1 **Title**

2 Selection for antibiotic resistance is reduced when embedded in a natural microbial

3 community

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Abstract

Antibiotic resistance has emerged as one of the most pressing, global threats to public health. In single-species experiments selection for antibiotic resistance occurs at very low antibiotic concentrations. However, it is unclear how far these findings can be extrapolated to natural environments, where species are embedded within complex communities. We competed isogenic strains of *Escherichia coli*, differing exclusively in a single chromosomal resistance determinant, in the presence and absence of a pig fecal microbial community across a gradient of antibiotic concentration for two relevant antibiotics: gentamicin and kanamycin. We show that the minimal selective concentration was increased by more than one order of magnitude for both antibiotics when embedded in the community. We identified two general mechanisms were responsible for the increase in minimal selective concentration: an increase in the cost of resistance and a protective effect of the community for the susceptible phenotype. These findings have implications for our understanding of the evolution and selection of antibiotic resistance, and can inform future risk assessment efforts on antibiotic concentrations.

Introduction

The emergence and spread of antimicrobial resistance (AMR) genes in bacterial pathogens has been identified as one of the major threats to human health by the World Health Organisation (WHO, 2014). Whilst AMR genes have been detected in ancient permafrost samples (D'Costa et al., 2011), anthropogenic use of antibiotics has caused a rapid increase in their prevalence (Knapp et al., 2010). A large body of theory and *in vitro* work has identified the role of ecological context, such as treatment regime and environmental heterogeneity, in AMR gene dynamics (Drlica, 2003; Drlica and Zhao, 2007; Gullberg et al., 2014, 2011). However, the majority of this work has not explicitly considered a crucial feature of microbial ecology: microbes are typically embedded within complex communities of interacting species. This is always the case within human and livestock microbiomes, in which antibiotic-imposed selection is likely to be particularly strong (Carlet, 2012). Here, we combine experiments and theory to determine how selection for AMR is influenced by the presence of other species derived from a natural gut microbial community. The focus of this study is selection for pre-existing resistance genes within a focal species, rather than selection on *de novo* variation arising through spontaneous mutations or acquired through horizontal gene transfer from another species.

Recent experimental studies suggest that selection for AMR genes in complex communities is occurring at antibiotic concentrations (the minimum selective concentration; MSC) that are much lower than those that prevent the growth of susceptible bacteria (minimum inhibitory concentration; MIC) (Lundström et al., 2016; Murray et al., 2018); as has been previously shown within single species *in vitro* (Gullberg et al., 2014, 2011; Liu et al., 2011). However, it is unclear how the presence of other microbial species affects the MSC. While the precise effect of other species is likely context-dependent, we hypothesise that the presence of the community will typically increase the MSC. Studies of single species suggest that resistant cells can afford protection to susceptible ones, through both, intracellular and extracellular degradation of antibiotics (Medaney et al., 2016; Sorg et al., 2016; Yurtsev et al., 2013), thus

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64   increasing relative fitness of susceptible strains and hence the MSC. However, excreted

65   metabolites can both potentiate or decrease antibiotic efficacy, thus decreasing or increasing

66   MSCs (Cao et al., 2012; Churski et al., 2012). Further, any costs associated with AMR may

67   be enhanced by increased competition for resources, as, for example, has been observed

68   with respect to resistance in flies to parasitoids (Kraaijeveld et al., 2001) and bacteria to

69   viruses (Gómez and Buckling, 2011).

70   To explore the potential effects of community context on AMR selection, we competed

71   isogenic *Escherichia coli* MG1655 strains, differing exclusively in a single chromosomal

72   resistance determinant, in the presence and absence of a microbial community across a

73   gradient of two different aminoglycoside antibiotics, kanamycin and gentamicin. We

74   embedded the *E. coli*, commonly found in the anaerobic digestive tract of warm-blooded

75   mammals (Tenaillon et al., 2010), within a pig faecal community in experimental anaerobic

76   digesters in an attempt to partially mimic a gut environment. We additionally employed

77   metagenomic analysis, community typing (16S) and mathematical modelling to provide

78   insights into mechanisms underpinning community effects on AMR selection.

79   **Results**

80   **Community context affects selection for gentamicin resistance**

81   Isogenic strains of the focal species *E. coli*, with and without gentamicin resistance, were

82   competed in the presence and absence of a pig faecal community across a 5 orders of

83   magnitude gradient of gentamicin concentrations. Independent of antibiotic concentration the

84   focal species increased in abundance during the 3 day evolution experiment from ~10% at

85   inoculation to above 90% relative abundance based on 16S sequencing (Fig S1A&B). Both

86   resistant and sensitive strains showed positive growth across the whole range of

87   concentrations and both treatments with cell counts increasing by 2.25 to 3.96 orders of

88   magnitude per day (Fig 1A).

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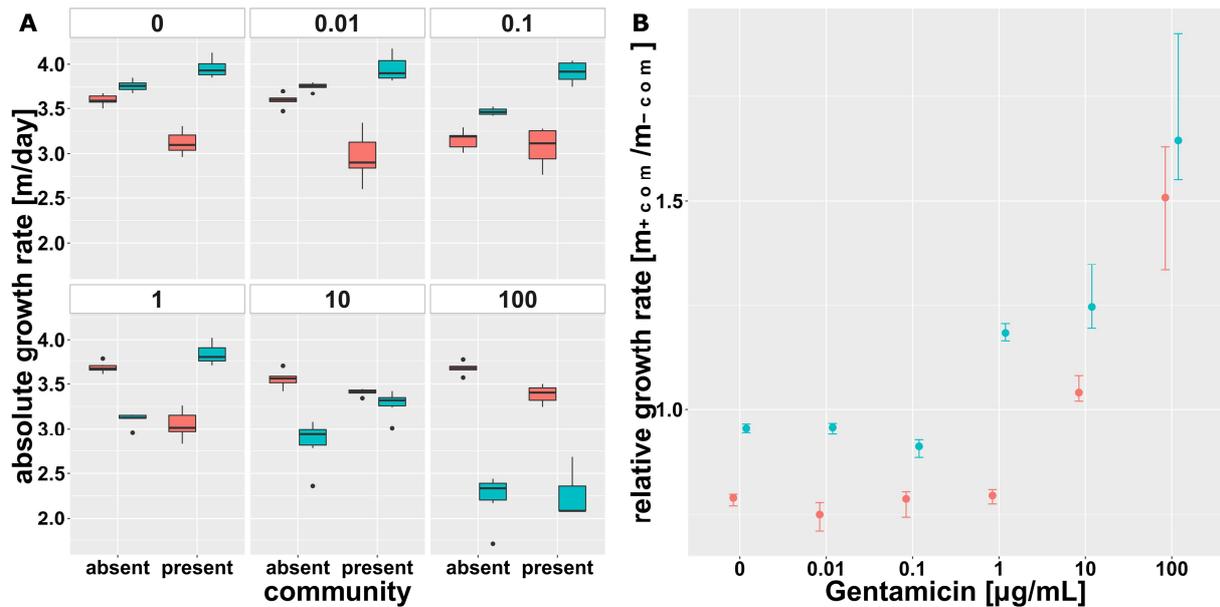
Fig 1. Malthusian growth parameter per day of the focal species' isogenic strains for gentamicin.

