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Combining phages and antibiotic to enhance antibiofilm efficacy against an *in vitro* dual species wound biofilm

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1 **Combining phages and antibiotic to enhance antibiofilm efficacy against an in**

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1 **ABSTRACT**

2   Chronic wound management is extremely challenging because of the persistence of biofilm-forming

3 pathogens, such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*, which are the prevailing bacterial

4 species that co-infect chronic wounds. Phage therapy has gained an increased interest to treat biofilm-

5 associated infections, namely when combined with antibiotics. Here, we tested the effect of gentamicin as a co-

6 adjuvant of phages in a dual species-biofilm wound model formed on artificial dermis. The biofilm-killing capacity

7 of the tested treatments was significantly increased when phages were combined with gentamicin and applied

8 multiple times as multiple dose (three doses, every 8 h). Our results suggest that gentamycin is an effective

9 adjuvant of phage therapy particularly when applied simultaneously with phages and in three consecutive doses.

10 The multiple and simultaneous dose treatment seems to be essential to avoid bacterial resistance development

11 to each of the antimicrobial agents.

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INTRODUCTION

Biofilm formation in wounds is considered a major barrier to successful treatments and contributes to the high global cost of chronic wound management [1]. It leads to impaired epithelialization, and microorganisms embedded in these biofilms show reduced susceptibility to antimicrobial agents [2], delaying the healing process [3]. *Pseudomonas aeruginosa* and *Staphylococcus aureus* are the most common species in chronic wounds [4,5]. These pathogens coexist in multi-species biofilms, and their association can result in higher virulence and increased tolerance to antimicrobial agents [6,7]. Phage therapy is a promising approach to tackle infectious diseases [8], However, several studies have raised concerns about phage therapy directed against biofilm-related infections [9], particularly due to the fast emergence of phage resistance [10]. Therefore, there has been an increased interest in using antibiotics as adjuvants of phage-therapy [11]. Gentamicin (GEN) is an aminoglycoside antibiotic that can be used for topical application to treat chronic wounds [12]. Recent clinical studies reveal that topical GEN application reduces the duration of wound healing [13], however, treatments should be limited in duration due to concerns about antibiotic resistance [14].

We have previously shown that the sequential combination of a *Pseudomonas*-specific phage EPA1 and GEN resulted in *P. aeruginosa* eradication in biofilms formed in standard laboratory conditions [15]. However, it is generally recognized that standard laboratory conditions do not always accurately reflect the infectious microenvironment, and the use of model systems that more closely resemble the *in vivo* situation is recommended [16].

In the present study, we designed new combined phage-antibiotic therapy protocols and application strategies, using phages targeting both *P. aeruginosa* and *S. aureus* with the combination of GEN as an adjuvant of phage therapy, in an *in vitro* artificial wound model.

RESULTS

Isolation and characterization of a new S. aureus infecting phage SAFA

A new *S. aureus* infecting virus, designated phage SAFA, was isolated from a sewage plant in Braga, Portugal. This phage has an icosahedral head that is 95 nm in diameter, and a contractile tail of approximately 232 × 23 nm in diameter, resembling the morphology of a myovirus (Figure S1). Phage SAFA could propagate on 13 out of 20 *S. aureus* strains investigated (65 %) with moderate to high Efficiency of Plating (EOP) (Table S1). This phage has a latent period of 25 min, and an average burst size of 64 progeny phages per infected cell (Figure S2).

Phage SAFA has a linear double-stranded DNA genome of 148,740 bp in size, and comparative genomics show that SAFA is very similar to many other staphylococcal phages of the *Kayvirus* genus. SAFA is presumably virulent and does not encode any genes associated with lysogeny or virulence. This suggests that SAFA is potentially safe for therapeutic purposes.

65 *Establishing dual-species biofilm on the artificial dermis*

66   To assess the anti-biofilm activity of the antimicrobials (phages and GEN), dual-species biofilms of *P.*

67 *aeruginosa* and *S. aureus* were formed in an *in vitro* wound model containing an artificial dermis (AD) (Figure

68 1A). After 24 h, biofilm populations consisted of 1.13×10^9 CFU/mL of *P. aeruginosa* and 2.43×10^8 CFU/mL of *S.*

69 *aureus* (Figure 2) [17–19]. Images of the colonized wound model show visible bacterial colonization on the upper

70 part of the dermis with a darkened colour change of growth medium after 24 h of biofilm formation (Figure 1B).

71 When the incubation time was extended to 48 h, an additional colour change in the medium and an increase in

72 surface colonization were observed (Figure 1C), concurrently, dermal fragmentation was evident (Figure 1D);

73 however, this phenomenon was not present in simultaneous treatments (SIM) of AD samples (Figure 1E).

