population was not large enough to include
232 less-susceptible mutants to ciprofloxacin.

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233 # Discussion

234 *P. aeruginosa* lung infections are increasingly difficult to treat with antibiotics calling for
235 therapeutic strategies to enhance bacterial killing. In this study, we developed an innovative use
236 of the HFIM to evaluate the potential benefit of combining ciprofloxacin with phages. The
237 HFIM allows the simulation of a clinically relevant ciprofloxacin concentration profile obtained
238 with oral treatment that leads in monotherapy to the selection of clones with increasing
239 resistance as previously reported [9–11]. The combination of ciprofloxacin and meropenem in
240 the HFIM suppressed the growth of resistant *P. aeruginosa* isolates, including hypermutable
241 strains [10,11]. In clinics, this combination requires IV administration and the hospitalization
242 of patients [2]. Moreover, it participates to the upscaling of drugs use and overall increases the
243 selection of MDR strains.

244 Instead of a second antibiotic, we evaluated the combination of ciprofloxacin with phages. A
245 unique local administration of one or two phages could not prevent the growth of phage-
246 resistant bacteria, as generally observed with *in vitro* tests. We observed that the growth of
247 resistant clones in presence of the two phages was delayed compared to single phages, in
248 agreement with increased fitness cost, as reported elsewhere [23,24].

249 In contrast to previous studies that combined a single addition of ciprofloxacin, with either
250 phage OKMO1, or PEV31, or a five-phages cocktail, we did not observe a modification of the
251 MIC for ciprofloxacin in any of the phage-resistant clones tested [17,25,26]. This suggests that
252 these clones may not be selected when a clinically relevant regimen of ciprofloxacin is used.
253 Moreover, the ciprofloxacin resistant clones remained largely susceptible to each of the two
254 phages as their frequency was the same as in the control (without treatment). Altogether, the
255 lack of correlation between the profiles of susceptibility and resistance to ciprofloxacin and the
256 two phages demonstrates their independent antibacterial activity.

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257 The combination of ciprofloxacin with one or two phages administered simultaneously on the
258 HFIM inoculated with the same bacterial density than individual treatments abolished the
259 growth of resistant clones during at least 72 h, a long-term performance compared to less than
260 30 h for the latter treatments.This strongly suggests that in these conditions no bacteria survived.
261 However, when the initial bacterial density was 1000 fold higher the growth of resistant clones
262 was detected at 24 h and rise up during the next 48 h, showing that this regimen was unable to
263 control a dense population of *P. aeruginosa*. When phages are administered first and the
264 ciprofloxacin 4 h later, the drop of bacteria aligned with the increase of phage concentrations.
265 The bacterial density reached the LOD until the end of each experiment, with low or high
266 inoculum, suggesting that no bacteria survived these regimens.
267 The *in vivo* efficacy of the combination of ciprofloxacin (a single oral dose simulating 750 mg
268 in human) with phages (a single intravenous administration of 10^{10} PFU) was previously tested
269 in an experimental *P. aeruginosa* endocarditis in rats and led to more frequent negative
270 vegetation cultures after 6 h than in rats receiving only phages or only ciprofloxacin [27]. Using
271 a murine model of *P. aeruginosa* pulmonary infection, a unique dry power insufflation of
272 ciprofloxacin and phages led to a reduction of nearly 6 $\log_{10}$ CFU in 24 h [28]. In these two
273 studies, a unique dose of ciprofloxacin was used associated to short time end-points. The data
274 we obtained with the HFIM suggest that the administration of antibiotics following their
275 recommended regimens could increase the efficacy of these combinations on longer time
276 points.
277 One of the limitations of our study relates to the lack of an immune component that could
278 enhance the overall efficacy of such regimen as the immune system was previously shown to
279 cooperate with phages during experimental pulmonary phage therapy [29]. Another limitation
280 is the lack of loss of phages over time as they remained trapped in the same compartment of
281 bacteria. However, the decay of phages in uninfected or infected lungs of mice was shown to

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282 be rather weak (below 1-log per day) compared to the overall density of phages in the HFIM
283 [30]. The data presented here advocate in favor of a translation to clinics that could ultimately
284 slow down the use of multiple antibiotics and therefore, the selection of MDR strains [17].
285

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401 **Table 1.** MIC of ciprofloxacin of the parental strain PAK and clones from samples exposed to
402 either ciprofloxacin, or phages, or their combination in the HFIM during 72 h.