Values are displayed across the antibiotic gradient and in absence and presence of the gut microbial community. (A) Average (±SD, n=6) logarithmic absolute growth per day for the resistant (red) and the susceptible (blue) strain. Note: A different inoculum size of the focal species in absence (~10^6 bacteria) and presence (~10^5 bacteria = 10% of total inoculum) of the community was used. (B) Ratio of absolute Malthusian growth parameters (with 95% confidence intervals based on 1000-fold bootstrap analysis) in presence and absence of the microbial community across the gradient of antibiotic concentrations.

There was a small competitive fitness cost (t-test against 1, p=0.0005) of gentamicin resistance in the absence of the community (ρ_r = 0.955 ± 0.014, mean ± SD), and this cost appeared to be greatly increased when the community was present (Fig 1B, 2) (ρ_r = 0.788 ± 0.016) (ANOVA corrected for multiple testing, p<0.01, F=360.36). As antibiotic concentration increased, this cost was offset by the benefit of resistance. However, the reduction in fitness of the resistant strain in the presence of the community remained fairly constant (significant differences, p<0.05 after controlling for multiple testing at concentrations between 0 and 10 μg/mL) up to 100 μg/mL gentamicin, at which point the community had no effect on relative fitness (ANOVA corrected for multiple testing, p=0.259, F=1.42).
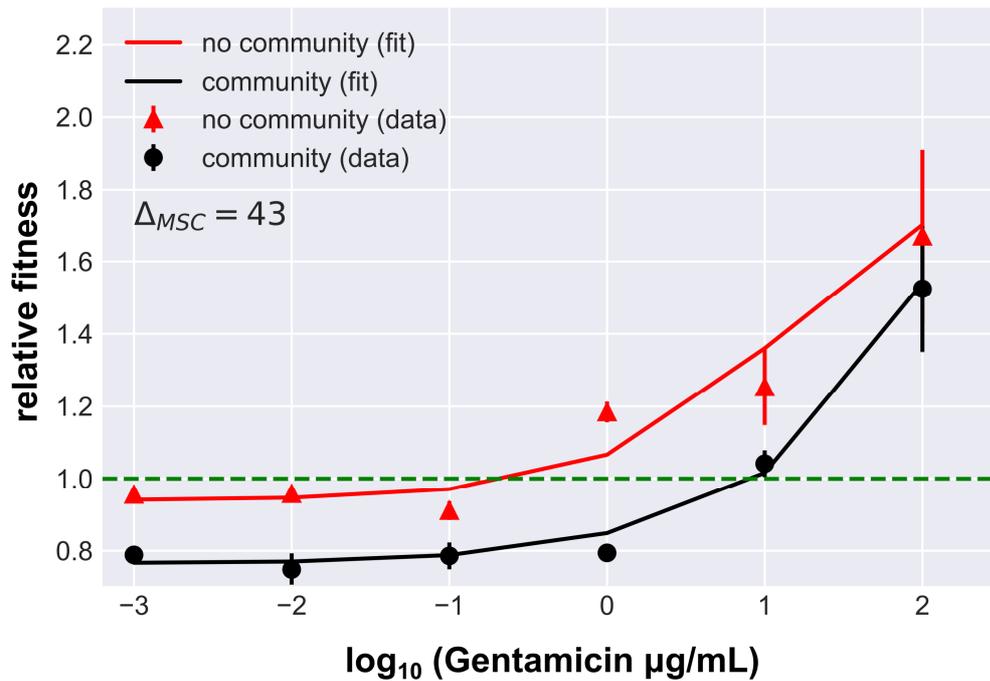
Fig 2. Relative fitness of the gentamicin resistant strain

Values (mean ± SD, n=6) in presence (black) and absence (red) of the community. Solid lines represent the best fit fitness curve through the mathematical model based on parameter estimates presented in Table 1. The dashed line indicates neutral selection at a relative fitness of $\rho_r = 1$, where the intercept with the fitness curve indicates the minimal selective concentration.

parameter	all	Gentamicin susceptible	resistant	community
φ		1.4	1.3	1.3
e_{ij}		1	2.3	3
$\alpha_{i,0}$		1.3	2.9	1.6
$\beta_{i,0}$		0.7	0.8	0.6
k_d	10^5	-	-	-
f_{max}	0.9	-	-	-

Table 1: Model parameter values for gentamicin fitness curves.

Community composition is altered across the gentamicin gradient

It is possible that changes in community composition across the antibiotic gradient may have contributed to the observed changes in selection for resistance caused by the community, notably between 10 and 100 µg/mL. The composition of the microbial community changed significantly from the collected faecal sample, to inoculum and further during the duration of

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121   the experiment (AMOVA, p<0.001, Fig S2A). Above 1 µg/mL gentamicin the previously

122   dominant Proteobacteria were outcompeted by Firmicutes (Fig S2B) leading to a significant

123   (AMOVA, p<0.01) separation of communities below and above this threshold concentration

124   in the NMDS plot (Fig S2B). However, there was no significant change in composition

125   between 10 and 100 µg/mL, suggesting that compositional changes did not play a major role

126   in community-imposed selection.