74 *Single-dose administration of sequential phages-antibiotic combination showed bacterial killing in dual-*

75 *species biofilm*

76   The activity of phage EPA1, phage SAFA, and GEN alone or in combinations was tested in the dual-species

77 biofilms. The six-hour treatments resulted in a modest reduction of the biofilm populations (Figure S3). Phage

78 EPA1 treatment reduced the *P. aeruginosa* population by 1.5 log reduction, while phage SAFA did not produce

79 a significant reduction in the *S. aureus* population when compared to the control. The anti-biofilm activity was

80 not altered when phages EPA1 and SAFA were applied simultaneously. Treatment with GEN alone led to a

81 modest reduction of the numbers of *P. aeruginosa* (1.0 log reduction) and *S. aureus* (0.9 log reduction) (Figure

82 S3).

83   In dual-species biofilms, after 24 h of treatments, phage EPA1 alone reduced the *P. aeruginosa*

84 population by 1.5 log reduction, however, phage SAFA did not significantly reduce the *S. aureus* population. The

85 killing activity of the simultaneous application of the two phages (EPA1+SAFA) was similar to their single

86 treatments (Figure 2). The effect of treatment with GEN alone was more pronounced after 24 h compared to 6

87 h treatment and resulted in a population reduction of 3.4 and 1.7 log reduction of *P. aeruginosa* and *S. aureus*,

88 respectively (Figure 2). When EPA1+SAFA and GEN were applied sequentially (first EPA1+SAFA, followed by GEN

89 6 h later), biofilm reductions of 4.8 and 2.3 log reduction were observed for *P. aeruginosa* and *S. aureus*,

90 respectively.

91 *Administration of multiple doses of phage(s) or/and antibiotic significantly reduced both P. aeruginosa and S.*

92 *aureus populations in dual-species biofilms*

93   To develop more efficient treatment strategies, both phages (EPA1+SAFA) and the antibiotic (GEN) were

94 administered in three doses (in different combinations and sequences) every 8 h for a total of 24 h (Table S2).

95 To explore the most efficient combinations, a total of 27 antimicrobial treatment regimens were designed and

96 tested on dual-species biofilms formed in 24-well plates. The most promising combinations (12 out of 27

97 treatments) were selected to test in the *in vitro* wound model (Figure 3, Table S2).

98   The pre-formed dual-species biofilms were initially exposed to either of three treatments for 8 h, i.e.

99 EPA1+SAFA, GEN, and the combination of EPA1+SAFA and GEN. After this first treatment, *P. aeruginosa*

100 populations were reduced by 0.8, 1.1, and 1.3 log reduction, while *S. aureus* populations were reduced by 0.2,

101 0.8, and 1.0 log reduction, respectively (Figure 3).The second dose resulted in additional biofilm reduction for *P. aeruginosa* and *S. aureus*, the total biofilm reductions at this stage ranged from 1.1 to 5.0 log reduction for *P. aeruginosa* and from 1.6 to 6.8 log reduction for *S. aureus* (Figure 3). The highest reduction for both species was observed when treatment with EPA1+SAFA was followed by GEN treatment, while the lowest reduction was observed when treatment with EPA1+SAFA was followed by another EPA1+SAFA treatment (Figure 3). The most pronounced reduction was obtained following multiple doses of EPA1+SAFA+GEN (SIM), with a 6.2 log reduction for *P. aeruginosa* and 5.7 log reduction for *S. aureus* (Figure 3). The combinations EPA1+SAFA/SIM/GEN, SIM/GEN/SIM, and SIM/SIM/GEN also led to more than 5 log reduction for both bacterial species (Figure 3). Some treatment regimens resulted in biofilm regrowth, most probably as a result development of resistance. This is particularly relevant in the case of multiple dose administration of the antibiotic and the phages alone.

Discussion

113 Increasing evidence suggests that phages are useful in the treatment of wound-associated infections, and phage therapy can be highly effective when administered appropriately, as demonstrated in standard laboratory conditions, as well as *in vivo* animal models and even in human patients (reviewed in [20,21]). Although treatments with single phages or phage cocktails have shown promising results [22–25], recent studies have suggested that the use of antibiotics as phage adjuvants are more effective against biofilm-related infections [26–29].

119 In the present study, we tested the anti-biofilm activity of two phages targeting *P. aeruginosa* and *S. aureus* alone and combined with gentamicin in different treatment regimens in an *in vitro* dual-species biofilm model of chronic wound infection [30,31] and found that the sequential treatment with phages (EPA1+SAFA) and antibiotic (GEN) led to significantly higher biofilm reductions than those obtained with single treatments.

123 The antimicrobial agents were also applied in multiple dose regimens with different combination strategies. The obtained reductions ranged from 1.9 to 5.2 log, suggesting that the order and frequency of application influence the treatment outcome.

126 The application of GEN as the first dose treatment, followed by phages usually led to low reductions. Phages rely on host mechanisms to facilitate their replication and antibiotics may adversely impact these essential mechanisms. For example, antibiotics that target the protein synthesis can alter the outcome of bacteria–phage interactions by interfering with the production of phage-encoded counter-defense proteins [32]. GEN targets protein synthesis and inhibits phage replication [15], therefore phage efficacy is compromised when it is added first. However, when GEN is applied simultaneously with phages, the rapid killing activity of phages can probably overcome the antagonistic effect of GEN on the activity of the phage against the biofilm, at least in the initial stages after application. Furthermore, the application of both antimicrobials in multiple doses can lead to a complementary effect in which phages target preferentially antibiotic resistant bacteria, and antibiotics kill phage resistant cells.