Experimental conditions	MIC (µg/mL)
Inoculum (5.7 log10 CFU/mL)	0.064
HFIM (72 h samples)	
Control (n=1)	0.064
Ciprofloxacin (n=2)	16; 16
LUZ19v (n=2)	0.128; 0.128
PAK_P1 (n=2)	0.064; 0.064
LUZ19v and PAK_P1 (n=2)	0.064:0.064
Simultaneous combination of ciprofloxacin and phages (n=2)	NBR
Delayed combination of ciprofloxacin and phages (n=2)	NBR
Simultaneous combination of ciprofloxacin and phages (n=2)	1; 2
Delayed combination of ciprofloxacin and phages (n=2)	NBR

403 Grey lines correspond to experiments performed with an inoculum of 8 log_{10} CFU/mL
404 NBR, no bacteria recovered
405

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406 **Table 2.** Frequencies of phage resistant clones from samples exposed to either ciprofloxacin,
407 or phages, or their combination in the HFIM during 72 h

Experimental conditions	Resistance to LUZ19v	Resistance to PAK_P1
Inoculum (5.7 log10 CFU/mL)	1.10^{-7}	6.10^{-5}
HFIM (72 h samples)		
Control (n=1)	8.10^{-5}	<LOD[b]
Ciprofloxacin (n=2)	1.10^{-5} ; 1.10^{-5}	6.10^{-6}; 3.10^{-6}
LUZ19v (n=2)	5.10^{-1}; $2.\ 10^{-1}$	8.10^{-5}; 1.10^{-7}
PAK_P1 (n=2)	3.10^{-7}; $<10^{-9}$	1; 6.10^{-1}
LUZ19v and PAK_P1 (n=2)	1.10^{-2}; 6.10^{-3}	2.10^{-1}; 8.10^{-3}
Simultaneous combination of ciprofloxacin and phages (n=2)	NBR[a]	NBR
Delayed combination of ciprofloxacin and phages (n=2)	NBR	NBR
Simultaneous combination of ciprofloxacin and phages (n=2)	1; <LOD[b]	<LOD[b]; 1
Delayed combination of ciprofloxacin and phages (n=2)	NBR	NBR

408 Grey lines correspond to experiments performed with an inoculum of 8 log_{10} CFU/mL

409 [a], no bacteria recovered

410 [b], limit of detection

411

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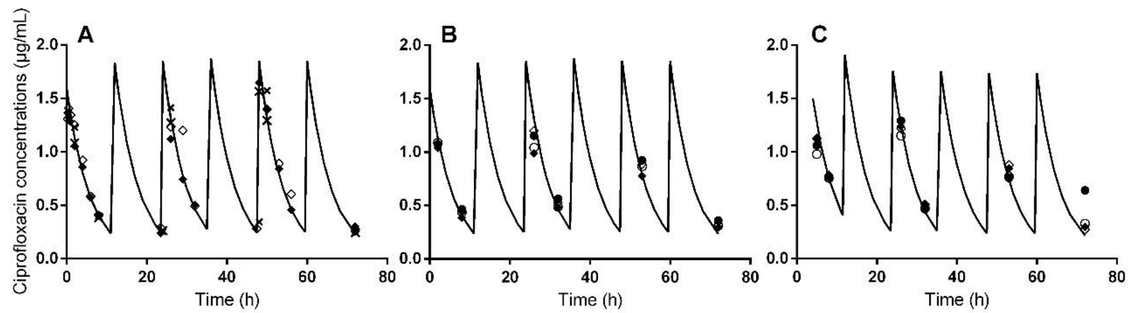
412

413 Figure 1



414

415 **Figure 1. The regimen of ciprofloxacin administered in the HFIM reproduces the regimen**

416 **of oral treatments.**

417 Expected (black line) and observed (diamonds and circles for standard and high inoculum,

418 respectively) concentration-time profiles of ciprofloxacin in the HFIM (inoculated with *P.*

419 *aeruginosa* strain PAK) after its administration twice a day for the following experiments: A,

420 ciprofloxacin alone. B, ciprofloxacin administered simultaneously with the two-phage cocktail.