127

128   *Community context imposes a cost of resistance*

129   To test the hypotheses derived from the numerical data we used numerical simulations of

130   our experimental set up to determine the likely mechanisms underpinning the observed

131   population dynamics in a common logarithmic growth model. We determined models based

132   on the key empirical findings in the absence of the community (specifically, that there is a

133   cost of resistance in the absence of antibiotics, and that antibiotics inhibit the growth of the

134   sensitive strain in a dose dependent manner), and then determined the most parsimonious

135   way in which the community could have altered the relative fitness of the resistant and

136   susceptible strains (Table 1). We found a good fit to the data simply by assuming that the

137   community imposed a greater competitive effect, constant across the antibiotic gradient, on

138   the resistant rather than the sensitive strain ($e_{rj} \gg e_{cj} > e_{sj}$; where e_{ij} is the competition

139   coefficient imposed on the focal population (resistant r, susceptible s and community c) by

140   the community). Note that the lack of effect of the community at high antibiotic

141   concentrations (100 µg/mL) was caused by there being very little growth of the susceptible

142   strain, and hence relative fitness was determined primarily by the growth of the resistant

143   strain.

144   The numerical simulation allowed us to estimate the change in MSC from absence to

145   presence of the community by deterministically evaluating the concentration at the intercept

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146   with neutral selection at a relative fitness of $\rho_r = 1$. We estimated a 43-fold increase in MSC

147   in the presence of the community (Fig 2).

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Community context affects selection for kanamycin resistance

150   As with gentamicin, the focal species increased in abundance during the 3 day competition

151   experiment from ~10% at inoculation to above 90% relative abundance (Fig S1C&D). Again,

152   both strains increased in abundance across both treatments and all concentrations of the 5

153   orders of magnitude antibiotic gradient with cell numbers increasing by 1.45 to 3.09 orders of

154   magnitude per day (Fig 3A). In the absence of this community, kanamycin resistance also

155   imposed a slight metabolic fitness cost on the resistant strain ($\rho_r = 0.915 \pm 0.036$) (Fig 3B).
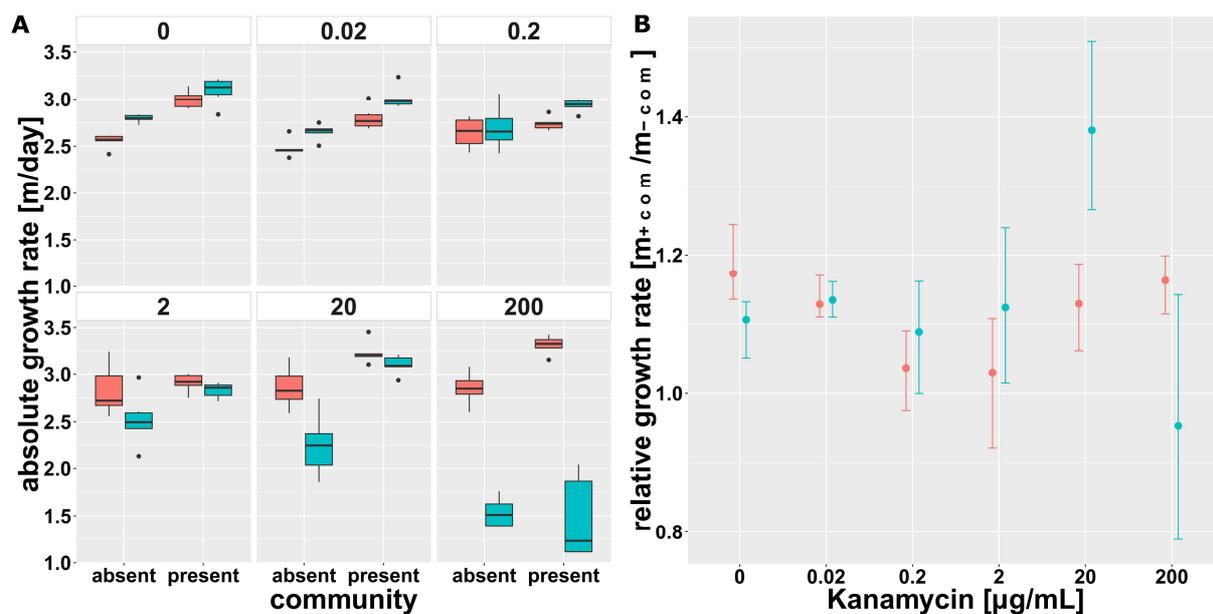
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157   **Fig 3. Malthusian growth parameter per day of the focal species' isogenic strains for kanamycin.**

158   Values are displayed across the antibiotic gradient and in absence and presence of the gut microbial community.
159   (A) Average (±SD, n=6) logarithmic absolute growth per day for the resistant (red) and the susceptible (blue)
160   strain. Note: A different inoculum size of the focal species in absence (~10^6 bacteria) and presence (~10^5
161   bacteria = 10% of total inoculum) of the community was used. (B) Ratio of absolute Malthusian growth
162   parameters (with 95% confidence intervals based on 1000-fold bootstrap analysis) in presence and absence of
163   the microbial community across the gradient of antibiotic concentrations.