136   The use of single antimicrobial agents in consecutive doses, be it phages or the antibiotic, was very

137   ineffective. In fact, when GEN was used in three consecutive treatments, a regrowth in the biofilm population

138   was observed (Figure 3). The same was observed for consecutive applications of phages (Figure 3). If phages do

139   not manage to kill a sufficient number of bacteria quickly, this may result in the proliferation of bacteriophage-

140   insensitive mutants (BIMs) [33,34]. Bacteria possess or can quickly develop different mechanisms to escape viral

141   infections, such as alteration or loss of receptors [10], secretion of substances that prevent phage adhesion to

142   the bacterial pathogen like outer membrane vesicles [35], blocking phage DNA injection, and inhibition of phage

143   replication and release [36]. Nonetheless, phages and antibiotics use different mechanisms of action [37]. This

144   feature can make their combination very effective against biofilms. When phages and antibiotics are used

145   simultaneously or sequentially, bacteria have a low chance of evolving resistance against both at the same time

146   [38].

147   The possible mechanisms involved in the biofilm treatment with multiple doses of antibiotics or phages

148   alone and in combination are summarized in Figure 4. Here we hypothesise that in a multi-dose treatment with

149   simultaneous application of phages and antibiotics, the bacterial population is exposed to multiple stresses at

150   the same time and is unlikely to be able to recover or evolve resistance.

151   Our work shows that, the *in vitro* wound model can be used to test the efficacy of phages against chronic

152   wounds and that results obtained in this *in vivo*-like model may differ from those obtained in other *in vitro*

153   models. This observation reiterates the importance of using relevant models that capture important aspects of

154   host physiology and the infectious microenvironment when evaluating innovative anti-biofilm strategies [16,39].

155   Our data indicate that gentamicin is an effective adjuvant of phage therapy, particularly when applied

156   simultaneously with phages in a multiple-dose treatment, to minimise the effect of resistance mechanisms.

157   Moreover, our results suggest that antibiotics can be effective adjuvants for phage therapy against chronic

158   wound infections. However, the order and frequency of the applied antimicrobials (phages or antibiotics) is

159   important for an optimal treatment outcome.

160   **MATERIAL and METHODS**

161   ***Bacterial strains and culture conditions***

162   The bacterial strains *P. aeruginosa* PAO1 (DSM22644) and *S. aureus* ATCC 25923 are reference strains

163   obtained from the German Collection of Microorganisms and Cell Cultures and American Type Culture

164   Collection, respectively. Seventeen additional clinical *S. aureus* isolates, and two culture collection strains were

165   kindly provided by the LPhage Laboratory in CEB (University of Minho, Braga, Portugal, Table S1) and were also

166   used in this study. All strains were grown in Tryptic Soy Broth (TSB, VWR Chemicals), Tryptic Soy Agar (TSA; VWR

167   Chemicals), or in TSA soft overlays (TSB with 0.6 % agar) at 37 °C. *Pseudomonas* isolation agar (PSA; Becton,

168   Dickinson) was used to enumerate *P. aeruginosa* cells, and mannitol salt agar (MSA; Neogene) was used to

169   enumerate *S. aureus* cells in dual-species biofilms.

170   ***Phage isolation and production***

171   Phage SAFA was isolated from effluent samples of raw sewage obtained in a waste-water treatment plant

172   in Braga, Portugal, using the enrichment protocol described before [40]. Briefly, 100 mL of the effluent was

173   mixed with 100 mL of double-strength TSB and with 10 μL of each of the exponentially grown *S. aureus* strains

174   (Table S1) and incubated at 37 °C, at 120 rpm (BIOSAN ES-20/60, Riga, Latvia) overnight. Suspensions were

175   further centrifuged (15 min, 9000× g, 4 °C), and the supernatants were filtered through a 0.22 μm

176   polyethersulfone (PES) membrane (ThermoFisher Scientific, Massachusetts, USA). The presence of phages was

177   confirmed by performing spot assays on bacterial lawns. The prepared plates were further incubated overnight

178   at 37 °C, and the presence of inhibition halos was observed. When phage plaques appeared, successive rounds

179   of single plaque purification were carried out until purified plaques were observed, reflected by a single plaque

180   morphology.

181   The purified phage was produced by using the double agar layer method, as described before [33]. Briefly,

182   100 μL of a phage suspension at 10^8 PFU/mL were spread on *P. aeruginosa* PAO1 or *S. aureus* ATCC 25923 lawns

183   for overnight incubation at 37 °C. If full lysis was observed, plates were further incubated at 4 °C for 6 h at 120

184   rpm (BIOSAN PSU-10i), with 2 mL of SM Buffer (100 mM NaCl, 8 mM MgSO4, 50 mM Tris/HCl, pH 7.5) to

185   resuspend the phage particles. The liquid phase was collected and centrifuged (15 min, 9000× g, 4 °C), and the

186   supernatants were filtered through a 0.22 μm PES membrane. Purified phages were stored at 4 °C for further

187   use.

