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1 Targeting antibiotic resistant bacteria with
2 phages reduces bacterial density in an
3 insect host

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11 insect model, phage therapy

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18 ABSTRACT

19 Phage therapy is attracting growing interest among clinicians as antibiotic resistance
20 continues becoming harder to control. However, clinical trials and animal model
21 studies on bacteriophage treatment are still scarce and results on the efficacy vary.
22 Recent research suggests that using traditional antimicrobials in concert with phage
23 could have desirable synergistic effects that hinder the evolution of resistance. Here,
24 we present a novel insect gut model to study phage-antibiotic interaction in a system
25 where antibiotic resistance initially exists in very low frequency and phage
26 specifically targets the resistance bearing cells. We demonstrate that while phage
27 therapy could not reduce the frequency of target bacteria in the population during
28 positive selection by antibiotics, it alleviated the antibiotic induced blooming by
29 lowering the overall load of resistant cells. The highly structured gut environment
30 had pharmacokinetic effects on both phage and antibiotic dynamics compared to in
31 vitro: antibiotics did not reduce the overall amount of bacteria, demonstrating a
32 simple turnover of gut flora from non-resistant to resistant population with little
33 cost. The results imply moderate potential for using phage as an aid to target
34 antibiotic resistant gut infections, and question the usefulness of in vitro inferences.

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40 INTRODUCTION

41 Problems arising from antibiotic resistant infections are set to increase worldwide.
42 As a consequence, bacteriophages (bacteria-specific viruses) have started to attract
43 serious consideration as antimicrobial agents after this approach was largely
44 forgotten for decades in the Western world [1, 2]. However, their efficacy as
45 therapeutic agents remains controversial [3]. Some of the in vivo work promises
46 therapeutic potential in insect [4], mouse [5], and human infections [6], and in vitro
47 experiments show overwhelming evidence of phages controlling bacterial population
48 sizes without adverse effects on non-target bacteria [7]. Despite this, the few
49 existing modern and properly controlled medical trials report varying success [3, 6,
50 8]. Key limitations of phage therapy include high specificity, ease at which bacteria
51 can evolve resistance, and localised activity in the body.

52 It has been argued that phages may be of particular therapeutic value when
53 combined with antibiotics, by constraining the emergence and spread of antibiotic
54 resistance [9]. A good example of recent success in combination treatment comes
55 from a difficult chronic *Pseudomonas aeruginosa* infection in human aortic graft [10].
56 Theoretically, phages may limit antibiotic resistance during treatment because of
57 reduction in population size and synergistic costs of resistance [9]. Moreover, with
58 cases where antibiotic resistance is carried on plasmids, phages can be used to
59 directly target the plasmid carrying cells [11]. However, this latter approach has only
60 been investigated in vitro [11, 12], which ignores a plethora of selection pressures
61 towards both the host bacteria and the phage, such as host immune system, spatial

62 structures within the tissues, nutrient availability and presence of native microbial
63 flora [13].

64 Here, we studied the effects of antibiotic and phage treatment to bacterial
65 load and frequency of resistant cells in a gnotobiotic insect gut model system, where
66 phages target the bacteria harbouring antibiotic resistance plasmids. We compare
67 these findings to in vitro context to assess the usefulness of inferring in vivo
68 dynamics from in vitro studies. A full factorial setup of antibiotics and phage were
69 orally administered to cabbage looper (*Trichoplusia ni*) larvae harbouring
70 *Enterobacter cloacae* gut bacteria and a low initial frequency of antibiotic resistance
71 plasmid. We show that targeting the tetracycline resistant cells with phage could not
72 prevent the increase in resistance frequency in the presence of antibiotics. However,
73 phage reduced the bacterial population size when plasmid was driven to high
74 frequency with antibiotic selection.

