Cross-feeding modulates antibiotic tolerance in bacterial communities.

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Running Title - Cross-feeding alters bacterial tolerance to antibiotics

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Abstract

1 Microbes frequently rely on metabolites excreted by other bacterial species, but little is known

2 about how this cross-feeding influences the effect of antibiotics. We hypothesized that when

3 species rely on each other for essential metabolites, the minimum inhibitory concentration (MIC)

4 for all species will drop to that of the "weakest link" - the species least resistant in monoculture.

5 We tested this hypothesis in an obligate cross-feeding system that was engineered between

6 *Escherichia coli, Salmonella enterica,* and *Methylobacterium extorquens.* The effect of

7 tetracycline and ampicillin were tested on both liquid and solid media. In all cases, resistant

8 species were inhibited at significantly lower antibiotic concentrations in the cross-feeding

9 community than in monoculture or a competitive community. However, deviation from the

10 "weakest link" hypothesis was also observed in cross-feeding communities apparently as result

11 of changes in the timing of growth and cross-protection. Comparable results were also observed

12 in a clinically relevant system involving facultative cross-feeding between *Pseudomonas*

13 *aeruginosa* and an anaerobic consortium found in the lungs of cystic fibrosis patients. *P.*

14 *aeruginosa* was inhibited by lower concentrations of ampicillin when cross-feeding than when

15 grown in isolation. These results suggest that cross-feeding significantly alters tolerance to

16 antibiotics in a variety of systems.

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Introduction

Antibiotic resistant bacteria pose a considerable public health threat worldwide; the World Health Organization reports that 25-50% of hospital-acquired pathogens are now multiple-drug-resistant (World Health Organization., 2014). Despite extensive research on the cellular mechanisms of resistance in many bacterial species (Friman et al., 2015; Hughes and Andersson, 2012), a growing body of research suggests that a single-species view of pathogen response to an antibiotic may be incomplete. Many infections are now known to involve multiple pathogens (de Vos et al., 2017; Tay et al., 2016) or interactions between pathogens and commensals, (Delhaes et al., 2012; Murray et al., 2014; Ramsey et al., 2011). As well, we still have little understanding of how ecological interactions between microbes influence the impact that antibiotics have on populations and communities of bacteria.

Growth in a polymicrobial consortium can influence a species' antibiotic tolerance by multiple mechanisms (Perlin et al., 2009; Yurtsev et al., 2016). First, resistant species can protect more sensitive species by degrading antibiotics. For example, β-lactamase production leads to the degradation of β-lactam antibiotics, thereby detoxifying shared growth medium (Medaney et al., 2016; Perlin et al., 2009). Additionally, secretions from one species can induce resistance mechanisms in others. Secretion of the signalling molecule indole has been shown to increase resistance by activating stress-response pathways in *S. enterica* (Vega et al., 2013) and efflux pump expression in *Pseudomonas putida* (Molina-Santiago et al., 2014). Community growth induces physiological changes in bacteria (Straight and Kolter, 2009), which may alter their antibiotic tolerance by increasing drug uptake or slowing their metabolic rate (Hong et al., 2014; Peng et al., 2015). Further, secretions that change environmental pH have been suggested to alter the impact of antibiotics (Bernier et al., 2011; de Vos et al., 2017). Spatial structure may also

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42   play a role in these protective interactions; a synthetic community of *Pseudomonas aeruginosa,*

43   *Pseudomonas protegens,* and *Klebsiella pneumoniae* was found to have greater tobramycin

44   resistance when grown as a multi-species biofilm versus single species biofilm or multispecies

45   planktonic culture (Lee et al., 2014). However, in many cases, the mechanisms underlying

46   communities' effects on resistance remain unclear (de Vos et al., 2017).

47   The primary mechanism of interaction between bacteria is through extracellular metabolites, yet

48   few studies have investigated how metabolic interactions in a bacterial community modulate the

49   impact of antibiotics (Manor et al., 2014). One such metabolic interaction, called cross-feeding,

50   occurs when metabolites produced by one organism are used as a nutrient or energy source by

51   another (Rosenzweig et al., 1994; Schink, 2002). This phenomenon is nearly ubiquitous in

52   microbial communities (Pande et al., 2014; Seth and Taga, 2014; Zelezniak et al., 2015) and is

53   thought to contribute to our inability to cultivate most bacterial species in isolation (Kaeberlein et

54   al., 2002; Oliveira et al., 2014). Cross-feeding has also been shown to play a critical role in the

55   human microbiome. For example, *Bifidobacterium* species in the gut cooperate with each other

56   to break down complex plant-based carbohydrates (Milani et al., 2015), which releases carbon

57   for butyrate- producing bacteria. Cross-feeding in the gut also increases the efficiency of glycan

58   breakdown by removing waste products such as H_2 (Koropatkin et al., 2012). Additionally,

59   cross-feeding can influence pathogen survival. For example, growth and virulence of the oral

60   pathogen *Aggregatibacter actinomycetemcomitans* depends on L-lactate generated by the

61   commensal *Streptococcus gordonii* (Stacy et al., 2016). Given the ubiquity and importance of

62   cross-feeding in human-associated microbial communities, greater investigation in to how cross-

63   feeding influences population and community responses to antibiotics is needed.

64   In this work, we test how cross-feeding changes the effect of antibiotics on bacteria in microbial

65   communities. We use the term tolerance to signify the ability of species to grow in a given

66   antibiotic concentration. Tolerance as we define it can change as a function of physiological state

67   or environmental conditions, while changes in resistance would require a change in DNA

68   sequence (Brauner et al., 2016). We hypothesize that when species depend on one another the

69   community tolerance (i.e. the level of antibiotic required to inhibit detectable growth of the

70   community as a unit) will be set by the tolerance of the 'weakest link', or the most antibiotic-

71   sensitive community member. Alternatively, community tolerance may be higher than that of the

72   weakest species in monoculture (the 'community protection' hypothesis), or lower (the

73   'community sensitivity' hypothesis). Higher than expected tolerance may occur if one or more

74   species in a community excretes a compound which either actively degrades antibiotics in the

75   medium (Perlin et al., 2009; Stiefel et al., 2015), or which activates tolerance mechanisms such

76   as efflux pump expression in neighboring species (El-Halfawy and Valvano, 2012; Molina-

77   Santiago et al., 2014). Lower than expected tolerance could result if sub-lethal concentrations of

78   antibiotic, while not sufficient to arrest or kill any one species, sufficiently disrupt cross-feeding

79   to inhibit community growth.