188   ***Electron microscopy***

189   Phage suspension was sedimented by centrifugation (25,000× g, 60 min, 4°C) using a ScanSpeed 1730R

190   centrifuge (Labogene, Lillerød, Denmark). The pellet was further washed in tap water by repeating the

191   centrifugation step. Subsequently, phage suspension was deposited on copper grids with a carbon-coated

192   Formvar carbon film on a 200 square mesh nickel grid, stained with 2 % uranyl acetate (pH 4.0) and examined

193   using a Jeol JEM 1400 transmission electron microscope (TEM) (Tokyo, Japan) [15].

194   ***Phage host range and efficiency of plating determination phage***

195   The host range of SAFA was determined with the spot test method [15] using the strains listed in Table

196   S1. Briefly, 100 μL of each overnight bacterial culture was added to 5 mL of TSB-soft agar and poured onto TSB

197   agar plates. 10 μL of serial 10-fold dilutions of the phage suspension was spotted on the bacterial lawns and

198   plates were incubated at 37 °C overnight. The efficiency of plating (EOP) was calculated by dividing the titer of

199   the phage (PFU/mL) obtained for each isolate by the titer determined in the propagating bacteria. EOP was

200   recorded as high (>10 %), moderate (0.01–9 %) or low (<0.01 %) [15].

201   ***Genome sequencing and in silico analysis***

202   The DNA of the *Staphylococcus* phage SAFA was extracted according to the standard phenol-chloroform-

203   isoamyl alcohol methods, as described elsewhere [41]. The DNA sample was used for library construction using

204   the Illumina Nextera XT library preparation kit. The generated DNA libraries were sequenced in the Illumina

205 MiSeq platform, using 250bp paired-end sequencing reads. Next, reads were assembled *de novo* with Geneious

206 R9, and manually inspected. SAFA genome was annotated using RAST [42]. The function of proteins was

207 manually inspected using BLASTP. tRNAscan-SE was used to predict tRNAs [43]. For comparative studies,

208 pairwise alignments were made using BLASTN or BLASTP.

Biofilm formation in microtiter plates

210 For the *in vitro* assessment of antimicrobial efficacy, 48 h old dual-species biofilm were formed in 24-

211 polystyrene well plates (Orange Scientific, Braine-l'Alleud, Belgium) as previously described [15]. Briefly, to

212 initiate biofilm formation, one bacterial colony (*P. aeruginosa* or *S. aureus*) was incubated in TSB overnight in an

213 orbital shaker (120 rpm, BIOSAN ES-20/60) at 37°C. For establishing mono-species biofilms, 10 µL of the starter

214 culture was transferred into 24-well plates containing 990µL of fresh TSB media. The plates were incubated for

215 24 h in an orbital shaker incubator (120 rpm, BIOSAN ES-20/60) at 37°C. After 24 h, half of the growth medium

216 (500µL TSB, 1:1, v:v) was replaced with fresh TSB and plates were incubated for an additional 24 h. For dual-

217 species biofilms, *S. aureus* cells were inoculated prior to *P. aeruginosa* addition. Thus, biofilms were initiated

218 with 10 µL of the overnight culture of *S. aureus* (~10^8 CFU/mL) in 990µL TSB and incubated for 24 h in an orbital

219 shaker (120 rpm) at 37°C. After that, half of the growth medium (500µL TSB, 1:1, v:v) was replaced with TSB

220 including 10 µL of the starter culture of *P. aeruginosa* (~10^8 CFU/mL, 1:49, v/v) and incubated for additional 24

221 h. In mono and dual-species biofilms, the supernatant was aspirated, and the wells were washed twice with

222 saline solution (0.9% NaCl (w/v)) to remove planktonic bacteria. Biofilms were scraped of the plate in saline

223 solution (1 mL) using a micropipette tip, and the number of culturable cells was determined using plate counts

224 [43].

Biofilm formation in the in vitro wound model

226 For the wound model, we used the previously prepared two-layer (upper and lower) AD substrate as

227 described elsewhere [33]. Dual-species biofilms were grown on an AD with minor modifications to the previously

228 described chronic wound biofilm model [31]. Briefly, ADs were placed in the 24-well microtiter plate, and 500

229 µL of Bolton Broth with 50% plasma (Sigma–Aldrich) and 5% freeze-thaw laked horse blood was added to the

230 ADs. Then, the same amount of growth medium was added into the wells. Next, 10 µL of the overnight culture

231 of *P. aeruginosa* and *S. aureus* (~10^8 CFU/mL) were spotted simultaneously on the upper part of each AD and

232 incubated at 37 °C overnight.