421 C, ciprofloxacin administered 4 h post two-phage cocktail. n=2 for each inoculum represented

422 by open and filled symbols. The concentrations in the peripheral compartment are represented

423 with crosses in panel A.

424

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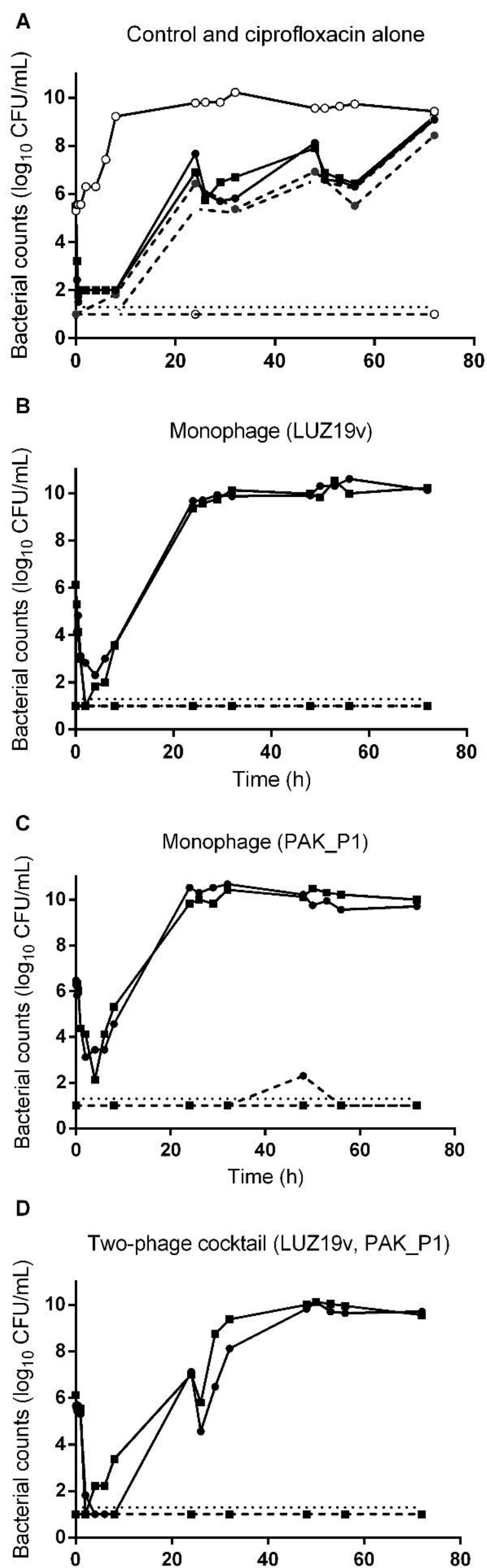
426 Figure 2



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428 **Figure 2. The growth of *P. aeruginosa* in the HFIM is not controlled by phages or**
429 **ciprofloxacin**
430 The concentration of *P. aeruginosa* strain PAK ($\log_{10}$ CFU/mL) in the HFIM from 1 h post-
431 inoculation to72 h after exposure to ciprofloxacin or phages. A, control experiment (n=1) and
432 ciprofloxacin alone (n=2). B, monophage (LUZ19v) (n=2). C, monophage (PAK_P1) (n=2). D,
433 two-phage cocktail (LUZ19v, PAK_P1) (n=2). Solid lines represent total bacterial populations
434 and dashed lines represent less-susceptible bacteria growing on agar containing 0.5 µg/mL of
435 ciprofloxacin. Square and circles represent independent experiments. The horizontal dotted line
436 corresponds to the LOD.
437
438

