Targeting antibiotic resistant bacteria with phage reduces bacterial density in an insect host

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ABSTRACT

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

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

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

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

76 **MATERIALS AND METHODS**

77 Although it's been argued that Lepidopterans lack a resident microbiome [14],
78 *Enterobacter cloacae* forms a persistent gut association with Lepidopteran larvae
79 [15] after oral inoculation. The strain was isolated from *P. xylostella* in the insectary
80 of University of Oxford. Two spontaneous antibiotic resistant mutants, ANC C2
81 (rif^R) and 11.1B ($strep^R$ + nal^R) were used in this experiment. The IncP-type
82 plasmid RP4, coding for tetracycline resistance, is usually conjugative [16], and
83 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

resistance and phage receptor genes. The mechanistic basis for low RP4 conjugation in these *E. cloacae* strains is unclear. Given that the phage remains infective, it could be that ANC C2 rifampicin-resistant mutant was an impotent recipient for the plasmid rather than that 11.1B(RP4) lacked the ability to express pili. Although rifampicin resistance mutations are most often polymerase related, known mechanisms in *Enterobacteriaceae* include elongated or abundant outer membrane LPS-chains [18] that could theoretically interfere with conjugation (but, see: [19]).

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

The *T.ni* were maintained as follows: The adults, fed with 100mM sucrose solution, were let to mate and lay eggs on paper strips in flight chambers. The eggs were placed in autoclaved, single-filter vented, 305 x 203 x 100mm Genesis™ containers for surgical instruments to maintain sterile conditions while allowing gas exchange. Hatched larvae were let to feed on Hoffman diet (288 g wheatgerm, 132 g caesin, 117 g sucrose, 70 g agar, 57 g dried brewers yeast, 37.5 g Wesson's salts, 6 g sorbic acid, 3.75 g cholesterol, 3.75 g methyl-4-hydroxybenzoate and 2 g Vanderzant

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

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

Phage survival in the gut was confirmed by overlaying homogenised larval faecal samples on ancestral 11.1B(RP4) lawn on soft agar and observing plaque formation (N=24).

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

RESULTS

In vitro, antibiotics and phage had synergistic effects on the total density of bacteria 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). Multiple comparisons showed that there was no phage effect in absence of antibiotics, but other pairwise differences were highly significant (p<0.001, Fig. 1. a). Thus, single treatment allowed compensatory growth of the non-target strain, but this effect was reduced for the plasmid carriers in the presence of antibiotics. The plasmid went to near fixation in the presence of antibiotics (F1, 147 44=8864.4, p<0.001) regardless of the phage (P and AB*P: p>0.05). This was contrary to previous studies with the same plasmid and phage in *E. coli* [12]. The increase in frequency was not the result of conjugational transfer, because unlike in *E. coli*, RP4 had very low conjugation rate in *E. cloacae*: transconjugants were present in only one of the 44 sampled in vitro populations (AB+P- treatment at a very low frequency). We also confirmed that the phage remained infective even with low conjugation. The cost of plasmid carriage in the absence of antibiotics manifested itself as complete plasmid extinction (at least below the detection threshold of <67 cfu/mL) in all sampled

populations (Fig 1. b). This was the case even though RP4 has been reported to be of low cost and rapidly evolve towards no cost through chromosomal compensatory mutations [21].

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

DISCUSSION

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

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

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

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

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

ETHICS

N/A

DATA ACCESSIBILITY

doi:10.5061/dryad.sc54383

AUTHOR CONTRIBUTIONS

All authors conceived the study and designed the experiments. LM did the experimental work and analysed the data. All authors contributed to writing, approved the final version of the manuscript, and agree to be held accountable for the work performed.

COMPETING INTERESTS

No competing interests

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Figure 1. Dynamics in vitro

The density of bacteria (a), and the frequency of plasmid bearing cells (b) in liquid culture after a 24 h factorial antibiotic (AB) and phage (P) treatments. Error bars are +/– 1 S.E., (**) denotes p<0.001 difference.