75 MATERIALS AND METHODS

76 Although it's been argued that Lepidopterans lack a resident microbiome [14],
77 *Enterobacter cloacae* forms a persistent gut association with Lepidopteran larvae
78 [15] (e.g. *Plutella xylostella*, *Mamestra brassicae* and *Trichoplusia ni*) after oral
79 inoculation. The strain was isolated from *P. xylostella* in the insectary of Department
80 of Zoology, University of Oxford. Two spontaneous antibiotic resistant mutants,
81 ANC C2 (rif^R) and 11.1B (strep^R + nal^R) were used in this experiment. The IncP-
82 type plasmid RP4, coding for tetracycline resistance, is usually conjugative [16],
83 and the lytic, plasmid dependent tectivirus PRD1 specifically infects RP4-bearing
84 cells by recognising the bacterial sex-apparatus [17]. However, in this system the

85 plasmid is essentially non-conjugative and functions as a carrier of tetracycline
86 resistance and phage receptor genes. The mechanistic basis for the poor RP4
87 conjugation in these *E. cloacae* strains is unclear. Given that the phage is still
88 infective, it could be that ANC C2 rifampicin-resistant mutant was an impotent
89 recipient for the plasmid rather than that 11.1B(RP4) lacked the ability to express
90 pili. Although rifampicin resistance mutations are most often polymerase related,
91 known mechanisms in Enterobacteriaceae include elongated or abundant outer
92 membrane LPS-chains [18] that could theoretically interfere with conjugation (but,
93 see: [19]).

94 The in vitro experiment used 200 μ l cultures on a 96-well plate with LB and
95 0.1% starting frequency of plasmid. 24 replicate populations were subjected to full
96 factorial antibiotic (AB) and phage (P) treatment: AB- P-, AB- P+, AB+ P-, and AB+
97 P+, respectively. The antibiotic treatments were set to 12 μ g/mL tetracycline and
98 phage starting density to 5×10^7 phage particles. The growth in optical density
99 (600nm) was recorded at 24 h with a spectrophotometer in 30°C. Plasmid frequency
100 and transconjugants were sampled from a subset (N=44) of populations at 24 h by
101 selective plating.

102 The *T.ni* were maintained as follows: The adults, fed with 100mM sucrose
103 solution, were let to mate and lay eggs on paper strips in flight chambers. The eggs
104 were placed in autoclaved, single-filter vented, 305 x 203 x 100mm Genesis™
105 containers for surgical instruments to maintain sterile conditions while allowing gas
106 exchange. Hatched larvae were let to feed on Hoffman diet (288 g wheatgerm, 132 g
107 caesin, 117 g sucrose, 70 g agar, 57 g dried brewers yeast, 37.5 g Wesson's salts, 6 g

108 sorbic acid, 3.75 g cholesterol, 3.75 g methyl-4-hydroxybenzoate and 2 g Vanderzant
109 vitamin mixture in 2750 ml water) [20]. The liquid medium was poured into a sterile
110 steel mould and solidified to diet cubes with approximately 25mm edges. The cubes
111 were placed on Petri dish covers inside the containers. All rearing and in vivo
112 bacterial work was carried out in 25 °C. For population maintenance, 50-60 pupae
113 were removed from their cocoons per flight chamber and let to emerge and mate to
114 start a new generation of hosts.

115 For inoculating the larval gut with *E. cloacae*, clones ANC C2 and 11.1B
116 bearing RP4 were grown over night in LB (30 °C, 180 rpm shaking), the latter diluted
117 1:10 with 0.85 % NaCl, and then mixed together in a 50:1 ratio. The frequency of the
118 plasmid bearing cells was counted by dilution plating as 0.072% and bacterial density
119 approximately 1.2×10^8 CFU. Diet cubes were dipped in this solution, dried for 5 min,
120 and 3rd instar larvae were allowed to feed on this bacterial diet for 30 h. The larvae
121 were then subjected to the AB- P-, AB- P+, AB+ P-, and AB+ P+ treatments.
122 Tetracycline (200 µg/mL final concentration) was added directly to the autoclaved
123 diet mix and 30 µl of 10^{10} pfu/mL filtered phage lysate was pipetted onto each face
124 of the phage treatment diet cubes. The experiment was carried out in two
125 independent batches (final N=156 larvae). After 96 h, the larvae were surface
126 sterilised with 70% alcohol and homogenised in 500 µl 0.85% NaCl with Qiagen
127 Tissuelyser II™. The homogenate was serially diluted and plated on 12 µg/mL
128 tetracycline, 12 µg/mL tetracycline + 100 µg/mL rifampicin, and LB plates to calculate
129 the frequencies of plasmid carriers, transconjugants, and total density of bacteria. 24
130 bacterial clones from the phage treatment serial dilutions on tetracycline were