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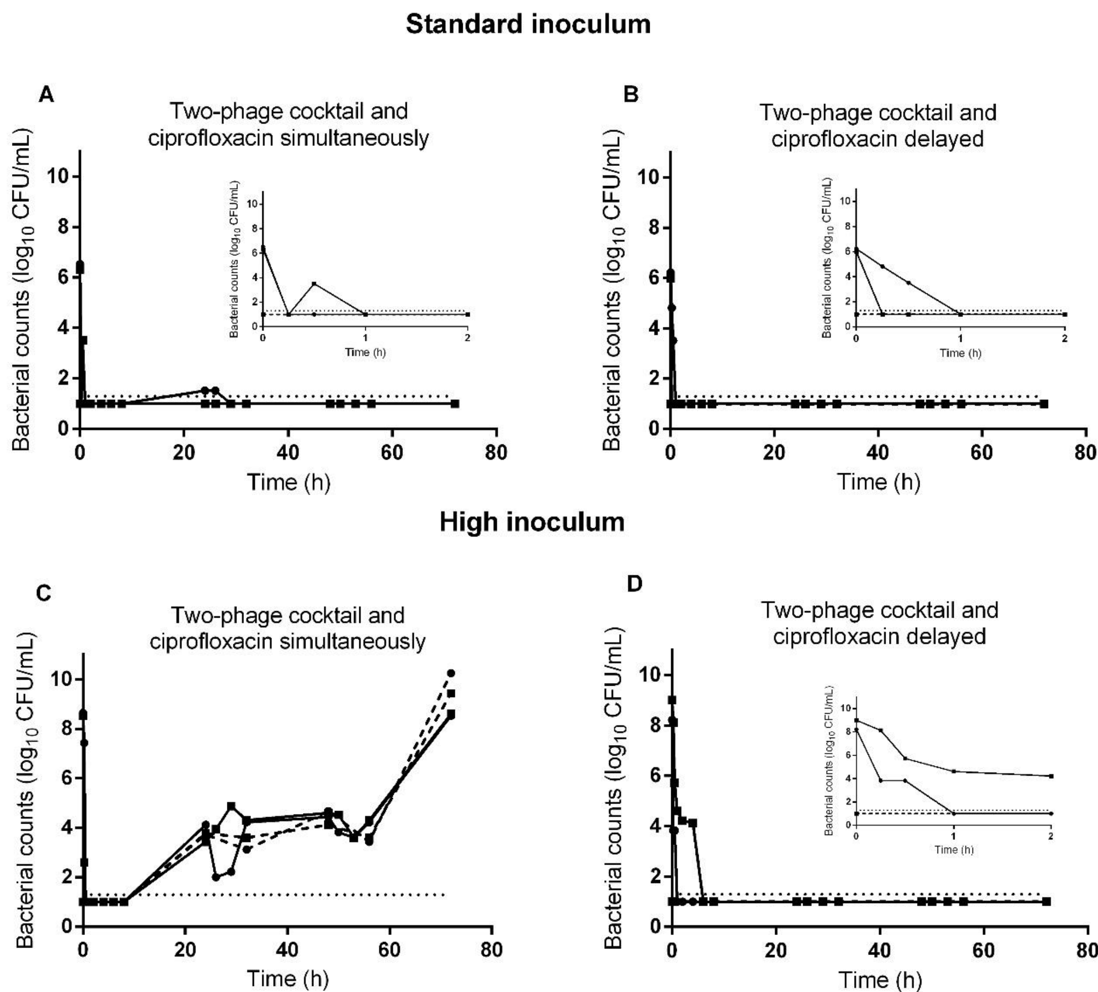
439 **Figure 3**



440

441 **Figure 3. The growth of *P. aeruginosa* in the HFIM is only controlled by the**
442 **combination of phages and ciprofloxacin**

443 Population of bacteria (mean±SD in log_{10} CFU/mL) in the HFIM over 72 h post exposure of a
444 standard (A and B) or high inoculum (C and D) of *P. aeruginosa* to the combination of
445 ciprofloxacin and phages. A and C, combination of simultaneous administrations of
446 ciprofloxacin with the two-phage cocktail (n=2). B and D, combination of the two-phage
447 cocktail with ciprofloxacin administrated 4 h post-phages (n=2). Solid lines represent total
448 bacterial populations and dashed lines represent less-susceptible bacteria growing on agar
449 containing 0.5 µg/mL of ciprofloxacin. Square and circles represent independent experiments.
450 The horizontal dotted line corresponds to the LOD.