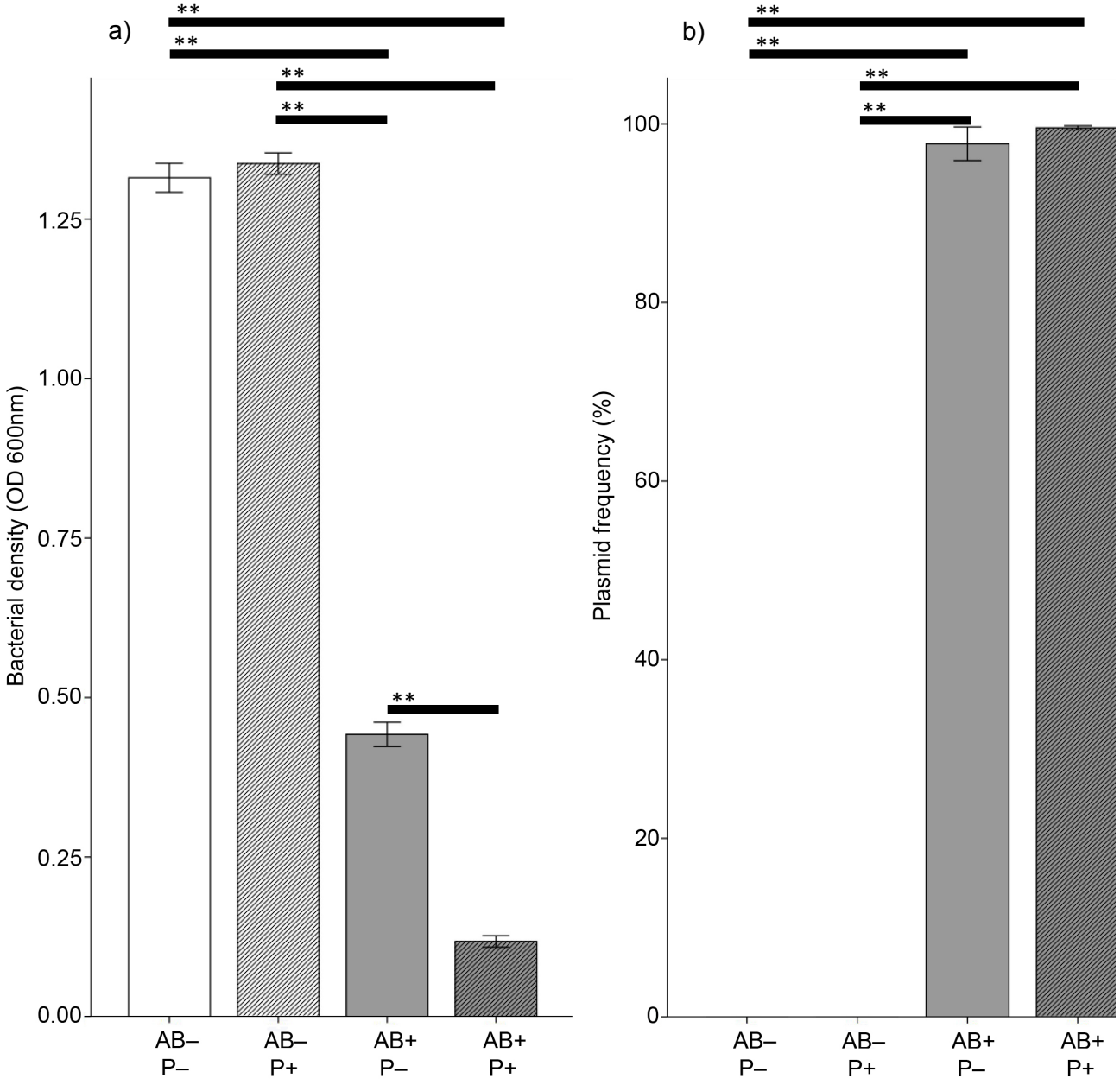
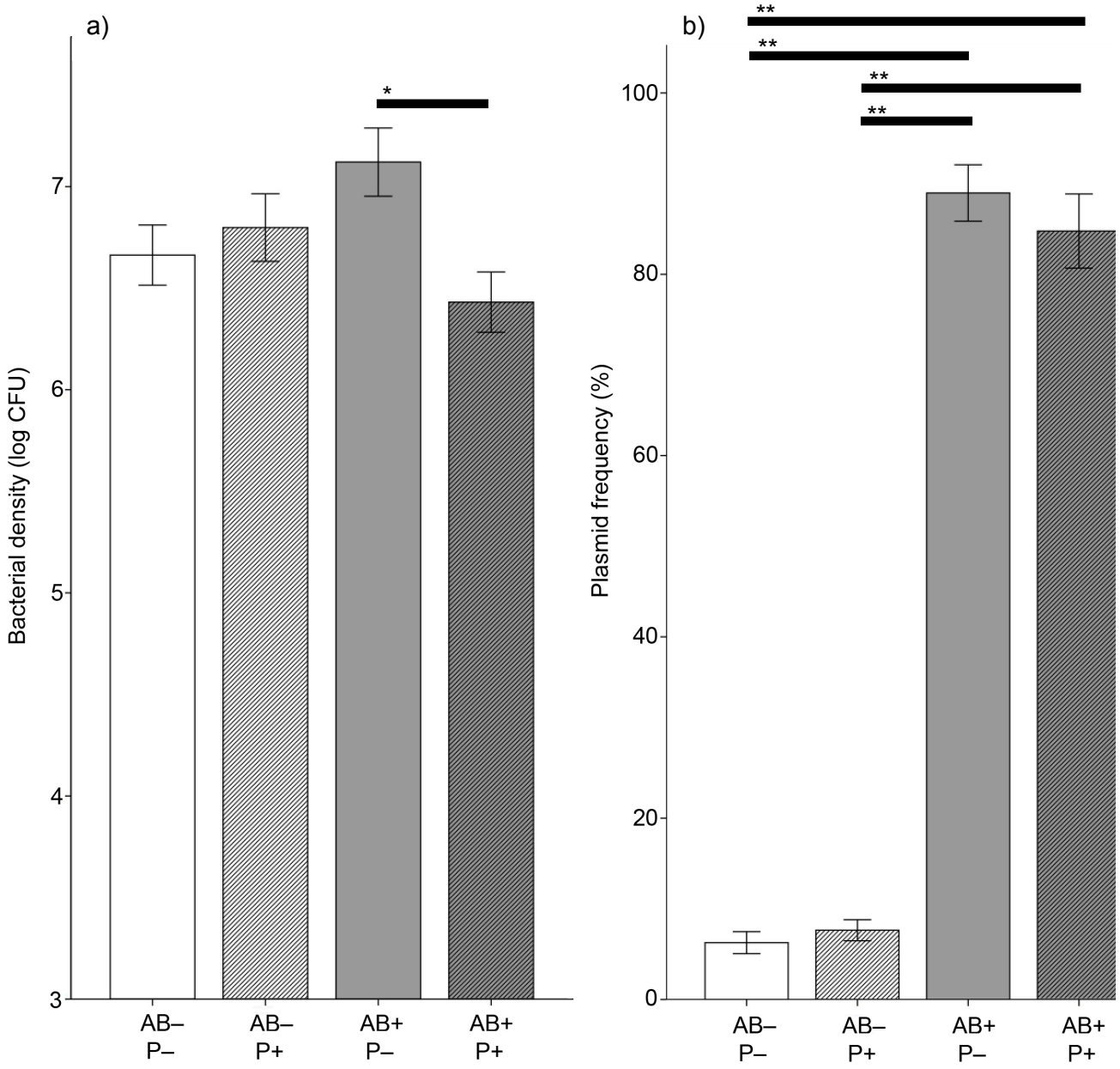


Figure 2. Dynamics in vivo

The overall density of bacteria (a) and the frequency of plasmid bearing cells (b) in the larval guts after a 96 h factorial antibiotic (AB) and phage (P) treatments. Error bars are +/– 1 S.E. (*) and (**) denote $p<0.05$ and $p<0.001$ differences, respectively.



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