131 tested for resistance against the ancestral phage with a phage plaque overlay assay.
132 Phage survival in the gut was confirmed by overlaying homogenised larval faecal
133 samples on ancestral 11.1B(RP4) lawn on soft agar and observing plaque formation
134 (N=24).

135 Treatment effects on densities and frequencies in both experiments were
136 analysed with SPSS statistics 21.0 with antibiotics, phage, and antibiotics*phage
137 interaction in ANOVA-GLM model. Post-hoc multiple comparisons were Bonferroni
138 corrected.

139 RESULTS

140 In vitro, antibiotics and phage had synergistic effects on the total density of bacteria
141 at 24h (AB: $F_{1, 96}=3528.0$, $p<0.001$, P: $F_{1, 96}=73.7$, $p<0.001$, AB*P: $F_{1, 96}=97.0$, $p<0.001$).
142 Multiple comparisons showed that there was no phage effect in absence of
143 antibiotics, but other pairwise differences were highly significant ($p<0.001$, Fig. 1. a).
144 Thus, single treatment allowed compensatory growth of the non-target strain, but
145 this effect was reduced for the plasmid carriers in the presence of antibiotics.
146 Contrary to our expectations and previous studies with the same plasmid and phage
147 in *E. coli* [12], the plasmid went to near fixation in the presence of antibiotics ($F_{1, 44}=8864.4$, $p<0.001$) regardless of the phage (P and AB*P: $p>0.05$). This is particularly
148 surprising, as unlike in *E. coli*, plasmid RP4 had very low rates of conjugation in *E.*
149 *cloacae*: transconjugants were present in only one of the 44 sampled in vitro
150 populations (AB+P- treatment at a very low frequency), while the phage remained
151 infective. The cost of plasmid carriage in the absence of antibiotics manifested itself
152 as complete plasmid extinction (at least below the detection threshold of <67

154 cfu/mL) in all sampled populations (Fig 1. b). This was the case even though RP4 has
155 been reported to be of low cost and rapidly evolve towards no cost through
156 chromosomal compensatory mutations [21].

157 In vivo patterns of plasmid dynamics were mainly similar to that observed in
158 vitro, but the effects smaller. The non-targeted strain could fully compensate for the
159 reduction in density of the competing strain (AB: $F_{1, 156}=0.1$, $p=0.77$), and phage had
160 no main effect on total bacterial load (P: $F_{1, 156}=3.0$, $p=0.085$). Phage however
161 lowered bacterial density in the presence of antibiotics (interaction: $F_{1, 156}=6.7$,
162 $p=0.011$), demonstrating that the treatment synergism was maintained in vivo (post
163 hoc AB+ P- and AB+ P+, $p=0.020$, Fig. 2. a). Phage-susceptibility screening of evolved
164 clones showed no emergence of resistance. Plasmid increased in frequency in all
165 treatments, but tetracycline selection drove the majority of the population to be
166 plasmid carriers with no effects of phage addition (effects on plasmid frequency, AB:
167 $F_{1, 156}=845.0$, $p<0.001$; P: $F_{1, 156}=0.6$, $p=0.43$; AB*P: $F_{1, 156}=0.4$, $p=0.55$ (Fig. 2. b). Thus,
168 phage therapy could not restrict the spread of antibiotic resistance in the *T. ni* gut.