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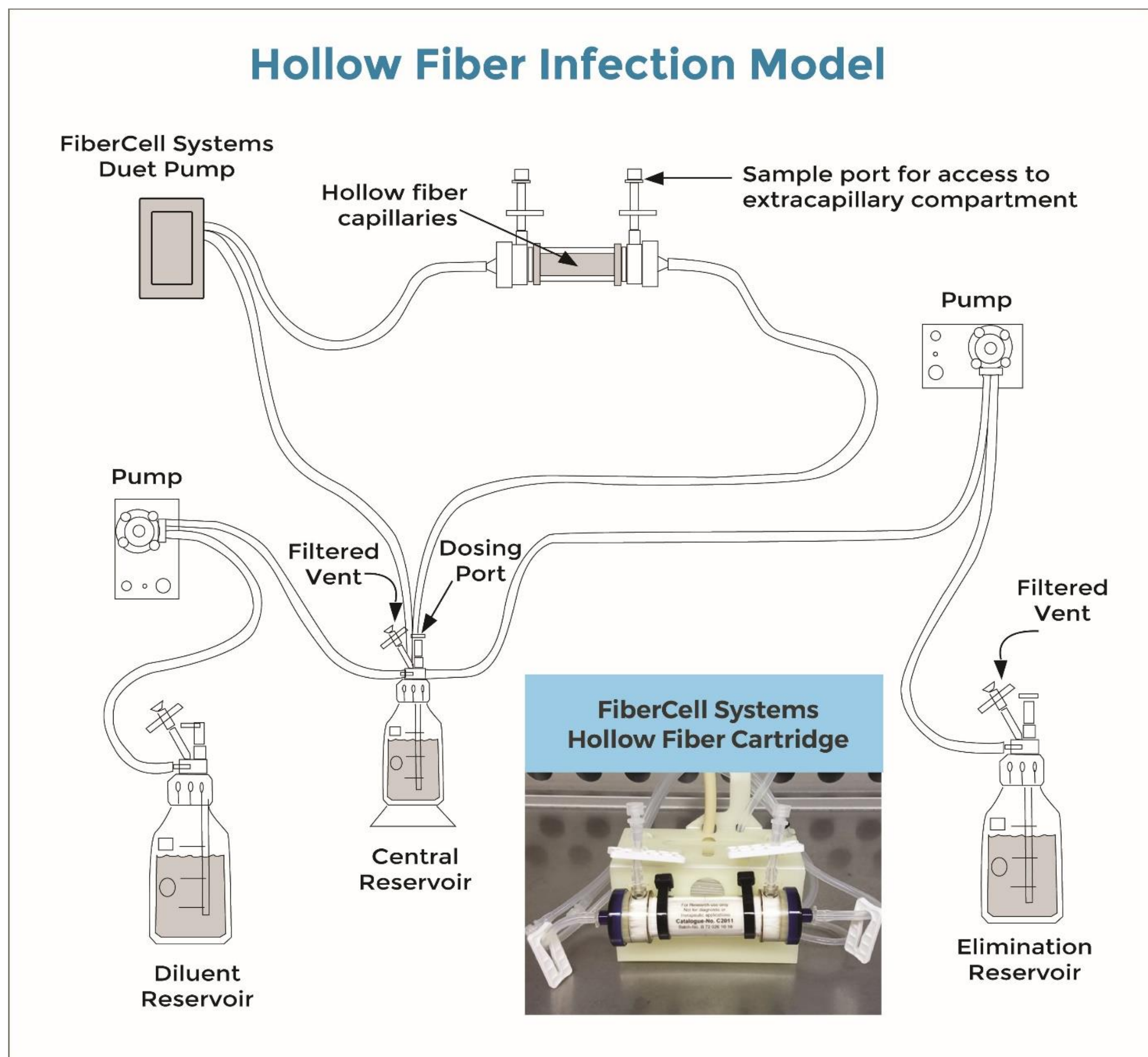
451 ## Supplementary figures



452

453 **Figure S1.** Schematic representation of the Hollow Fiber Infection Model kindly provided by
454 FiberCell Systems®. Bacteria and phages were trapped in the extracapillary space of the
455 cartridge (peripheral compartment) (see also embedded photo). Ciprofloxacin was added to the
456 central reservoir and freely circulated through the cartridge and bacteria by means of the
457 Fibercell Systems Duet pump® (FiberCell Systems, Inc., Frederick, MD, USA). Ciprofloxacin
458 concentrations decreased over time after drug administrations, due to the continuous addition
459 of a diluent (MHB) by means of another set of pumps