164

165   However, unlike gentamicin, the community did not increase the general cost of resistance.

166   Indeed, the community had no significant effect on the relative fitness of the resistant strain

167   except at a concentration of 20μg/mL (ANOVA corrected for multiple testing, p=0.002,

168   F=15.58) (Fig 4). There was a clear fitness advantage for the resistant strain in the absence

169   of the community at this concentration (ρ_r = 1.288 ± 0.149; t-test against 1, p=0.0052), while

170   in the presence of the community this difference in relative fitness while still significant (t-test

171   against 1, p=0.0088) was considerably lower (ρ_r = 1.034 ± 0.020). At 200 μg/mL kanamycin,

172   close to the susceptible strains MIC, the resistant strain had an equally high relative fitness

173   regardless of the presence of the community (ANOVA corrected for multiple testing,

174   p=0.079, F=3.84).
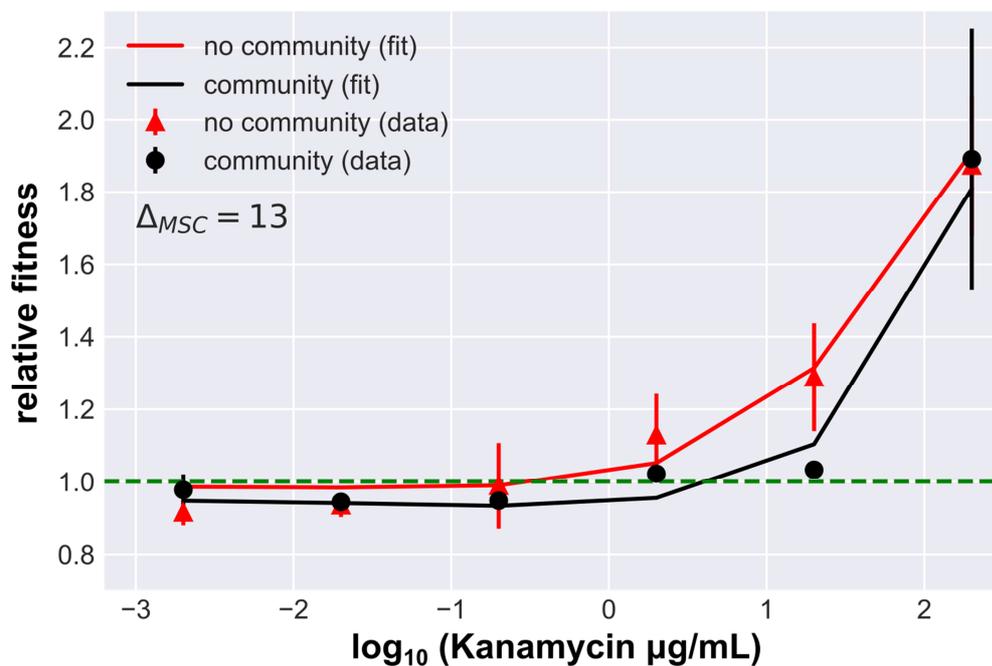
175

176   **Fig 4. Relative fitness of the kanamycin resistant strain**

177   Values (mean ± SD, n=6) in presence (black) and absence (red) of the community. Solid lines represent the best
178   fit fitness curve through the mathematical model based on parameter estimates presented in Table 2. The
179   dashed line indicates neutral selection at a relative fitness of ρ_r = 1, where the intercept with the fitness curve
180   indicates the minimal selective concentration.

181

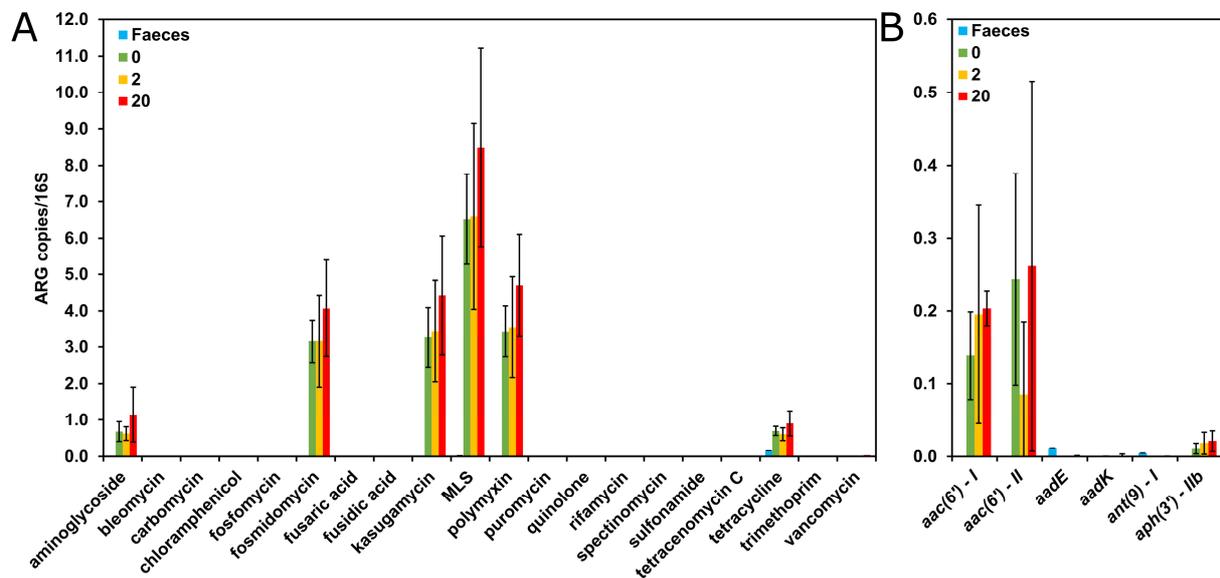| Kanamycin				
parameter	all	susceptible	resistant	community
φ	-	1.9	1.8	1.3
e_{ij}	-	1.7	1.3	1.6
$\alpha_{i,0}$	-	1.0	1.4	1.6
$\beta_{i,0}$	-	0.6	0.4	0.5
k_d	10^5	-	-	-
f_{max}	0.9	-	-	-

Table 2. Model parameter values for kanamycin selection curve.

