Author(s): Mikonranta, Lauri; Buckling, Angus; Jalasvuori, Matti; Raymond, Ben

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1 # Targeting antibiotic resistant bacteria with

2 # phages reduces bacterial density in an

3 # insect host

4   Lauri Mikonranta[1,2*], Angus Buckling[1], Matti Jalasvuori[3], Ben Raymond[1]

5   [1]University of Exeter, Biosciences, Penryn Campus, Penryn, Cornwall, TR10 9FE, UK.

6   [2]University of York, Department of Biology, Wentworth Way, York, YO10 5DD, UK.

7   [3]University of Jyväskylä, Department of Biological and Environmental Science,

8   Nanoscience Center, PL 35, 40014, Jyväskylä, Finland

9   *Corresponding author: lauri.mikonranta@york.ac.uk.

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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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INTRODUCTION

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

It has been argued that phages may be of particular therapeutic value when combined with antibiotics, by constraining the emergence and spread of antibiotic resistance [9]. A good example of recent success in combination treatment comes from a difficult chronic Pseudomonas aeruginosa infection in human aortic graft [10]. Theoretically, phages may limit antibiotic resistance during treatment because of reduction in population size and synergistic costs of resistance [9]. Moreover, with cases where antibiotic resistance is carried on plasmids, phages can be used to directly target the plasmid carrying cells [11]. However, this latter approach has only been investigated in vitro [11, 12], which ignores a plethora of selection pressures 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 μl 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 μg/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 ($F_{1,}$

148    $_{44}=8864.4$, p<0.001) regardless of the phage (P and AB*P: p>0.05). This is particularly

149   surprising, as unlike in E. coli, plasmid RP4 had very low rates of conjugation in E.

150   cloacae: transconjugants were present in only one of the 44 sampled in vitro

151   populations (AB+P- treatment at a very low frequency), while the phage remained

152   infective. The cost of plasmid carriage in the absence of antibiotics manifested itself

153   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

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

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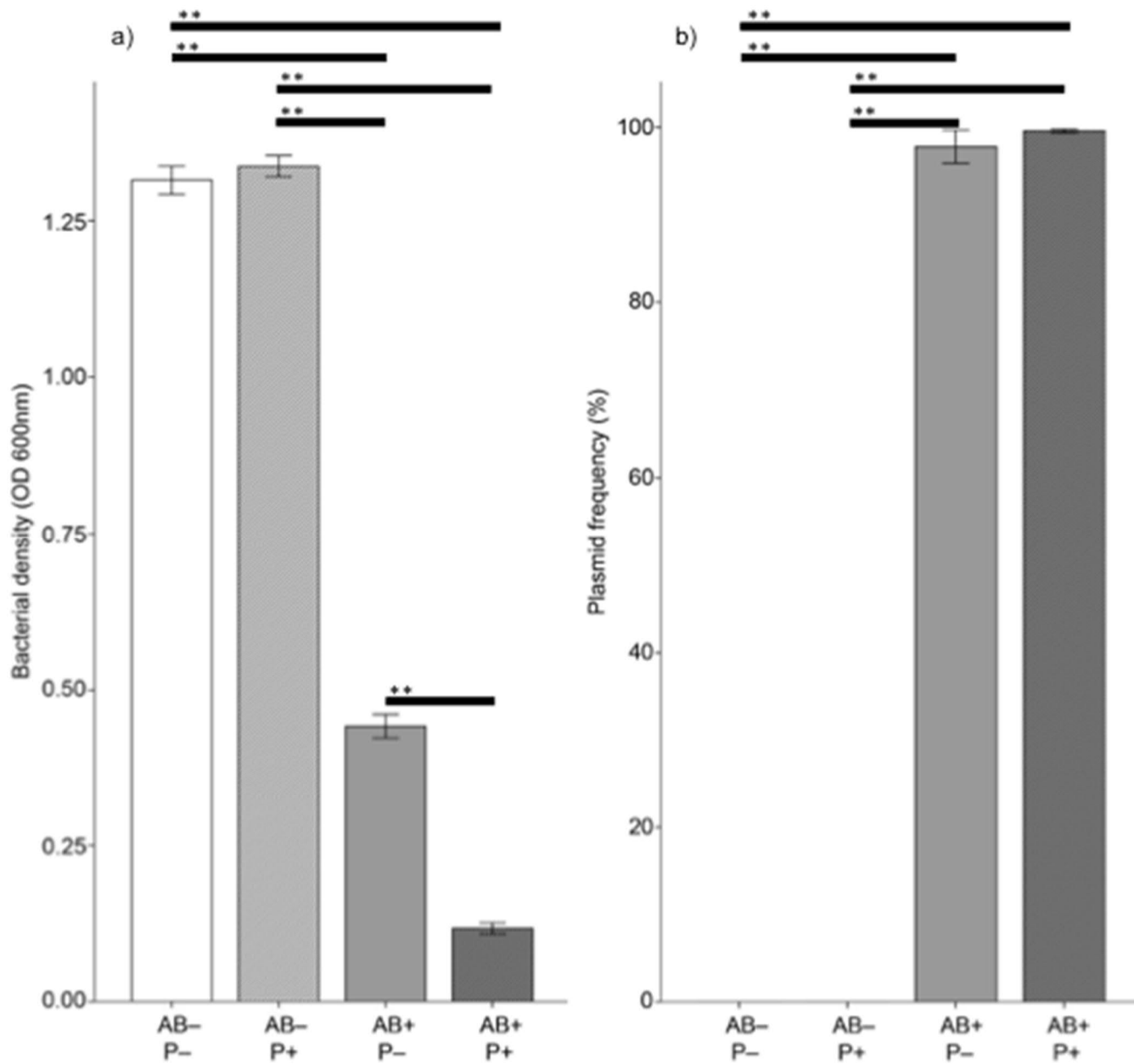
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246   Figure 1. Dynamics in vitro