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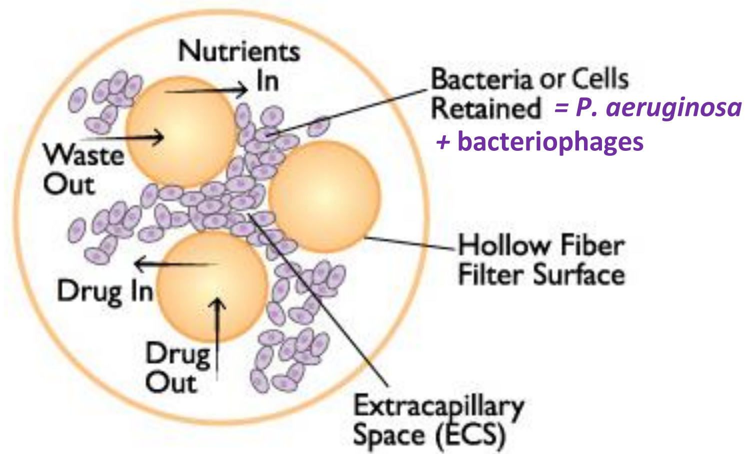

460

461 **Figure S2.** Schematic representation of the cross-section of the cartridge of the HFIM, kindly
462 provided by FiberCell Systems®. Phages were directly introduced in the extracapillary space
463 containing *P. aeruginosa* to simulate local administration. Both phages and *P. aeruginosa*
464 remained trapped in this extracapillary space during the experiments. The drug, here
465 ciprofloxacin, freely circulates through the fibers and was distributed both in the central
466 reservoir and the extracapillary spaces of the cartridge.

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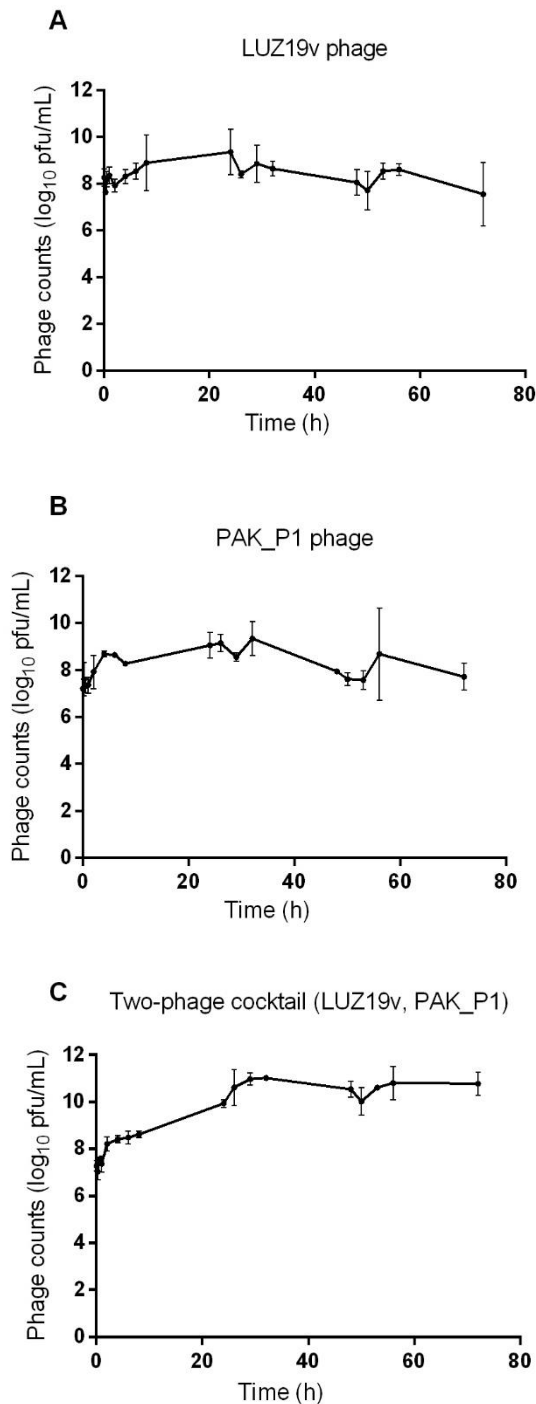
467 Figure S3



468

469 **Figure S3.** Total population of phages (mean±SD in log_{10} PFU/mL) in the HFIM over 72 h in
470 the different experiments. A, phage LUZ19v alone (n=2). B, phage PAK_P1 alone (n=2). C,
471 phages LUZ19v and PAK_P1 (n=2).