Biofilm challenge

234 Dual-species biofilms formed on AD were treated with the antimicrobials; alone, in simultaneous

235 (EPA1+SAFA+GEN) or sequential combinations (first EPA1+SAFA and then GEN with 6 h delay) for 24 h. Briefly,

236 10 µL of antimicrobials were added to the AD at final concentration of 4 µg/mL (MIC of GEN for *P. aeruginosa*

237 PAO1) and at MOI of 1 for phages. Plates were incubated at 37 °C for 24 h. Then, treated and untreated (control)

238 ADs were transferred into tubes containing 10 mL saline solution, the sessile cells were removed from the AD

239 by three cycles of vortexing (30 s) and sonication (30 s; Branson 3510; Branson Ultrasonics Corp, Danbury, CT)

240 and the number of CFU/biofilm was determined by plate counting.

241   To develop more efficient treatment strategies, 27 different treatment variables were initially tested on

242   dual-species biofilms formed on 24-well polystyrene plates (Table S2). Briefly, biofilms were washed twice with

243   the saline solution and GEN (at 1x MIC for *P. aeruginosa*, 4 μg/mL) and EPA1+SAFA (at MOI 1) were applied in

244   TSB according to the order as described in Table S2. Following the CFU counting, the most promising variables

245   were selected and tested on dual-species biofilm formed on ADs. The same protocol was applied to treat and

246   enumerate the cells as described above in AD treatment. However, instead of the single-dose treatment, the

247   multiple dose treatments were applied every 8 h for a total of 24 h, and the number of viable cells was

248   enumerated by plate counting.

249   *Statistical analysis*

250   In all the assays, averages and standard deviations were determined based on 3 independent

251   experiments (n = 3) performed in duplicate. The results of the assays were compared using two-way analysis of

252   variance (ANOVA) by applying the Tukey's multiple comparisons tests using Prism 9.0.0 for Windows. Plots were

253   obtained using Prism 9 (GraphPad, La Jolla, CA, USA). Means and standard deviations (SD) were calculated with

254   the software. Differences among conditions were considered statistically significant when $p < 0.001$.

255   *Accession number*

256   SAFA genome was deposited in GenBank database under the accession number OP651044.

CRediT authorship contribution statement:

Competing interests:

The funding agencies had no role in study design, data collection, analyses, the decision to publish, or the preparation of the manuscript.

Ethical Approval:

Not required.

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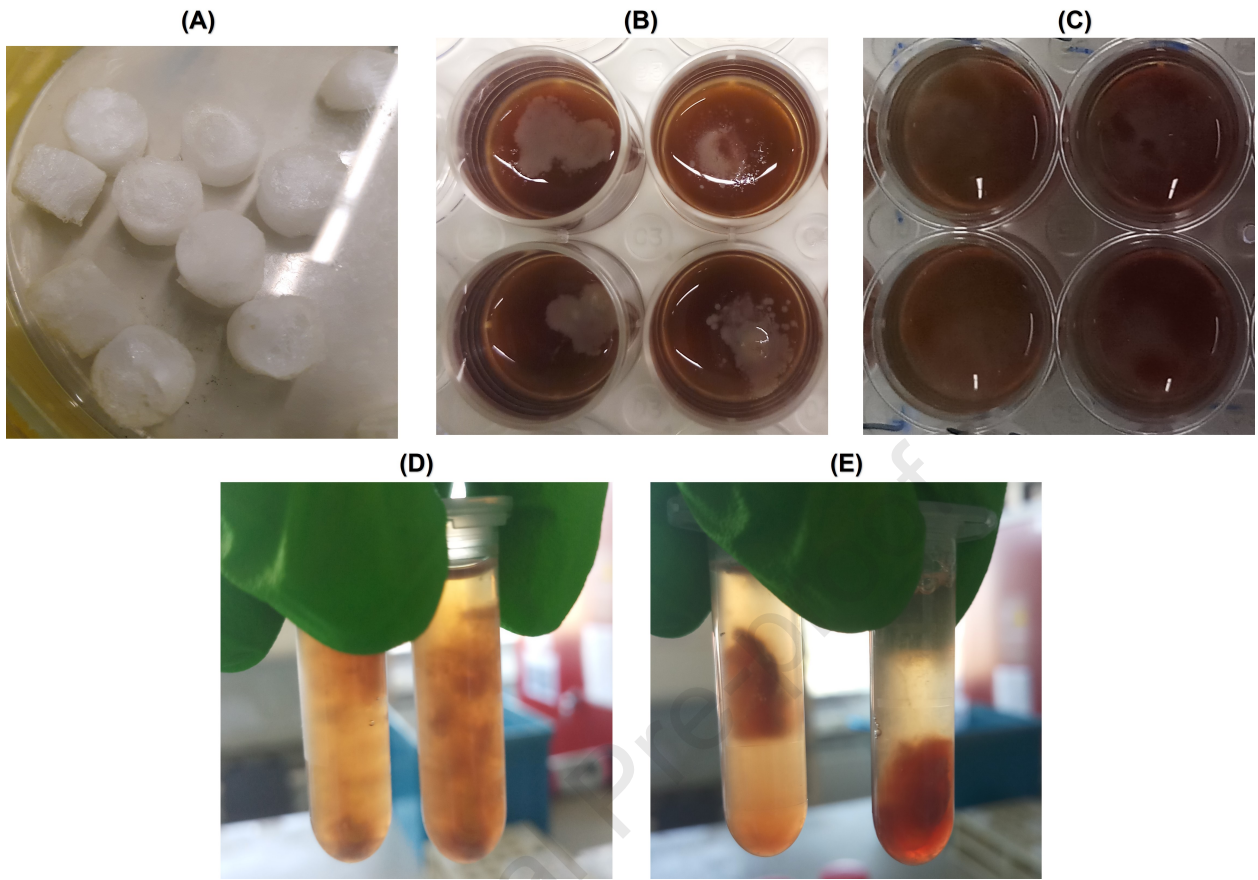414   https://doi.org/10.1093/nar/gkab688.