80   We tested the impact of cross-feeding on tolerance in a variety of conditions. We started with an

81   engineered obligate mutualism involving *Escherichia coli, Salmonella enterica* serovar

82   Typhimurium*,* and *Methylobacterium extorquens* (**Figure 1**, Harcombe et al, 2014). The

83   tolerance of the mutualism was compared to the tolerance of each species in monoculture. These

84   tests were done with ampicillin and tetracycline because the drugs have different mechanisms of

85   action (Perlin et al., 2009, Hong et al., 2014), and because ampicillin can be enzymatically

86   degraded (Munita and Arias, 2016), allowing the potential for cross-protection of sensitive

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87   species (Perlin et al., 2009). Further, we tested the impact of these antibiotics in both liquid

88   media and agar plates to test the influence of spatial structure. Finally, to test the generality of

89   our findings we investigated the effect of cross-feeding on tolerance in a model relevant for

90   cystic fibrosis. This system involves the pathogen *P. aeruginosa*, and a previously defined

91   anaerobic consortium of bacteria that provide carbon for the pathogen when growing on mucin

92   (Flynn et al., 2016).

93   Across all treatments and in both the model and clinically relevant systems, resistant bacteria

94   were inhibited by lower concentrations of antibiotic when cross-feeding than when growing

95   independently. However, we found that cross-feeding can provide protection to more sensitive

96   bacteria. For both ampicillin and tetracycline, cases arose in which tolerance was higher than

97   predicted based on measurements of tolerance in monoculture. Our results demonstrate that

98   metabolic interactions impact antibiotic tolerance in a community, and suggest that antibiotic-

99   resistant pathogens may be treated by targeting their less tolerant metabolic partners.

100

101   **Methods**

102   **Bacterial strains and media**

103   The three species community contained strains of *Escherichia coli, Salmonella enterica* and

104   *Methylobacterium extorquens* all of which have been described previously (Harcombe et al.,

105   2014). Briefly, the *E. coli* str. K12 contains a Δ*metB* mutation. The *S. enterica* mutant excretes

106   methionine as a result of mutations in *metA* and *metJ* (Douglas et al., 2017, 2016; Harcombe,

107   2010). The *M. extorquens* AM1 Δ*hprA* mutant is unable to assimilate carbon from C1

108   compounds (Marx, 2008). In lactose minimal medium, the three species rely on each other for

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109 essential metabolites. *E. coli* secretes acetate byproducts which the other species rely on for a

110 carbon source. *M. extorquens* releases ammonia byproducts which provide a source of nitrogen

111 for other species. *S. enterica* secretes methionine, which is essential because our *E. coli* strain is

112 auxotrophic for this amino acid. Each species has a unique fluorescent label integrated into its

113 genome: cyan fluorescent protein (CFP) for *E. coli*, yellow fluorescent protein (YFP) for *S.*

114 *enterica,* and red fluorescent protein (RFP) for *M. extorquens.* Bacteria were grown in minimal

115 Hypho media containing varying amounts and types of carbon and nitrogen, depending on the

116 medium type (see **Figure 1**). *Pseudomonas aeruginosa* strain PA14 (Rahme et al., 1995) was

117 obtained from D.K. Newman (Caltech). The anaerobic consortium (composed primarily of

118 *Prevotella sp., Veillonella sp., Fusobacterium sp., and Streptococcus sp.*) was derived from

119 human saliva and was enriched using porcine gastric mucin as previously described (Flynn et al.,

120 2016).

121

Liquid media experiments

123 Bacteria were inoculated along an antibiotic gradient to measure the minimum inhibitory

124 concentration (MIC) for monocultures and co-cultures. The OD of each culture was taken to

125 ensure cells were in mid-log phase (OD~0.2-0.3) and cultures were diluted 1/200. Cells were

126 inoculated into relevant wells on a 96-well plate, with fresh Hypho and varying concentrations of

127 an antibiotic. The inoculate size for a species was kept constant at $\sim10^4$ in monoculture and

128 community (i.e. community treatments started with 3x more total cells than monocultures).

129 Ampicillin was used at 0, 0.2, 0.5, 1, 2, and 5 µg/mL for *E. coli*, *S. enterica*, and the

130 communities, and at 0, 2, 5, 10, 20, and 50µg/mL for *M. extorquens*; these concentrations

131 provided the best range of sublethal to lethal ampicillin concentrations. Tetracycline was used at

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132   0, 1, 2, 3, 5, and 10µg/mL for *E. coli*, *M. extorquens*, and the community, and 0, 10, 20, 30, 50,

133   and 100µg/mL were used for *S. enterica*. 96-well plates were placed into a Tecan InfinitePro 200

134   at 30°C for 120 hours. Measurements of OD600 were taken every 15 minutes to track overall

135   bacterial growth, and fluorescence measurements were taken to track the growth of individual

136   species. The minimum inhibitory concentration (MIC) was defined as the lowest antibiotic

137   concentration at which no growth was seen by three times the lag time of the antibiotic-free

138   control.

139

140   **Solid media antibiotic susceptibility experiments**

141   Resistance on plates was determined by measuring the diameter of the zone of inhibition around

142   an antibiotic containing disk. For monocultures, 150µL of log-phase culture (OD=0.5) was

143   spread on Hypho plates (1% agar) in a lawn; for communities, 150µL of culture from each

144   species was mixed, spun down, and re-suspended in 150µL of the appropriate community

145   medium before plating onto Hypho plates. Discs of filter paper 6mm in diameter were inoculated

146   with 25 µg antibiotic, and left to dry for 10 minutes. Discs were applied to the center of plates

147   with bacteria, and plates were incubated at 30°C for 48 hours (*E. coli*, *S. enterica*, competitive

148   community) or 72 hours (*M. extorquens*, cooperative community), depending on how long it

149   took for cells outside the zone of inhibition to become confluent. The diameter of the zone of

150   clearing was measured three times for each plate and averaged to provide a single plate

151   measurement.