169 DISCUSSION

170 Here, we investigated the effect of plasmid-targeting phage on the spread of
171 antibiotic resistance plasmid in vitro and in an insect gut model. Different selective
172 environment in the gut made treatment effects less pronounced compared to the
173 liquid culture: for example, the increased complexity in spatial structures may have
174 protected the plasmid from extinction in the absence of antibiotics, and thus allowed
175 more non-resistant bacteria to coexist in the presence of tetracycline. This happened
176 even though the antibiotic concentration in the diet was over 16 times higher than in

177 the liquid culture and the experiment length 4 times longer, allowing more time for
178 potential fixation and/or extinction to happen. Phage driven reduction in the
179 bacterial population size in the presence of tetracycline was also more moderate in
180 vivo, but it is also notable that no detectable phage resistance emerged in the
181 sampled populations. Surprisingly, antibiotics did not reduce the overall bacterial
182 density in the gut, but rather replaced non-resistant cells with resistant ones while
183 population size remained the same. In other words, antibiotics increased the
184 relative fitness of the resistant strain without reducing the absolute fitness [22]. This
185 was in contrast with liquid culture where the plasmid went even closer to fixation in
186 the presence of antibiotics, but resulted in lower total bacterial density, which
187 suggests a greater growth cost associated with antibiotic resistance in vitro. We
188 cannot attribute the inversion of fitness effects between in vivo and in vitro
189 conditions (increase in plasmid frequency in vivo, and extinction in vitro in absence
190 of antibiotics) directly to the plasmid, because it could also be due to the differences
191 in the mutant strains. The result still highlights the limitations of making inferences
192 from test tube dynamics.

193 In the absence of antibiotics neither in vitro nor in vivo regimes showed a
194 phage effect on the bacterial population size, which was not surprising because of
195 very low initial frequency of phage-susceptible (i.e. plasmid bearing) cells. Most
196 importantly, when antibiotic selection increased the frequency of phage-susceptible
197 cells by spreading antibiotic resistance, phage decreased the total density of
198 bacteria. This effect was bigger in vitro, but also clearly visible in the insect gut. The
199 gut lumen is a favourable environment for bacterial biofilm formation [23], which

200 can offer non-resistant cells spatial protection from antibiotics [24] and/or from
201 phage [25]. On the other hand, it could be that the observed phage efficacy to
202 reduce bacterial load in vivo is partly due to their known ability to eradicate biofilms
203 [26, 27], which are essential for bacterial persistence in the insect gut [23].

204 One potential epidemiological implication of viable phages in the faeces is
205 that the viral therapeutic agent could restrict ongoing pathogen transmission or be
206 transmitted with the target pathogen [28]. The model system presented in this study
207 allows the manipulation of structure and connectivity of the population of the
208 treated organism and would be an interesting future endeavour towards
209 epidemiological effects of phage therapy. Our findings have implications for
210 therapeutic use of phage, especially considering synergistic evolutionary effects with
211 antibiotics [9]. However, antibiotics overwhelmed the selection imposed by the
212 phages, meaning that the relative effect remained rather small. This could be
213 improved with using more efficient phages or phage cocktails in a way that can
214 target wider niche space in vivo by, for example, biofilm specificity [27]. It has also
215 been shown that the effect of phage on plasmid-maintenance can depend on the
216 type of antibiotics that are used [29]. To conclude, within-organism
217 pharmacokinetics and the spatial, temporal, and resource-related dynamics are very
218 different compared to a test tube, having major effects on cost of resistance,
219 magnitude of selection, and treatment efficacy even in a highly simplified and low
220 diversity model system.

221 ETHICS

222 N/A

223 DATA ACCESSIBILITY

224 doi:10.5061/dryad.sc54383

225 AUTHOR CONTRIBUTIONS

226 All authors conceived the study and designed the experiments. LM did the
227 experimental work and analysed the data. All authors contributed to writing and
228 approved the final version of the manuscript.