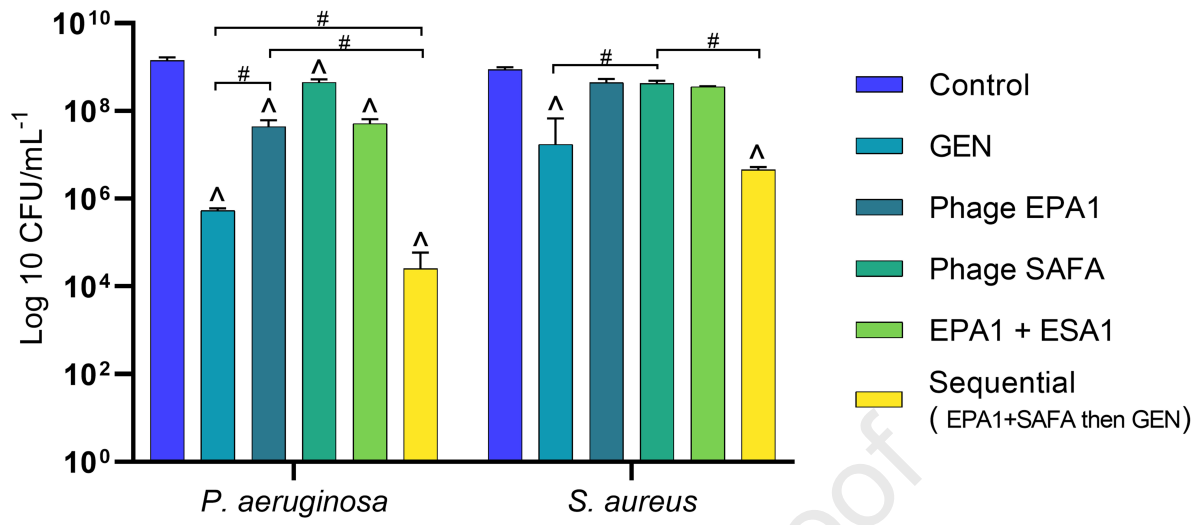415

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Figure Legends

417

418 **Figure 1.** Macroscopic images of wound biofilm model used. (**A**) AD (**B**) *P. aeruginosa* and *S. aureus* infected AD after
419 24 h of biofilm formation. (**C**) *P. aeruginosa* and *S. aureus* infected AD (non-treated control) after 48 h of biofilm formation
420 (**D**) Untreated control (48 h) dermis after being transferred to 2 mL microcentrifuge tubes containing saline solution (**E**)
421 Treated AD (48 h, the treatment details are in section 0) after being transferred to 2 mL microcentrifuge tubes containing
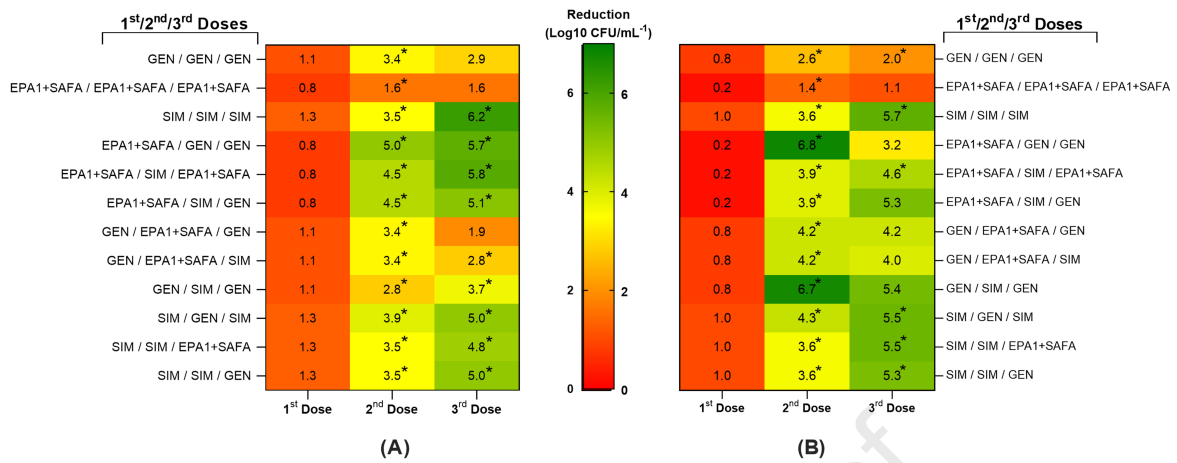422 saline solution.