152

153   **Fluorescence microscopy**

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154   Fluorescent microscope images were obtained using a Nikon AZ100 Multizoom macroscope

155   with a C1si Spectral confocal attachment, 4x objective lens at 3.40x magnification using Nikon

156   NIS Elements software. The 457nm argon laser was used to visualize CFP, the 514nm laser was

157   used for YFP, and the 561nm laser was used for RFP. Disc diffusion Petri plates were placed on

158   the stage and images from 2x10 fields of view (for monocultures) or 2x12 fields of view (for

159   communities) were obtained and stitched together using Elements software. Images for each

160   fluorescent protein were overlaid using Fiji image analysis software (Schindelin et al., 2012).

161

162   **Testing β-lactamase production**

163   Nitrocefin discs (Sigma-Aldrich, 49862) were used to determine if *M. extorquens* was producing

164   a β-lactamase. For solid medium, cells were scraped off agar plates and suspended in the

165   appropriate liquid medium; the OD600 of the suspension was then determined and diluted to an

166   OD600 of ~0.5, to match the OD600 of liquid cultures. Discs were placed on a microscope slide

167   and 15µL of liquid culture or diluted solid medium suspension was added to the disc. After 60

168   minutes, a color change from yellow to purple/pink indicated the production of a β-lactamase

169   that hydrolysed the nitrocefin in the disc (O'Callaghan et al., 1972). As a positive control, an *E.*

170   *coli* strain carrying a pBR322 plasmid, which contains a *bla* β-lactamase gene was also tested,

171   and the plasmid-free *E. coli* strain was used as a negative control.

172

173   *Pseudomonas aeruginosa* **cross-feeding model**

174   Antibiotic tolerance assays were performed in minimal medium containing 1 mM magnesium

175   sulfate, 60 mM potassium phosphate (pH 7.4), 90 mM sodium chloride, trace minerals (Marsili

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176   et al., 2008) and supplemented with autoclaved and dialyzed pig gastric mucin (30g/L, Sigma-

177   Aldrich) for co-cultures or glucose (12 mM) for *P. aeruginosa* monoculture. Ampicillin was

178   added at indicated concentrations. For mucin fermenting community assays, cultures were

179   inoculated from freezer stock characterized previously (Flynn et al., 2016) and allowed to grow

180   under anaerobic conditions containing carbon dioxide, hydrogen and nitrogen (5:5:90) at 37°C

181   for 48 hrs. For *P. aeruginosa* assays, cultures were inoculated from overnight cultures grown in

182   LB and allowed then to grow while shaking aerobically at 37°C for 16 hrs. Optical densities

183   were determined using a Biotek Synergy H1 plate reader. Optical densities are given as mean

184   and standard deviation of three replicates.

185   Cross-feeding assays were performed as described previously (Flynn et al., 2016). Briefly, a

186   mucin fermenting community from freezer stock was inoculated into the minimal mucin medium

187   described above and allowed to grow for 48 hrs anaerobically at 37°C. This culture was used to

188   inoculate the lower phase (1:100 dilution) which contained 2 mL of minimal mucin medium, 1%

189   agar, and supplemented with ampicillin as indicated. After solidification of the mucin fermenting

190   agar cultures in 16mm glass culture tubes, *P. aeruginosa* PA14 was added to buffered media

191   containing no mucin and 0.7% agar to 1/1000 from an LB overnight culture. This mixture was

192   then added to the top of the mucin fermenting community. After agar solidification, the tubes

193   were placed in 37°C. After 60 hrs, the top agar section (containing PA14) was removed,

194   homogenized by pipette in sterile saline, serially diluted, and plated on Luria Broth agar plates to

195   enumerate PA14 in each culture.

196

197   **Statistical analyses**

198   For liquid and solid media assays, at least eight replicates of each treatment were obtained for

199   each antibiotic. Pairwise comparisons between monocultures and co-cultures were conducted

200   using a Mann-Whitney U test with an applied Bonferroni correction for ten multiple

201   comparisons. For *P. aeruginosa* cross-feeding assays, triplicate experiments were performed for

202   each antibiotic concentration and community type. Normalized CFU values were calculated by

203   dividing CFU at each antibiotic concentration by the CFU at 0ug/mL ampicillin. Comparisons of

204   normalized values at each concentration were performed using a Mann-Whitney U test.

205

206   **Results**

207   **Dependence on other species reduces the amount of antibiotic necessary to inhibit resistant**

208   **bacteria in liquid media.**

209   In monoculture experiments, one species was at least 25 times more resistant than others, though

210   the most resistant species was different for each antibiotic. When grown in the presence of

211   ampicillin, *M. extorquens* had a median MIC of 100 µg/ml, which was significantly higher than

212   the 2 µg/ml and 1 µg/ml necessary to inhibit *E. coli* and *S. enterica* respectively (**Figure 2A**,

213   *P*<0.0001 for each). For tetracycline, *S. enterica* had a median MIC of 50 µg/ml (**Figure 2B**).

214   This was significantly higher than the median MIC of 5µg/ml in *M. extorquens* (*P* <0.0001) and

215   2 µg/ml in *E. coli* (*P* <0.0001).

216   When bacteria were grown in an obligate mutualism rather than in monocultures, the amount of

217   antibiotic needed to inhibit resistant species decreased substantially. Fifty-fold less ampicillin

218   was needed to inhibit the cooperative community (1µg/mL) than to inhibit *M. extorquens* in

219   monoculture (**Figure 3A,** *P* < 0.0001). Similarly, the median MIC of tetracycline for *S. enterica*

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220   decreased significantly from 50 μg/ml in monoculture to 4 μg/ml in the cooperative community

221   (**Figure 3B**, *P* <0.0001).

222   We next distinguished the effect of species interactions from community complexity by

223   measuring the MIC of the bacterial community when the species were not reliant on one another

224   for resources (**Figure 1B, Figure 2**). The median MIC for resistant bacteria was not

225   significantly different in the competitive community than it was in monoculture (for *M.*

226   *extorquens* in ampicillin **Figure 3A**, *P* >0.90; for *S. enterica* in tetracycline, **Figure 3B**, *P*=0.80).

227   Therefore, the decreased tolerance of resistant species to these antibiotics was a result of the

228   metabolic interdependence, rather than simply the presence of other species.