229 COMPETING INTERESTS

230 No competing interests

231 FUNDING

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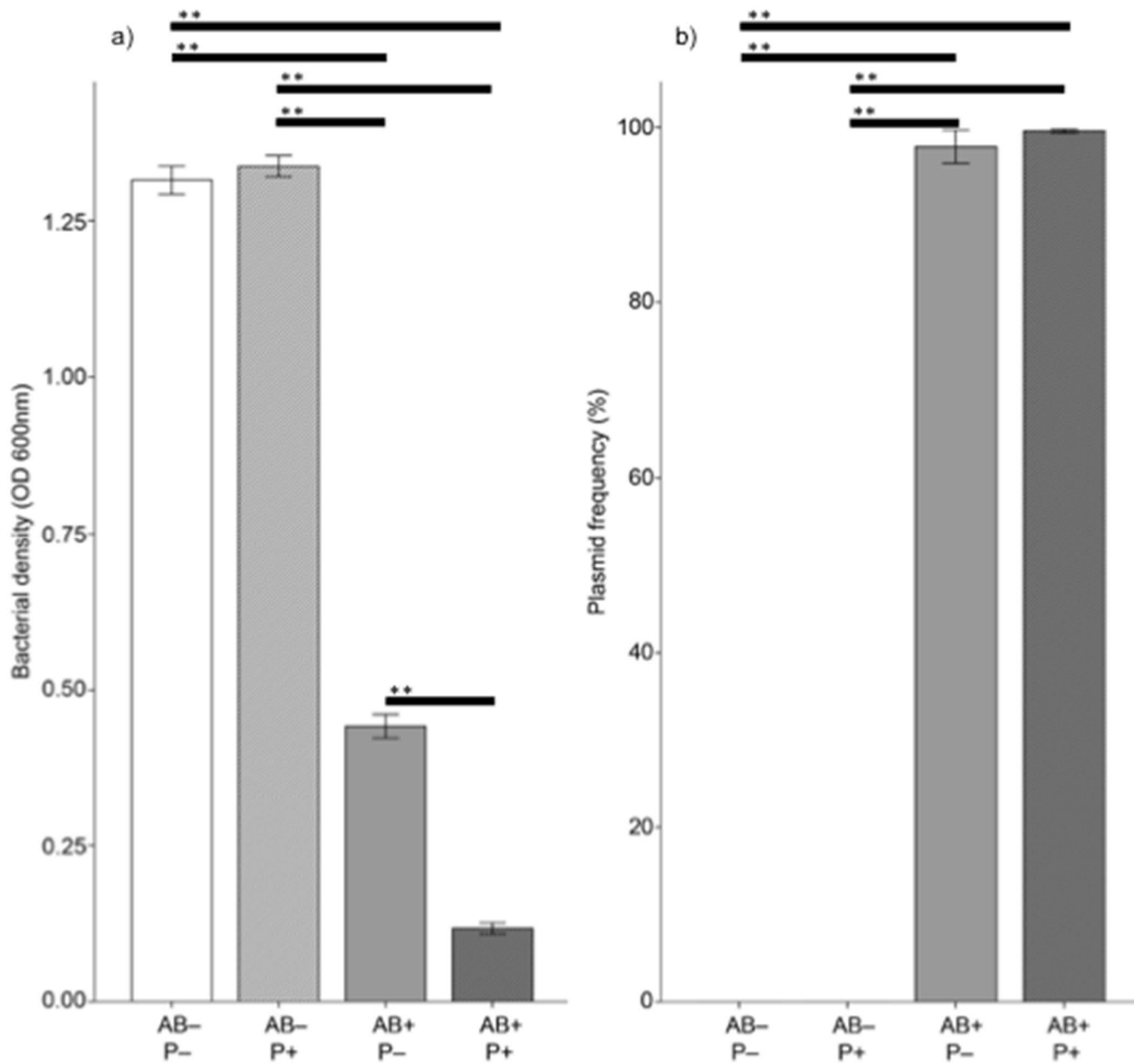
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246 Figure 1. Dynamics in vitro

247 The density of bacteria (a), and the frequency of plasmid bearing cells (b) in

248 liquid culture after a 24 h factorial antibiotic (AB) and phage (P) treatments.