472

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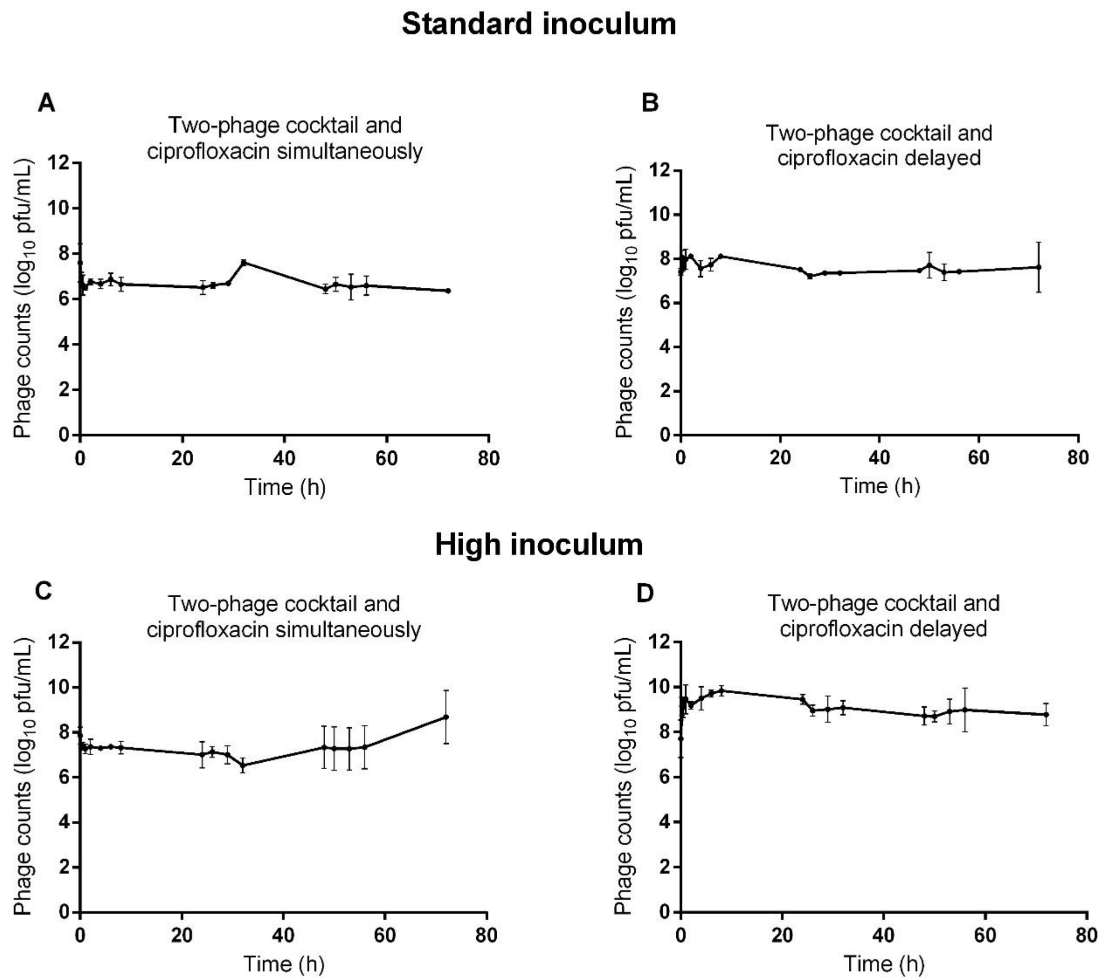
473 **Figure S4**



474

475 **Figure S4.** Total population of phages (mean±SD in log_{10} PFU/mL) in the HFIM over 72 h in
476 the experiments with a standard (A and B) or high inoculum (C and D) of *P. aeruginosa*. A
477 and C, combination of simultaneous administrations of ciprofloxacin with the two-phage
478 cocktail (n=2). B and D, combination of the two-phage cocktail with ciprofloxacin
479 administrated 4 h post-phages (n=2).