Community and antibiotic resistance composition remain stable across the kanamycin gradient

As with the gentamicin experiment, a significantly shift in community composition from collected faecal sample, to inoculum and further during the duration of the kanamycin experiment (AMOVA, p<0.001, Fig S3A) was observed. However, across the whole gradient of antibiotics, Firmicutes (Fig S3B) remained the dominant phylum with no significant changes in community composition as a result. As such, compositional changes again cannot explain the impact of the community on focal strain fitness under selection at 20 μg/mL only. We additionally carried out metagenomic analysis for the 0, 2 and 20 μg/mL kanamycin treatments to determine whether relative abundance of resistance genes had changed within the community, despite the fact that there were no changes in community composition. Resistance to aminoglycoside (ANOVA, p=0.04) and other classes of antibiotics (fosmidomycin, kasugamycin, macrolides, polymyxin and tetracycline (ANOVA, all p<0.01)) significantly increased in the community of all reactors compared to the original faecal community independent of antibiotic concentrations (Fig 5A). However, there was no significant difference between kanamycin concentration and the abundance (ANOVA, p=0.15) of aminoglycoside resistance in general (Fig 5A) or any specific aminoglycoside resistance subtypes (Fig 5B) suggesting that relative fitness of the focal species was not influenced by the community resistome. Unsurprisingly, since no antibiotic concentration

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203   dependent selection for aminoglycoside resistance was observed within the community, no

204   significant co-selection for resistance to any other classes of antibiotics was observed either.

205

206   **Fig 5. Detected resistance genes.**

207   Type (A) and aminoglycoside subtype (B) relative abundance (resistance gene number normalized with 16S
208   rRNA copy number), in original faecal community and in final reactor community at 3 kanamycin concentrations
209   (mean ± SD; n_{feces}=2, n_{Kn0}=6, n_{Kn2}=6 n_{Kn20}=5). Only genes detected with the ARGs-OAP pipeline are shown. MLS
210   = Macrolides, Lincosamides, Streptogramines

211

212   *Presence of the community can enhance growth of susceptible E. coli population at*

213   *intermediate antibiotic concentrations*

214   Numerical simulations showed that, unlike for gentamicin resistance, a community-imposed

215   increase in the cost of kanamycin resistance was unable to explain why the benefit to the

216   drug resistant focal *E. coli* strain was reduced in the presence of the community at

217   intermediate drug concentrations ($e_{rj} = e_s$). This suggested different interactions between *E.*

218   *coli* and the rest of the community, and we speculated that the community might have

219   provided a protective effect against kanamycin for the susceptible *E. coli*. Growth data

220   demonstrated this to be the case: the presence of the community decreased or had no

221   significant impact on the growth rate of either the susceptible or resistant *E. coli*, except at

222   20 µg/mL where the growth rate of the susceptible, but not the resistant strain, was

223   significantly increased by the presence of the community (Fig 3B). We investigated if a

11

224 protective effect of the community was sufficient to explain the observed data by fitting

225 numerical simulations where the dose-response parameters $\alpha_{s,r}$ and $\beta_{s,r}$ were explicitly

226 dependent on the (time-dependent) density of the community (as listed in Table 2). The

227 resulting model provided a good fit to the experimental data, suggesting that community

228 protection was driving the observed population dynamics with a 12-fold increase in MSC.

229

Discussion

231 In this study we investigated how being embedded within a semi-natural community (a pig

232 gut derived community in an anaerobic digester) affects selection for AMR within a focal

233 species (*E. coli*). For two antibiotics commonly fed to agricultural animals (gentamicin and

234 kanamycin), we find the presence of the community selects against resistance, resulting in

235 1-2 orders of magnitude higher minimal selective concentrations for antibiotic resistance.

236 This suggests that recent *in vitro* single strain based estimates of MSCs (Gullberg et al.,

237 2014, 2011; Liu et al., 2011) are likely much lower than would be observed *in vivo* and might

238 explain why in certain ecosystems no selection for antibiotic resistance was observed in

239 focal strains (Flach et al., 2018).

240 The primary mechanisms responsible for this community-imposed reduction in selection for

241 resistance differed for the two tested drugs, yet are likely fairly general based on their

242 ecological origin. For gentamicin, the community increased the fitness costs reflected by

243 reduced growth rates that are associated with resistance in the absence of antibiotics. These

244 elevated costs were retained at similar levels across the antibiotic gradient, up until doses

245 were so high that only the resistant strain grew (similar behaviour above a certain threshold

246 concentration has previously been described for single strain systems (Andersson and

247 Hughes, 2011, 2010) and our results show that his holds true in a community context).

248 Resource limitation – directly manipulated or though competition - has been found to

249 increase costs against a range of stressors in a range of organism, from resistance of

250   plasmodium to antimalarial drugs (Wale et al., 2017) to phage resistance in bacteria (Gómez

251   and Buckling, 2011). This is presumably because resource limitation has a more pronounced

252   effect on resistant genotypes (Song et al., 2014).

253   For kanamycin, community-imposed selection against resistance was only apparent at

254   intermediate antibiotic concentrations. The absolute growth rate of the susceptible strain was

255   significantly increased at intermediate concentrations in presence of the community. Our

256   model fitting suggests this is because of a protective effect of the community. The protective

257   effect might have only been observed at intermediate concentrations since low

258   concentrations were insufficient to detectably lower the relative fitness of the susceptible

259   strain, while at high concentrations the protective effect was too small to be detectable. Such

260   protective effects have been reported extensively within-species (Medaney et al., 2016;

261   Yurtsev et al., 2013), as well as more recently within more complex communities (Sorg et al.,

262   2016), either because of extra- or intracellular modification of antibiotics. Other common

263   mechanisms known to increase a strains resistance to antibiotics in communities involve

264   flocculation (Kümmerer, 2009) or biofilm formation (Drenkard and Ausubel, 2002; Mah et al.,

265   2003), but might here only play a minor role due to the shaking conditions.