249 Error bars are ± 1 S.E., (**) denotes $p < 0.001$ difference.



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253 Figure 2. Dynamics in vivo

254 The overall density of bacteria (a) and the frequency of plasmid bearing cells

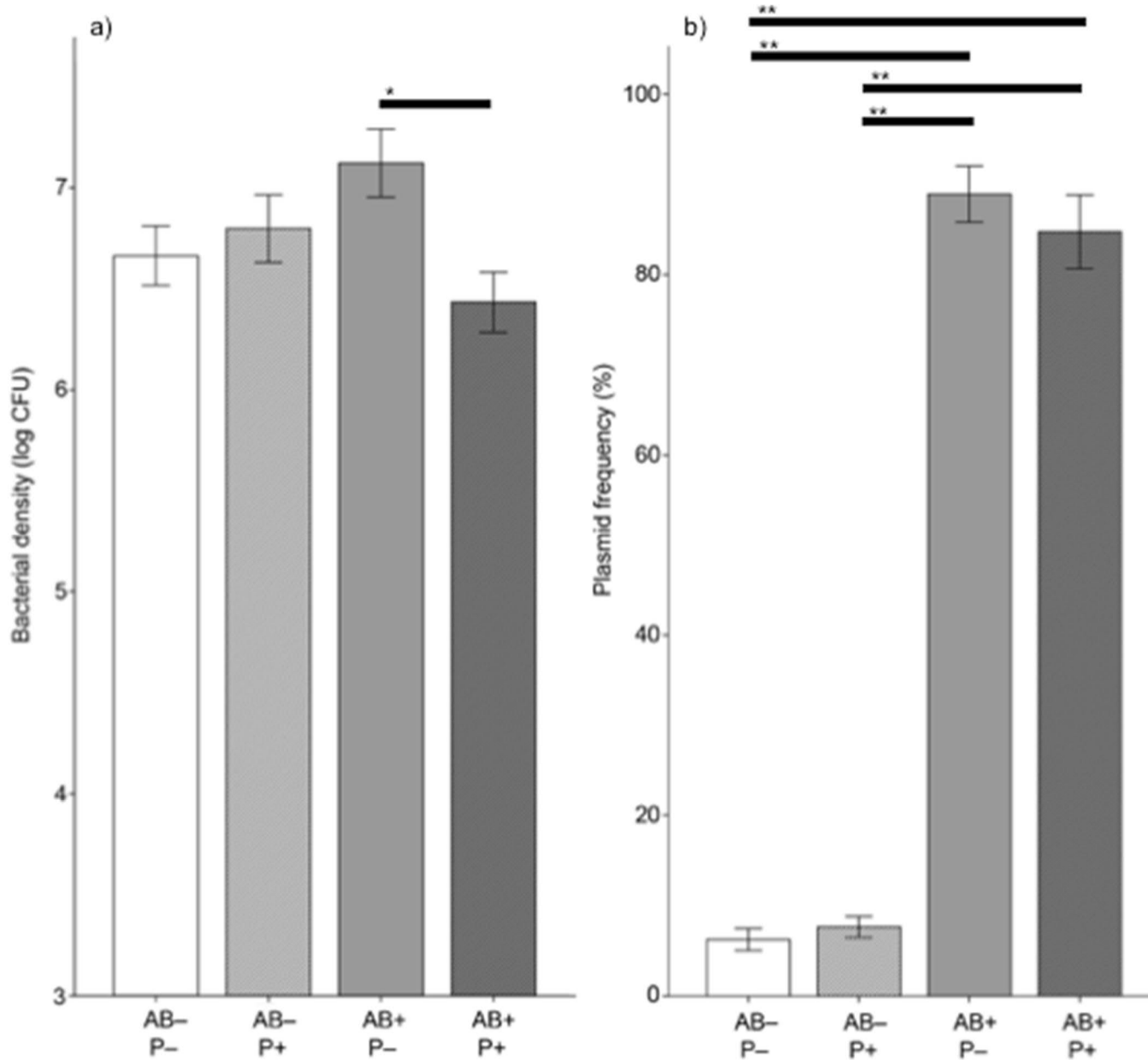
255 (b) in the larval guts after a 96 h factorial antibiotic (AB) and phage (P)

256

treatments. Error bars are ± 1 S.E. (*) and (**) denote $p < 0.05$ and $p < 0.001$

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differences, respectively.



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