229   We also repeated monoculture and competitive community experiments using acetate as the sole

230   carbon source for *S. enterica* and *M. extorquens* to ensure that the change in carbon source

231   between treatments was not responsible for changes in tolerance (**Supplementary figure 1**). We

232   found that the same qualitative patterns were observed with *M. extorquens* showing high

233   tolerance to ampicillin and *S. enterica* demonstrating high tolerance to tetracycline in both

234   monoculture and competitive community. However, we found that growth was less robust, and

235   that high initial concentrations of acetate may be toxic to *M. extorquens*.

236

237   **Tolerance of tetracycline was higher than expected in the cross-feeding community**

238   Unexpectedly, the MIC of tetracycline for the cooperative community was higher than the least

239   resistant monoculture, *E. coli* (median monoculture MIC 2μg/mL, median cooperative

240   community MIC 4μg/mL, *P* =0.0111). This effect was not due to higher starting cell density in

241   community (**Supplementary fig. 3A**). We therefore suspected the increased tolerance of *E. coli*

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242   in cooperative community might be due to increased lag times for *E. coli* in the cooperative

243   community. As tetracycline is known to rapidly break down, we predicted that the slower growth

244   in community might be protecting *E. coli* by allowing time for tetracycline to break down before

245   growth started. Consistent with this prediction, the time to detectable *E. coli* growth was

246   significantly longer in cooperative community than it was in monoculture ($P = 0.003$), and the

247   MIC of *E. coli* in monoculture increased if media containing tetracycline was allowed to sit for

248   20 hours before cells were added (**Supplementary figure 3B).** Therefore, we concluded that the

249   increased tolerance of *E. coli* to tetracycline in cooperative community was likely influenced by

250   delayed *E. coli* growth relative to monoculture.

251   The competitive community appears to decrease the effective resistance of *M. extorquens* to

252   tetracycline. In monoculture, the tetracycline MIC for *M. extorquens* was 5μg/ml, while in the

253   competitive community *M. extorquens* growth was not observed even in the absence of antibiotic

254   (**Figure 3B**). This indicates that competitive exclusion, rather than antibiotic tolerance, governs

255   the growth of *M. extorquens* in this context. *M. extorquens* was not competitively excluded in the

256   ampicillin treatments because it can grow at high ampicillin concentrations where its better

257   competitors cannot. (**Supplementary figure 5**). This suggests that growth patterns of *M.*

258   *extorquens* in tetracycline are governed by competitive ability rather than resistance, while in

259   ampicillin *M. extorquens* experiences competitive release.

260

261   **Resistant bacteria are also constrained by sensitive partners in structured environments**

262   We next tested whether growth on agar plates (rather than growth in liquid media) altered the

263   impact that species interactions had on antibiotic resistance. We grew confluent bacterial lawns

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264   and measured the zone of clearing around an antibiotic disc at the center. All discs contained

265   2.5μL of 10mg/mL antibiotic. We hypothesized that spatial structure might enhance the ability of

266   resistant bacteria to protect metabolic partners from degradable antibiotics like ampicillin, and

267   thereby eliminate the reduction of tolerance in the cross-feeding system.

268   Resistance on agar largely mirrored results from the liquid media. As in liquid, *M. extorquens*

269   was the most resistant monoculture on ampicillin, and *S. enterica* was the most resistant

270   monoculture in tetracycline (**Figure 4**). Note that small clearing diameters signify high

271   resistance, so the relative rankings in **Figure 4** are the inverse of **Figure 3**. We also once again

272   observed that less antibiotic was needed to inhibit resistant bacteria in cooperative community

273   than was needed in monoculture. Ampicillin cleared growth of *M. extorquens* out to a diameter

274   of 32.7 mm in cooperative community, but only out to 15.7 mm in monoculture (**Figure 4A**, P =

275   0.002). Similarly, on tetracycline, *S. enterica* had a median diameter of clearing of 9.92 mm in

276   monoculture and 33.7 mm in the cooperative community (**Figure 4B**, P <0.0001). In the

277   competitive community, the zones of clearing matched those of the most resistant monoculture.

278   Using acetate as the carbon source for the competitive community (in addition to lactose) and *S.*

279   *enterica* and *M. extorquens* monocultures showed qualitatively similar results (**Supplementary**

280   **figure 2**).

281   Though *M. extorquens* had lower tolerance for ampicillin in cooperative community, we did

282   observe cross-protection of the more sensitive species. The cooperative community had a

283   significantly smaller zone of inhibition than either *S. enterica* or *E. coli* monocultures (**Figure**

284   **4A,** P <0.0001 for *S. enterica* and P =0.01 for *E. coli*). Fluorescent images indicate that

285   inhibition of both sensitive species was reduced in the presence of *M. extorquens* (**Figure 5**). In

286   both cooperative (**Figure 5D**) and competitive (**Figure 5E**) community, *E. coli* and *S. enterica*

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287   grew closer to the disk of ampicillin in co-culture than they did in monoculture (**Figure 5A,B**).

288   We found that protection was not due to an increase in initial cell density on community verses

289   monoculture plates (**Supplementary figure 6A**), and that *M. extorquens* was responsible for

290   providing the protection (**Supplementary figure 5B**). Consistent with the observed cross-

291   protection, ampicillin degrading β-lactamases were found in the genome of *M. extorquens*.

292   Further, nitrocefin disks were used to demonstrate β-lactamase activity when *M. extorquens* was

293   grown on agar in the presence of ampicillin (**Supplementary Figure 7**).

294

Metabolic dependency reduces antibiotic tolerance of a pathogen

296   To test whether our findings extend to medically relevant systems, we investigated how co-

297   culturing influences the effective tolerance of *Pseudomonas aeruginosa* to ampicillin. *P.*

298   *aeruginosa* is commonly found in the lung and is a leading cause of morbidity and mortality in

299   people with cystic fibrosis (Langton Hewer and Smyth, 2017) . Further, it was recently

300   demonstrated that *P. aeruginosa* can cross-feed on carbon generated by mucin-degrading

301   anaerobes (Flynn et al., 2016) . In addition to its medical relevance, this system is distinct from

302   our previous system in that the cross-feeding is not obligate (*P. aeruginosa* growth on mucin

303   decreases but is not abolished by the absence of the anaerobes). We tested how ampicillin

304   influenced the growth of *P. aeruginosa*, when grown alone on glucose versus in a cross-feeding

305   co-culture.