266   The mechanisms discussed above all underlie the selection for standing variation in pre-

267   existing resistance genes, rather than selection on *de novo* variation arising through

268   spontaneous mutations or horizontal gene transfer from other species. For de novo

269   chromosomal mutations, the community is likely to further limit the spread of resistance,

270   because the reduced population sizes of the focal strains in the presence of the community

271   increases the chance that more costly mutations will be fixed (Perron et al., 2007). In

272   contrast, being embed in a community might enhance the spread of resistance. First, there

273   will be a greater source of resistance genes available to the focal species. Second, selection

274   against resistance acquired through horizontal gene transfer at low antibiotic concentrations

275   might follow different dynamics. While chromosomal resistance might be outcompeted and

276   subsequently lost, resistance genes embedded on conjugative plasmids can persist or even

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277 increase in abundance, as a consequence of their sometimes extremely broad host ranges

278 and high transfer frequencies (Arias-Andres et al., 2018; Klümper et al., 2017, 2015;

279 Musovic et al., 2014; Shintani et al., 2014). In controlled single strain experiments plasmid

280 born resistance proved more costly than chromosomal resistance (Gullberg et al., 2014).

281 However, in more complex scenarios selection for mobile genetic element borne resistance

282 usually depends not only on the single acquired resistance gene, but a combination of other

283 linked traits encoded by the MGE as part of the communal gene pool (Norman et al., 2009).

284 Thus, difficulties in making general predictions on the selection dynamics of horizontally

285 acquired resistance in microbial communities arise that merit future research efforts.

286 In summary, we show that selection for antimicrobial resistance was influenced by being

287 embed in a "natural" microbial community, such that the MSC was increased by more than

288 one order of magnitude for two different antibiotics. Further to reducing relative fitness of

289 resistance, being embedded in a community would also reduce absolute fitness, which has

290 been argued to sometime be the major driver of spread of resistance (Day et al., 2015).

291 To determine MSCs that are relevant in environmental settings it is thus crucial to test for

292 selection in a complex community context, rather than in single strain systems.

293 Understanding under which concentrations selection for and thus long-term fixation of newly

294 acquired resistance mechanisms is occurring is crucial for future mitigation of the spread of

295 resistance genes as well as their potentially pathogenic hosts (Larsson et al., 2018; Smalla

296 et al., 2018). Our results further stress the need to preferentially use narrow spectrum

297 antibiotics in clinical therapy to maintain a healthy microbiome within the patient that can

298 more easily recover after antibiotic administration (Palleja et al., 2018), thus decreasing the

299 likelihood of positive selection for pathogens that might have acquired resistance when

300 embedded in a community.

301

Material and Methods

Pig faecal community

Pig faeces were collected from four Cornish Black pigs without previous exposure to antibiotics in April 2016 on Healey's Cornish Cyder farm (Penhallow, Cornwall, United Kingdom). Two hundred grams of faeces from each pig were pooled, mixed with 400ml each of sterile glycerol and 1.8 g/L NaCl solution. The mixture was homogenized for 3 min in a Retsch Knife mill Gm300 (Retsch GmbH, Haan, Germany) at 2000 rotations per minute (rpm), filtered through a sieve (mesh size ~1mm^2), centrifuged at 500 rpm for 60 s at 4°C and the liquid supernatant fraction was collected and frozen at -80°C as the inoculum.

Pig fecal extract

Two hundred grams of faeces from each pig were pooled, mixed with 800 mL of sterile 0.9 g/L NaCl solution. The mixture was homogenized for 3 minutes in a Retsch Knife mill Gm300 (Retsch GmbH, Haan, Germany), at 2000 rotations per minute, filtered through a sieve (mesh size ~1 mm^2) and the liquid fraction was collected. The extract was then centrifuged (3500 rpm, 20 minutes, 4°C), the supernatant collected and autoclaved (121°C, 20 min). The autoclaved extract was centrifuged again (3500 rpm, 20 minutes, 4°C) and the supernatant collected and used as a nutrient supplement.

Strains

The focal species, *E. coli* MG1655, was chromosomally tagged with a TN7 gene cassette encoding constitutive red fluorescence, expressed by the *mCherry* gene (Remus-Emsermann et al., 2016) to ensure that *E. coli* can be detection and distinguished from other community members after competition based on red fluorescence. The kanamycin resistant, red fluorescent variant containing resistance gene *aph(3')-IIb* encoding an aminoglycoside 3′-phosphotransferase was created previously (Klümper et al., 2015, 2014).

328   To create the gentamicin resistant mutant the strain was further tagged through

329   electroporation with the pBAM delivery plasmid containing the mini-TN5 delivery system

330   (Marti-nez-Garci-a et al., 2014; Martínez-García et al., 2011) for gentamicin resistance gene

331   *aacC1* encoding a gentamicin 3′-*N*-acetyltransferase (Kovach et al., 1995). Successful

332   clones were screened for gentamicin resistance (30 µg/mL) and for the chosen clone a

333   single strain growth curve in LB medium was measured to ensure that the cost of the

334   resistance gene was lower than 10% compared to the susceptible strain to ensure

335   competitive ability.

336

Competition experiments

338   Competition experiments as well as initial growth of focal species strains were performed in

339   25 mL serum flasks with butyl rubber stoppers. As growth medium 10mL of sterile Luria

340   Bertani broth supplemented with 0.1% pig faecal extract, 50 mg/L Cysteine-HCl as an

341   oxygen scrubber and 1 mg/L Resazurin as a redox indicator to ensure anaerobic conditions

342   (Großkopf et al., 2016), was added to each reactor, heated in a water bath to 80°C and

343   bubbled with 100% N_2 gas until the oxygen indicator Resazurin turned colourless. After

344   cooling down to 37°C the appropriate concentration of antibiotic (AB) was added from a

345   1000x anaerobic stock solution.

346   Two isogenic pairs of the focal species, the susceptible, red fluorescent *E. coli* strain with

347   either its gentamicin or kanamycin resistant counterpart, were competed across a gradient of

348   six antibiotic concentrations (Gentamicin [µg/mL]: 0, 0.01, 0.1, 1, 10, 100; Kanamycin

349   [µg/mL]: 0, 0.02, 0.2, 2, 20, 200). Strains as well as the community (100 µL of frozen stock)

350   were grown separately under anaerobic conditions in triplicate reactors, replicates were

351   combined, harvested through centrifugation, washed twice in 0.9% anaerobic NaCl solution

352   and finally resuspended in 0.9% NaCl solution, adjusted to OD_{600} 0.1 (~10^7 bacteria/mL) and

353   subsequently used in competition experiments. Isogenic strains were mixed at 1:1 ratio (no

354   community treatment), and that mix further added at 10% ratio to 90% of the faecal

355   community (community treatment). Approximately 10^6 bacteria of either mix were transferred

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356 to 6 replicate reactors of each of the antibiotic concentrations and grown at 37˚C with 120

357 rpm shaking for 24h which allowed growth up to carrying capacity. 100µL of each reactor

358 were then transferred to a fresh bioreactor, grown for 24h, transferred for a final growth cycle

359 and finally harvested for subsequent analysis.