423 **Figure 2.** The number of *P. aeruginosa* and *S. aureus* CFU recovered after single-dose treatment of 24 h old dual-
424 species biofilms. EPA1+SAFA: phage EPA1 and SAFA were applied simultaneously at MOI of 1. Sequential means that phage
425 EPA1 and SAFA were applied simultaneously at MOI of 1; subsequently GEN was applied (4 µg/mL, i.e. the MIC for *P.*
426 *aeruginosa*) with a 6 h delay. (^) Statistical differences between the control and treated biofilms. (#) Statistical differences
427 between the compared treatment groups. Statistical differences were determined by two-way repeated-measures analysis
428 of variance (ANOVA) with Tukey's multiple comparison tests (p <0.001). Values are the average of three technical repeats in
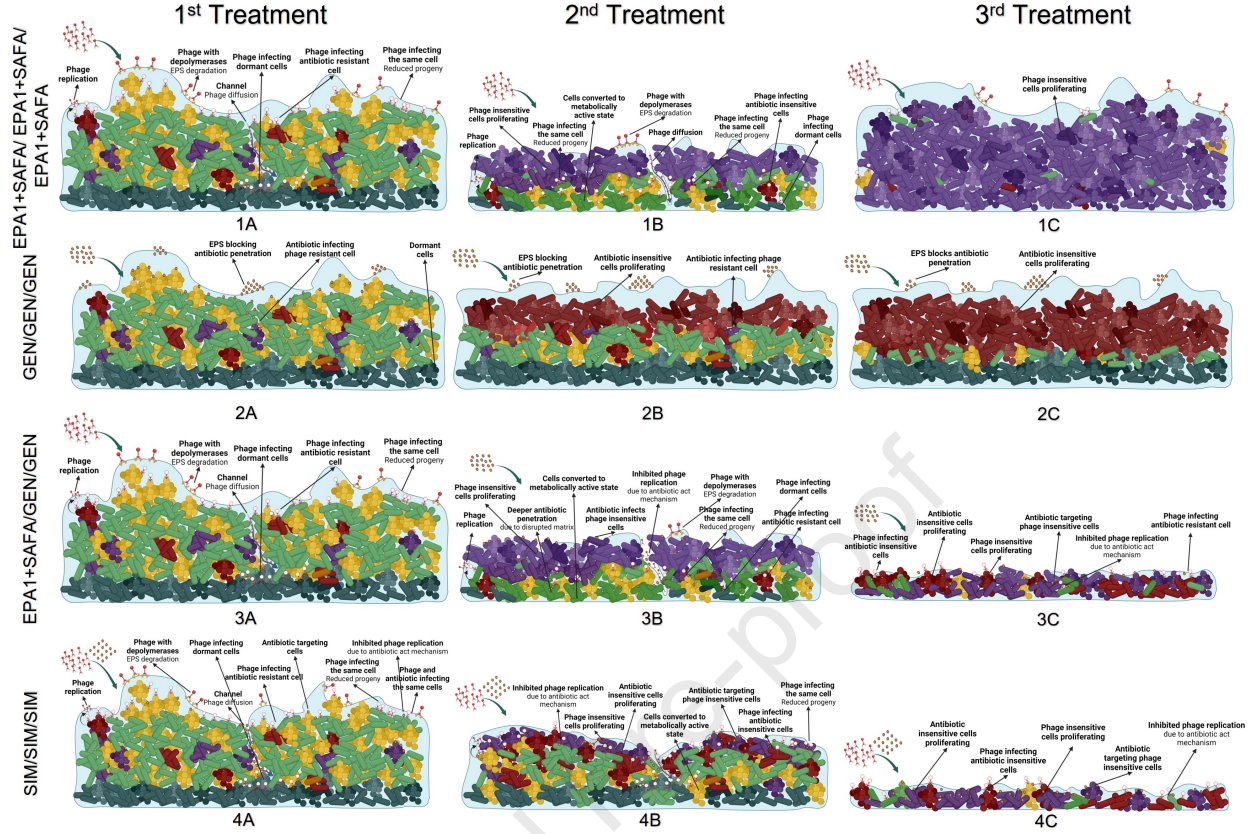429 duplicate, error bars indicate standard deviation.

430 **Figure 3.** Heat map representing the log reduction of (A) *P. aeruginosa* and (B) *S. aureus* in dual-species biofilm after
431 multiple treatments. The middle legend bar indicates the colour change according to log reduction reductions, with log
432 reduction reductions increasing from red to green. first dose, second dose, and third dose indicate the order of treatment.
433 The 24 h old dual-species biofilms were treated for 24 h in total (3 treatments of 8 h). The prefix "SIM" indicates the
434 simultaneous application of phage EPA1, SAFA (at MOI of 1) and GEN (4 µg/mL, i.e. the MIC for *P. aeruginosa)* treatments.
435 (^) Statistical differences between the control and treated biofilms. (*) Statistical differences between the current and
436 previous dose-treated biofilms. Statistical differences were determined by two-way repeated-measures analysis of variance
437 (ANOVA) with Tukey's multiple comparison tests (p <0.001). Values are the average of three technical repeats in duplicate.