306   Consistent with previous findings (Krogfelt et al., 2000), *P. aeruginosa* was highly resistant to

307   ampicillin in monoculture (**Figure 7A**). No observable decrease in final yield was observed even

308   out to 25 μg/ml of the drug. In contrast, the mucin-degrading anaerobes were inhibited by 5

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309   µg/ml of ampicillin. Consistent with expectations, 5µg/mL ampicillin was sufficient to

310   significantly reduce the density of *P. aeruginosa* grown in co-cultures on mucin, as compared to

311   *P. aeruginosa* growth alone (P =0.0334). Similar results were observed if the *P. aeruginosa*

312   monoculture was grown on mucin rather than glucose, however the maximum growth was

313   substantially lower (**Supplementary Figure 8**). These data suggest that applying antibiotic to

314   inhibit the growth of cross-feeding partners can inhibit resistant species even in a non-obligate

315   cross-feeding system of clinical significance.

316

317   **Discussion**

318   Our results demonstrate that metabolic dependency between microbial community members

319   plays a critical role in mediating the effect of antibiotics. In both mass-action (liquid) and

320   structured (solid) environments we observed that bacterial species which show high levels of

321   tolerance to a given antibiotic in monoculture are inhibited at much lower concentrations in an

322   obligate mutualism. This was true for two antibiotics with different modes of action. Further, the

323   effect of metabolic dependence on antibiotic tolerance extended to a medically relevant system

324   with facultative cross-feeding. *P. aeruginosa* growth was reduced by substantially lower

325   concentrations of ampicillin when the pathogen was cross-feeding off of mucin- degrading

326   anaerobes that were sensitive to the drug. The constraint that cross-feeding places on the

327   tolerance of bacteria was consistent across drugs and microbial systems.

328   We have shown that the ability of a given bacterial species to grow in the presence of an

329   antibiotic is a combination of its intrinsic tolerance and the tolerance of species on which it relies

330   for metabolites. Dependence on other bacteria reduced the MIC of bacteria with high resistance

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331   in monoculture. This change in MIC was driven by inhibition of a beneficial partner rather than a

332   change in the resistance of the focal species. The effective tolerance of a cross-feeding network,

333   therefore, is generally set by the 'weakest link' species; that is, the species with the lowest

334   resistance to the antibiotic, whose tolerance in community usually matches its monoculture

335   tolerance This suggests that antibiotics will often be more effective at controlling polymicrobial

336   communities where there is extensive metabolic interdependence, and that tolerance in cross-

337   feeding communities can be approximated from monoculture studies.

338   Unexpectedly, we did see deviations from our weakest link hypothesis. *E. coli* had a higher

339   tetracycline MIC in the cross-feeding community than in monoculture, suggesting that cross–

340   feeding provided some protection to *E. coli*. This slight, but significant, increase was likely

341   driven by an increase in time to detectable *E. coli* growth when cross-feeding. Tetracycline

342   breaks down rapidly, so delaying growth likely allowed microbial populations to experience

343   reduced antibiotic concentrations. Similar antibiotic dynamics may often occur in clinical or

344   environmental settings, (Yılmaz and Özcengiz, 2017, Oka et al., 1989), where metabolically

345   inactive "persisters" commonly survive antibiotic treatment by delaying growth (Brauner et al.,

346   2016). Community context further altered tolerance by enabling cross-protection of less tolerant

347   species by more tolerant partners. On agar plates with ampicillin, both *E. coli* and *S. enterica*

348   were able to grow closer to the antibiotic disk in the presence of *M. extorquens*. This protection

349   was likely caused by degradation of the antibiotic as a result of β-lactamase activity in *M.*

350   *extorquens*. Our results are consistent with previous observations that spatial structure can allow

351   bacteria to lower local antibiotic gradients sufficiently to permit growth of sensitive isolates

352   (Perlin et al., 2009). It is important to note, however, that there were limits on the extent of cross-

353   protection in our community. The cross-feeding community increased the tolerance of *E. coli*

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354   and *S. enterica* but, the tolerance of *M. extorquens* was still lower in the cooperative community

355   than it was in monoculture. Cross-protection may reduce the magnitude of the constraints placed

356   on resistant species by their more sensitive metabolic partners, but it does not eliminate this

357   constraint.

358   This study also demonstrates some issues which can arise when measuring the effect of

359   antibiotics in microbial communities. It has previously been shown that MIC is a troublesome

360   metric that can be influenced by factors such as changes in initial microbial density, or metabolic

361   state (Artemova et al., 2015; Brauner et al., 2016). In our study, it was not possible to measure an

362   MIC of tetracycline for *M. extorquens* in the competitive community, as *M. extorquens* was

363   always outcompeted. The competitive release of *M. extorquens* in ampicillin treated competitive

364   communities again deviates from standard patterns for MIC. Our results highlight that the

365   community context further complicates the challenges associated with interpreting MIC

366   measurements, especially for polymicrobial communities.

367   The constraint that cross-feeding places on antibiotic tolerance also extended to a polymicrobial

368   community relevant to cystic fibrosis. It should be noted that this system involved facultative

369   cross-feeding, so inhibiting anaerobes only reduced the yield of *P. aeruginosa* by two-fold.

370   While this reduction is substantially smaller than the complete elimination of growth in the

371   obligate system, two-fold changes may be medically relevant (Brotons et al., 2016; Cox et al.,

372   2016; Pan et al., 2016; Priest et al., 2017; Weber et al., 2010). More broadly, the constraint in

373   this treatment speaks to the generality of our findings. Even in scenarios with less extreme

374   metabolic dependency, the impact of antibiotics can be magnified when highly resistant species

375   are cross-feeding from less resistant species. Given that metabolic interactions are common in

376   infection contexts (Murray et al., 2014; Ramsey et al., 2011), this work suggests that even

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377   narrow-spectrum antibiotics, designed to target a single species, may have ripple effects

378   throughout a metabolically interconnected community.

379   Our results highlight that mutualistic networks are highly susceptible to environmental change.

380   This result is consistent with work in other ecological systems from plant-pollinator to insect-

381   symbiont (Gehring et al., 2014; Kikuchi et al., 2016; Silverstein et al., 2015). Integrating these

382   ecological concepts into a microbial perspective may allow greater precision in our medical

383   practices. Broad-spectrum reductions of bacteria in the gut can cause long-lasting negative health

384   outcomes such as facilitating infections by *Clostridium difficile* (Chang et al., 2008). To develop

385   precision treatments we need to be able to predict the impact of a drug on a focal population, and

386   how a drug will affect off-target members of a microbial community. Our work highlights that

387   precision microbiome management will require not only improved pharmacology but also a more

388   comprehensive understanding of ecological interactions in microbial systems.