360

361 **Fitness assay**

362 From each reactor after 3 days (T_3), as well as the inocula (T_0), a dilution series in sterile

363 0.9% NaCl solution was prepared and plated on LB and LB+AB (30 µg/mL Gm or 75 µg/mL

364 Kn). For appropriate dilutions total and resistant red fluorescent *E. coli* colonies were

365 counted under the fluorescence microscope. Plating of the susceptible strain on LB+AB

366 plates further did not lead to any growth of spontaneous mutants. The relative Fitness (ρ) of

367 the resistant (r) compared to the susceptible strain (s) strain was subsequently calculated

368 based on their individual growth rate (γ) throughout the competition experiment:

$$\rho = \frac{\gamma_r}{\gamma_s} = \frac{\log\left(10^6 \times n_r^{T_3}/n_r^{T_0}\right)}{\log\left(10^6 \times n_s^{T_3}/n_s^{T_0}\right)} = \frac{\log\left(10^6 \times n_r^{T_3}/n_r^{T_0}\right)}{\log\left(10^6 \times (n_{total}^{T_3} - n_r^{T_3})/(n_{total}^{T_0} - n_r^{T_0})\right)}$$

369 Statistical significant testing (n=6) was performed using a one-tailed t-test against neutral

370 selection (ρ=1) and ANOVA corrected for multiple testing to compare the relative fitness of

371 different samples.

372

373 **DNA extraction & sequencing**

374 Bacteria from each reactor, as well as inoculum and original pig faecal community were

375 harvested through centrifugation of 2 mL of liquid, followed by DNA extraction using the

376 Qiagen PowerSoil kit as per the manufacturer's instructions. The quality and quantity of the

377 extractions was confirmed by 1% agarose gel electrophoresis and dsDNA BR (Qubit)

378 respectively.

379 16S rRNA gene libraries were constructed using multiplex primers designed to amplify the

380 V4 region (Kozich et al., 2013). Amplicons were generated using a high-fidelity polymerase

381 (Kapa 2G Robust), purified with the Agencourt AMPure XP PCR purification system and

382 quantified using a fluorometer (Qubit, Life Technologies, Carlsbad, CA, USA). The purified

383 amplicons were pooled in equimolar concentrations based on Qubit quantification. The

384 resulting amplicon library pool was diluted to 2 nM with sodium hydroxide and 5 mL were

385 transferred into 995mL HT1 (Illumina) to give a final concentration of 10 pM. 600 mL of the

386 diluted library pool was spiked with 10% PhiXControl v3 and placed on ice before loading

387 into Illumina MiSeq cartridge following the manufacturer's instructions. The sequencing

388 chemistry utilized was MiSeq Reagent Kit v2 (500 cycles) with run metrics of 250 cycles for

389 each paired end read using MiSeq Control Software 2.2.0 and RTA 1.17.28.

390 Metagenomic libraries were created using the KAPA high throughout Library Prep Kit (Part

391 No: KK8234) optimized for 1ug of input DNA with a size selection and performed with

392 Beckman Coulter XP beads (Part No: A63880). Samples were sheared with a Covaris S2

393 sonicator (available from Covaris and Life Technologies) to a size of 350bp. The ends of the

394 samples were repaired, the 3' to 5' exonuclease activity removed the 3' overhangs and the

395 polymerase activity filled in the 5' overhangs creating blunt ends. A single 'A' nucleotide was

396 added to the 3' ends of the blunt fragments to prevent them from ligating to one another

397 during the adapter ligation reaction. A corresponding single 'T' nucleotide on the 3' end of

398 the adapter provided a complementary overhang for ligating the adapter to the fragment

399 ensuring a low rate of chimera formation. Indexing adapters were ligated to the ends of the

400 DNA fragments for hybridisation on a flow cell. The ligated product underwent size selection

401 using the XP beads detailed above, thus removing the majority of un-ligated or hybridized

402 adapters. Prior to hybridisation the samples underwent 6 cycles of PCR to selectively enrich

403 those DNA fragments with adapter molecules on both ends and to amplify the amount of

404 DNA in the library. The PCR was performed with a PCR primer cocktail that anneals to the

405 ends of the adapter. The insert size of the libraries was verified by running an aliquot of the

406 DNA library on a PerkinElmer GX using the High Sensitivity DNA chip (Part No: 5067-4626)

407 and the concentration was determined by using a High Sensitivity Qubit assay. All raw

408 sequencing data has been submitted to ENA under study accession number PRJEB29924.

409

16S Analysis

Sequence analysis was carried out using mothur v.1.32.1 (Schloss et al., 2009) and the MiSeq SOP (Kozich et al., 2013) as accessed on 07.08.2017 on http://www.mothur.org/wiki/MiSeq_SOP. Sequences were classified based on the RDP classifier (Wang et al., 2007). Diversity was assessed based on observed OTUs at 97% sequence similarity. NMDS plots for the community were created after removing all sequences of the focal species *E. coli* based on the Bray-Curtis dissimilarity metric (Bray and Curtis, 1957). Further sample similarity was tested using analysis of molecular variance (AMOVA) a nonparametric analogue of traditional ANOVA testing. AMOVA is commonly used in population genetics to test the hypothesis that genetic diversity between two or more populations is not significantly different from a community created from stochastically pooling these populations (Anderson, 2001; Gravina and Vijg, 2010).