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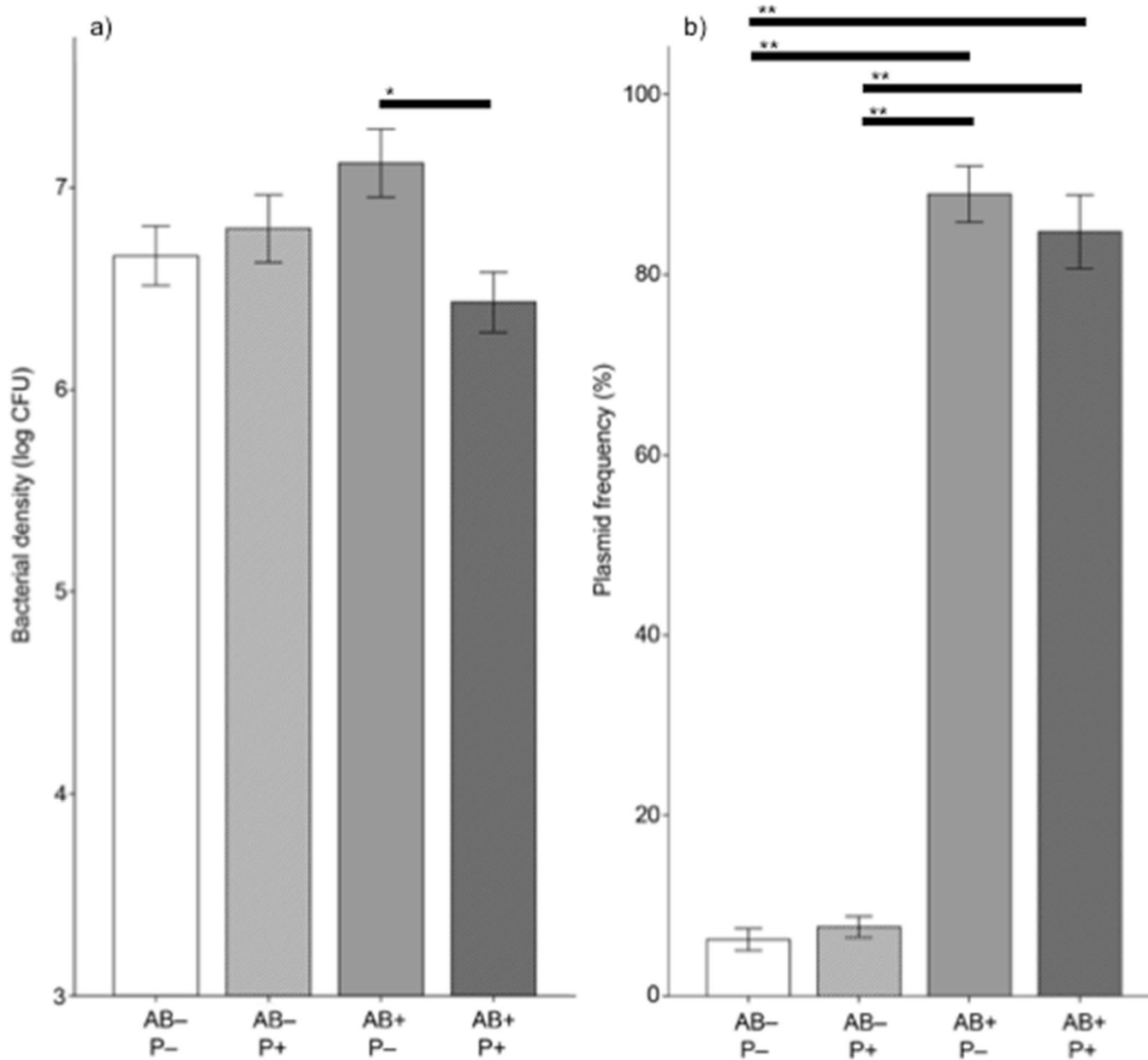
255   (b) in the larval guts after a 96 h factorial antibiotic (AB) and phage (P)

256   treatments. Error bars are +/– 1 S.E. (*) and (**) denote $p < 0.05$ and $p < 0.001$

257   differences, respectively.

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