389

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394

395   **<u>Conflicts of interest</u>**

395   The authors declare no conflicts of interest.

396   **<u>References</u>**

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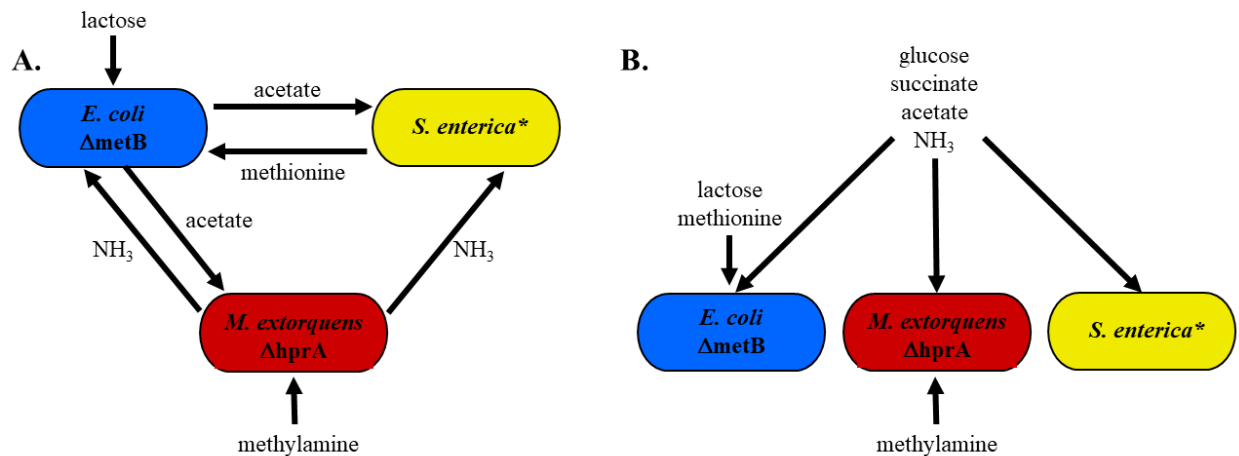
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Figure 1. Cooperative and competitive model communities. **A.** Cooperative community.

Methylamine and lactose are supplied in the growth medium as a nitrogen and a carbon source,

respectively. *E. coli* consumes lactose and excretes acetate as a carbon source for *S. enterica* and

M. extorquens. *S. enterica* secretes methionine for the methionine auxotroph *E. coli*. *M.*

extorquens, which has a deletion in *hprA* which renders it unable to assimilate carbon from

methylamine, provides nitrogen to the community via methylamine breakdown. **B.** Competitive

community. Growth medium contains all metabolites necessary for growth of each individual

species such that no cross-feeding is necessary to support growth.

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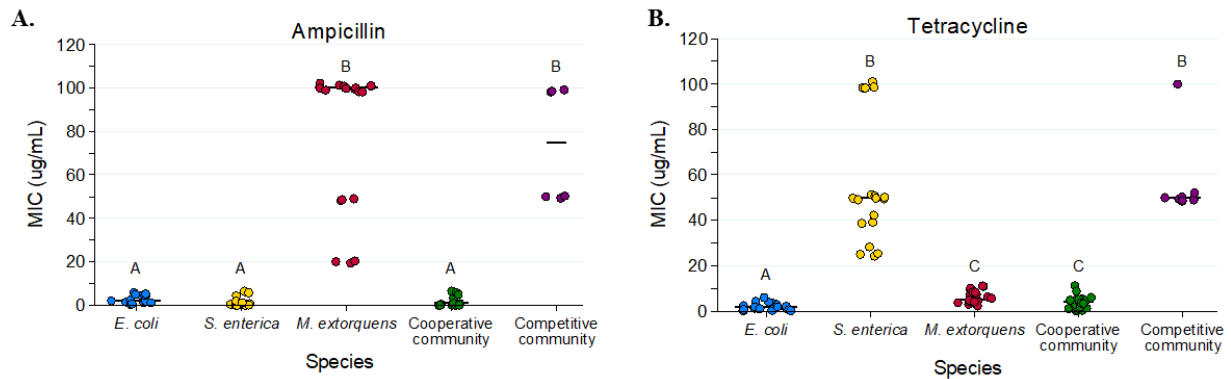
Figure 2. Minimum inhibitory concentration (MIC) values for each monoculture and community type in ampicillin (**A**) and tetracycline (**B**) based on OD600. Bars represent median values of MIC. At least eight replicates were performed for each species/antibiotic combination. Pairwise comparisons of median MIC were performed using a Mann-Whitney U test, with a Bonferroni correction applied for ten pairwise multiple comparisons. Letters above data points indicate significant differences between groups.