480

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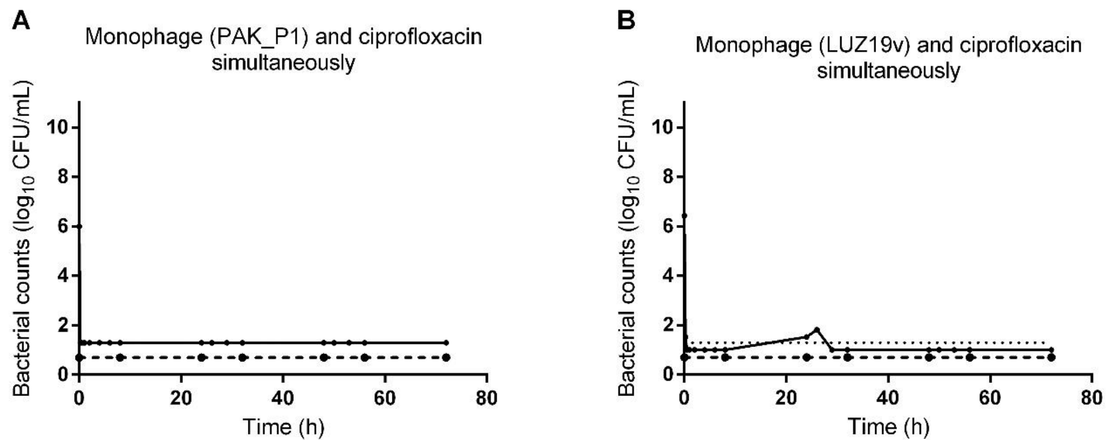
482 Figure S5



483

484 **Figure S5.** Population of bacteria (mean±SD in $\log_{10}$ CFU/mL) in the HFIM over 72 h after
485 exposure to the combination of ciprofloxacin and a single phage. A, combination of
486 simultaneous administration of ciprofloxacin with LUZ19v (n=1). B, combination of
487 simultaneous administration of ciprofloxacin with PAK_P1 (n=1). Solid lines represent total
488 bacterial populations and dashed lines represent less-susceptible bacteria growing on agar
489 containing 0.5 µg/mL of ciprofloxacin. The horizontal dotted line corresponds to the LOD.

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491 **Supplementary file**

492

493 Samples for ciprofloxacin quantification were withdrawn from the central compartment at 0.25,
494 0.5, 1, 2, 4, 6, 8, 24, 26, 29, 32, 48, 50, 53, 56, and 72 h, and from the extracapillary space of
495 the cartridge at 2, 8, 24, 26, 48, 54, and 72 h (Supplementary file). Samples were centrifuged at
496 3000 g for 10 min, and the supernatant was stored at -20°C. One hundred microliters of water
497 containing the marbofloxacin internal standard at 5 µg/mL were added to 100 µL of calibrators,
498 quality controls, or samples. The mixture was vortexed at 1400 rpm for 2 min at 10°C and
499 centrifuged at 20000 g for 10 min. The supernatant (20 µL) was injected into an Acquity ultra
500 performance liquid chromatography (UPLC®) coupled to a UV detector (Waters, Milford, MA,
501 USA). Ciprofloxacin was eluted at 0.3 mL/min on an Acquity UPLC BEH C18 column (2.1x50
502 mm, 1.7 µm) equipped with a frit (0.2 µm, 2.1 mm) and set at 40°C under the following gradient
503 conditions: t0 90% A (H_2O acidified with 0.1% HCOOH) 10% B (acetonitrile); t(4 min) 60%
504 A and 40% B. The return to initial conditions was held for 1 min. Wavelength detection was
505 set at 278 nm. Chromatographic data were monitored by Empower software (Waters, Milford,
506 MA, USA). The method was validated from 0.05 to 5 µg/mL of ciprofloxacin with a linear
507 model weighted by $1/X^2$ (X=concentration). Precisions and accuracy were checked by injecting
508 six replicates of QC samples over three days, at the limit of quantification (0.05 µg/mL); 0.075
509 µg/mL; 0.75 µg/mL, and 4 µg/mL. Accuracies ranged from 92% to 107%, with intra-day and
510 inter-day CV precisions below 5% and 13%, respectively. The limit of quantification was
511 validated at 0.05 µg/mL, with an accuracy of 96% and intra- and inter-day CV precision lower
512 than 6%.

513