Metagenomic analysis

Metagenomic samples, as well as a reference genome for the focal species *E. coli* MG1655, were analysed using the ARG-OAP pipeline for antibiotic resistance genes detection from metagenomic data using an integrated structured antibiotic resistance gene database (Yang et al., 2016). This resulted in the abundance of different resistance gene classes and subtypes within these groups normalized by 16S rRNA copy number. Antibiotic resistance genes detected in the *E. coli* reference genome were subtracted from the total number of hits per 16S copy based on the abundance of *E. coli* 16S/total 16S. Further, all antibiotic resistance gene numbers were normalized to the amount of pig faecal community 16S per total 16S copy.

Antibiotic inhibition testing

To test if any degradation of antibiotics occurred in any of the reactors, 500µL of filter sterilized (0.22 µm^2 pore size) supernatant of each reactor were applied to a 6mm Grade AA paper disk (Whatman, Maidstone, UK), and a paper disk-agar diffusion assay(Raahave,

19

438   1974) was performed on LB medium supplemented with 1% of an overnight culture of the

439   susceptible strain. After 24h incubation at 37˚C images of the halo zones were taken using a

440   Leica S8APO stereomicroscope (Leica, Wetzlar, Germany). The area of halo zones was

441   determined by image analysis in Inkscape (version 0.91, http://www.inkscape.org/). Three

442   technical replicate disks for each of the six replicate reactors were averaged, for a total of 18

443   measurements per concentration.

444

445   **Mathematical model**

446   In order to illustrate possible mechanisms underlying the data for bacterial fitness in the

447   presence / absence of the community for varying concentrations of gentamicin and

448   kanamycin, we described our experimental setup mathematically. For this we first developed

449   a discrete-time mathematical model for the growth of the susceptible and drug-resistant

450   bacteria, *s* and *r*, respectively, in the presence or absence of the community, *c*.

451

452   *Bacterial growth*

453   The discrete-time model describing the growth of the bacteria *i*, *i=s,r,c*, is governed by the

454   following iterative model

$$n_i^{t+1} = n_i^t\big(1 + \phi_i(1 - g_i)(1 - f_i)\big),$$

455   where n_i^{t+1} is the size of the population of strain *i* at time *t*+1, and ϕ_i is the maximum growth

456   rate in the absence of competition and drug pressure. The reduction in growth due to

457   density-dependent regulation / resource limitation, given as

$$g_i = \frac{\sum_j e_{ij}\, n_j}{k_d},$$

458   with k_d as the carrying capacity and e_{ij} being the competition coefficient, describing how

459   much the presence of an allospecific strain *j* impacts the competitive fitness of strain *i*. The

20

460   reduction in bacterial growth due to drug pressure, f_i, is governed by a generalised logistic

461   function

$$f_i = \min\left(f_{\max}, \frac{1}{1 + e^{\alpha_i - \beta_i \ln c}}\right)$$

462   where c is the drug concentration (in μg/mL), α_i and β_i are the parameters describing the

463   dose-response relationship for strain i, and $f_{\max}$ =0.9 is the maximum growth inhibition.

464

465

466   _Model simulation and relative fitness calculation_

467   Starting from an initially small number of bacteria in fresh medium, we ran the model for 30

468   generations, at which point the bacterial population had reached carrying capacity, and

469   diluted the population accordingly. The bacteria were again allowed to grow for 30

470   generations before being diluted and grown for a final 30 generations. At this point we

471   calculated the relative fitness of the resistant strain as

$$\rho = \frac{\gamma_r}{\gamma_s} = \frac{\log(10^6 \times n_r^{90}/n_r^0)}{\log(10^6 \times n_s^{90}/n_s^0)}.$$

472

473   _Community-dependent change in drug resistance / susceptibility_

474   The kanamycin data seem to suggest that the benefit of the drug resistant bacteria is

475   reduced in the presence of the community at medium to high drug concentrations pointing

476   towards a decrease in the susceptibility of the susceptible strain in a community context. We

477   captured this scenario by making the dose-response parameters $\alpha_{s,r}$ and $\beta_{s,r}$ explicitly

478   dependent on the density of the community by increasing the resistance of susceptible

479   strain, s, i.e.

480

21

481
$$\alpha_s(t) = \alpha_{s,0}\left(1 + \frac{1.3\,n_c^t}{n_c^t + 10^3}\right),$$

$$\beta_s(t) = \beta_{s,0}\left(1 + \frac{0.35\,n_c^t}{n_c^t + 10^3}\right),$$

482   where $\alpha_{i,0}$ and $\beta_{i,0}$ are the time-independent dose-response parameters (Table 2). The effect

483   of density dependence is further illustrated in Figure SI4.

484

485   **Parameter estimations**

486   For each drug (gentamicin and kanamycin) we obtained a set of parameter values that

487   resulted in a good overall fit between the model simulations and the data, where the data

488   comprised the observed relative fitness for both sets of experiments (i.e. bacteria grown in

489   the presence and absence of the community) for six different drug concentrations. To allow

490   for logarithmic regression the non-antibiotic control was assumed as one order of magnitude

491   lower than the lowest concentration used in the experiment. The parameter values were

492   determined by minimising the root-mean-square error using an optimisation algorithm akin to

493   simulated annealing (Kirkpatrick et al., 1983). The aim here was not to perform rigorous

494   parameter estimation but rather to find a set of parameters that, given specific model

495   constraints and assumptions, resulted in model behaviours that qualitatively agreed with

496   both the observed dynamics over the repeated growth cycles and the empirically determined

497   fitness values. In fact, our method failed to find a unique set of values that consistently gave

498   the best fitting model, which suggests that the available data was insufficient to determine

499   the global maximum. However, the qualitative relationships between individual parameters

500   and between the parameters comparing the two antimicrobials were fairly consistent

501   between model runs. Tables 1 and 2 list the sets of parameters as used in Figures 1-2.

502

Acknowledgments

Author contributions

UK, LZ, AB and WG conceived the study and designed experiments; UK performed experimental work; MR performed mathematical modelling; UK, XY and TZ performed sequencing analysis; UK, AB, WG analysed data and wrote the manuscript.

Competing interests

The authors declare no competing interests.

Materials & Correspondence

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