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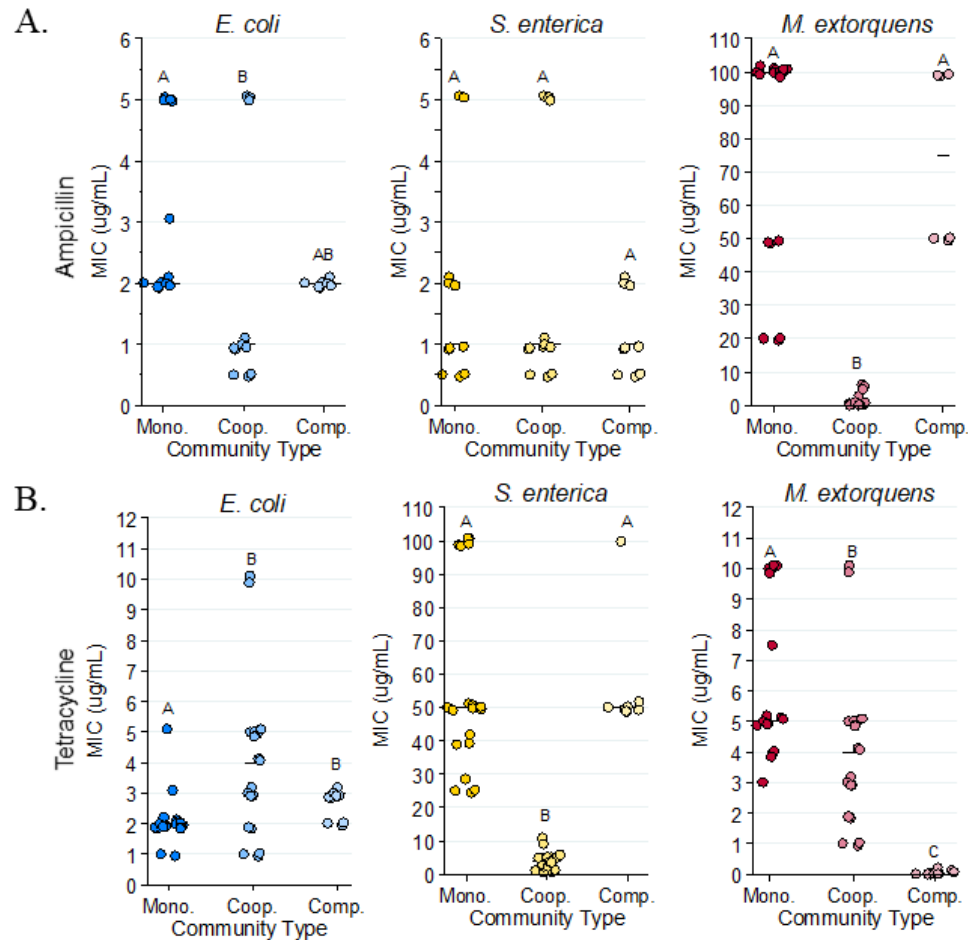
Figure 3. Minimum inhibitory concentration (MIC) values for each species in monoculture,

cooperative community, and competitive community in ampicillin (**A**) and tetracycline (**B**).

MICs were calculated based on fluorescence. Pairwise comparisons of median MIC were

performed using a Mann-Whitney U test, with a Bonferroni correction applied for three multiple

comparisons. Letters above data points indicate significant differences between groups.

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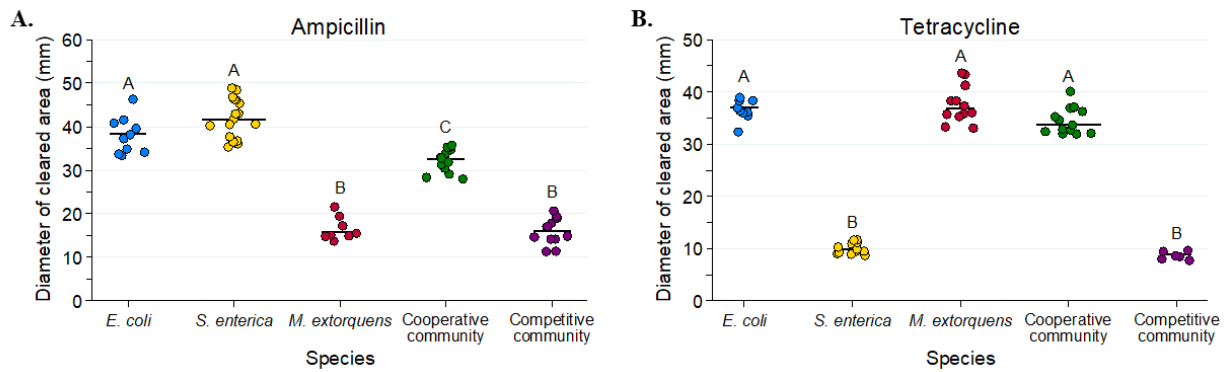
Figure 4. Diameters of zones of clearing for ampicillin (**A**) and tetracycline (**B**) disc diffusion assays. Pairwise comparisons of the zone of clearing for each monoculture and community was performed with a Mann-Whitney U test with Bonferroni adjustment for multiple comparisons. Significant differences are noted by different letters above each bar.

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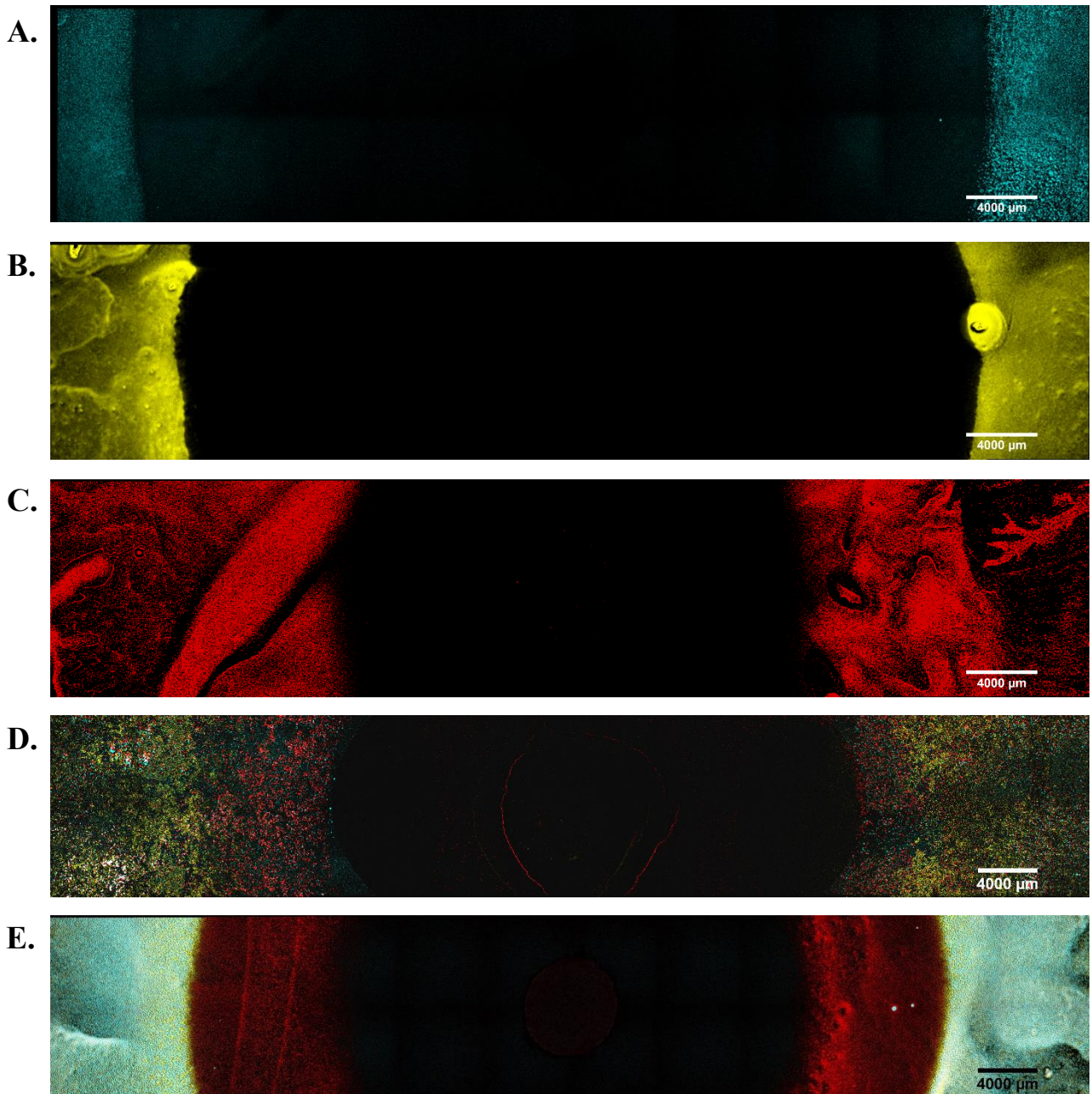
612 **Figure 5**. Fluorescent microscopy images of Petri plates with ampicillin antibiotic discs. *E. coli*