438 **Figure 4.** Schematic presentation of antimicrobial treatments. The row A represents first dose treatment; row B
439 represents second dose treatment; row C represents third dose treatment. EPA1+SAFA/EPA1+SAFA/EPA1+SAFA (1A, 1B, 1C)
440 represent multiple dose treatment regimens of phages at a MOI of 1. In the first dose treatment, phages disrupt and
441 penetrate the biofilm matrix and infect the bacteria cells, helping the penetration of larger molecules such as nutrients. The
442 additional second and third doses of phage treatment continue to target phage-sensitive cells. However, BIM cells proliferate
443 and dominate the biofilm population. GEN/GEN/GEN (2A, 2B, 2C) represent 3 multiple dose treatment regimens of GEN at
444 MIC for P. aeruginosa, 4ug/mL. In the first dose treatment, GEN infects sensitive cells in the upper layer of biofilm. However,
445 single GEN treatment results in GEN-insensitive cell proliferation. The evolved bacteria can proliferate and dominate the
446 biofilm population, rendering the second and third antibiotic treatments ineffective. EPA1+SAFA/GEN/GEN (3A, 3B, 3C)
447 represent multiple dose treatment regimens of antimicrobials: EPA1+SAFA, GEN, and GEN, respectively. In the first dose of
448 treatment, phages disrupt and penetrate the biofilm matrix and infect the bacteria cells. it helps the penetration of larger
449 molecules such as nutrients and antibiotics. However, initial phage treatment induces BIM cell proliferation. The following
450 GEN treatments targets proliferating BIMs and GEN-sensitive cells. Nonetheless, GEN treatments can inhibit phage
451 replication and result in reduced phage efficiency. SIM/SIM/SIM (4A, 4B, 4C) represent multiple dose treatment regimens of
452 the simultaneous combination of EPA1+SAFA and GEN at MOI of 1 and MIC value (4ug/mL, i.e. the MIC for P. aeruginosa).
453 Phages disrupt and penetrate the biofilm matrix and infect the bacteria cells. it helps the penetration of larger molecules
454 such as nutrients and antibiotics. Phages and antibiotics use different mechanisms of action. Following the first dose of
455 treatment, the proliferating phage- or GEN-insensitive cells are targeted by another antimicrobial agent, which is supplied
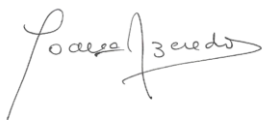456 to the environment by the second and third doses of treatment.

(A)

(B)

(C)

(D)

(E)

1st/2nd/3rd Doses

	1st Dose	2nd Dose	3rd Dose
GEN / GEN / GEN	1.1	3.4*	2.9
EPA1+SAFA / EPA1+SAFA / EPA1+SAFA	0.8	1.6*	1.6
SIM / SIM / SIM	1.3	3.5*	6.2*
EPA1+SAFA / GEN / GEN	0.8	5.0*	5.7*
EPA1+SAFA / SIM / EPA1+SAFA	0.8	4.5*	5.8*
EPA1+SAFA / SIM / GEN	0.8	4.5*	5.1*
GEN / EPA1+SAFA / GEN	1.1	3.4*	1.9
GEN / EPA1+SAFA / SIM	1.1	3.4*	2.8*
GEN / SIM / GEN	1.1	2.8*	3.7*
SIM / GEN / SIM	1.3	3.9*	5.0*
SIM / SIM / EPA1+SAFA	1.3	3.5*	4.8*
SIM / SIM / GEN	1.3	3.5*	5.0*

Reduction (Log10 CFU/mL^{-1})

(A)

1st/2nd/3rd Doses

	1st Dose	2nd Dose	3rd Dose
GEN / GEN / GEN	0.8	2.6*	2.0*
EPA1+SAFA / EPA1+SAFA / EPA1+SAFA	0.2	1.4*	1.1
SIM / SIM / SIM	1.0	3.6*	5.7*
EPA1+SAFA / GEN / GEN	0.2	6.8*	3.2
EPA1+SAFA / SIM / EPA1+SAFA	0.2	3.9*	4.6*
EPA1+SAFA / SIM / GEN	0.2	3.9*	5.3
GEN / EPA1+SAFA / GEN	0.8	4.2*	4.2
GEN / EPA1+SAFA / SIM	0.8	4.2*	4.0
GEN / SIM / GEN	0.8	6.7*	5.4
SIM / GEN / SIM	1.0	4.3*	5.5*
SIM / SIM / EPA1+SAFA	1.0	3.6*	5.5*
SIM / SIM / GEN	1.0	3.6*	5.3*

(B)

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

29th January 2023

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On behalf of all authors