613 fluoresces blue (CFP), *S. enterica* fluoresces yellow (YFP) and *M. extorquens* fluoresces red

614 (RFP). (**A**) *E. coli* monoculture (**B**) *S. enterica* monoculture (**C**) *M. extorquens* monoculture (**D**)

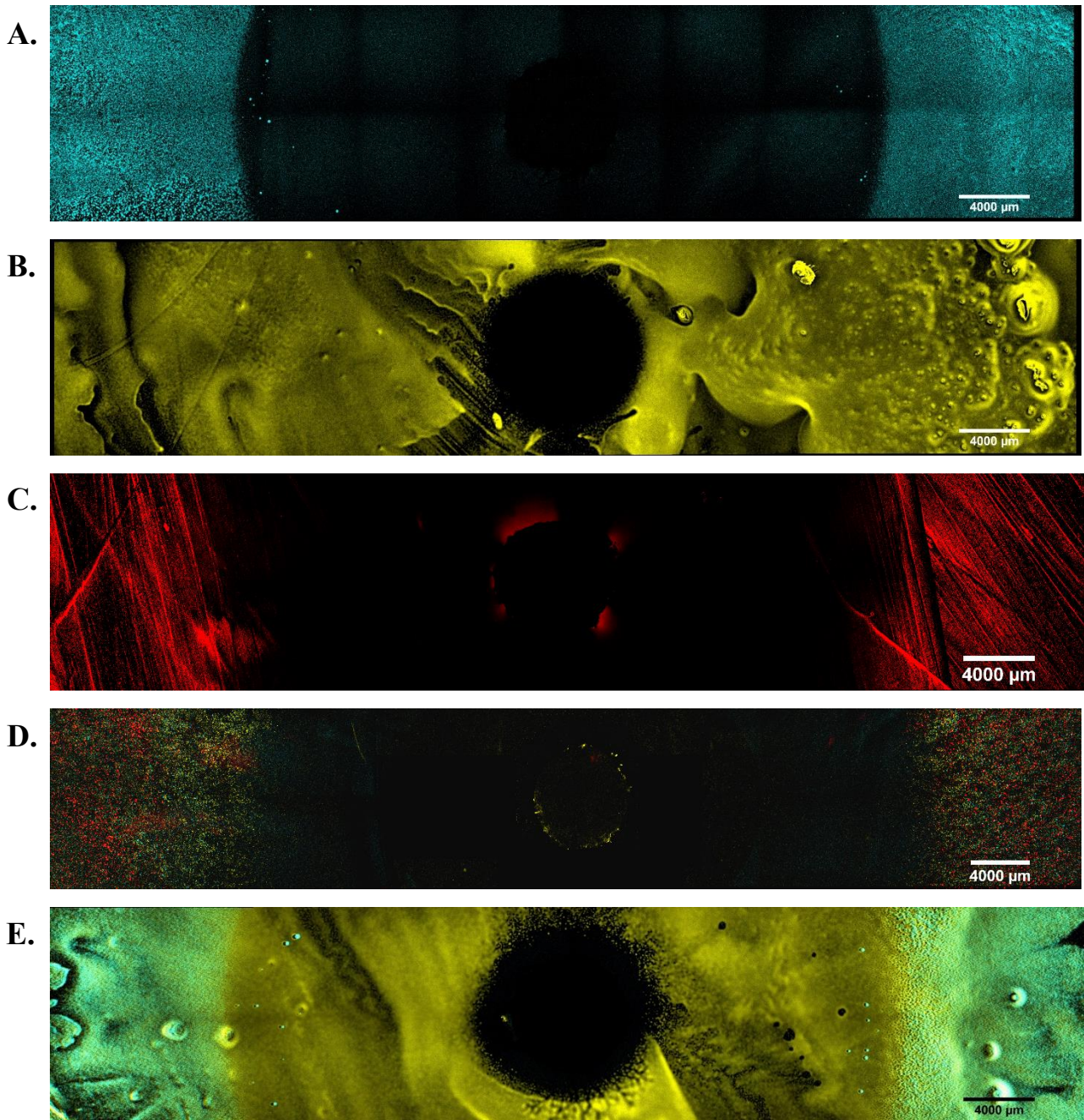615 cooperative community (**E**) competitive community.

Figure 6. Fluorescent microscopy images of Petri plates with tetracycline antibiotic discs. *E. coli* fluoresces blue (CFP), *S. enterica* fluoresces yellow (YFP) and *M. extorquens* fluoresces red (RFP). (**A**) *E. coli* monoculture (**B**) *S. enterica* monoculture (**C**) *M. extorquens* monoculture (**D**) cooperative community (**E**) competitive community. No RFP signal was observed for the competitive community with a tetracycline disc.
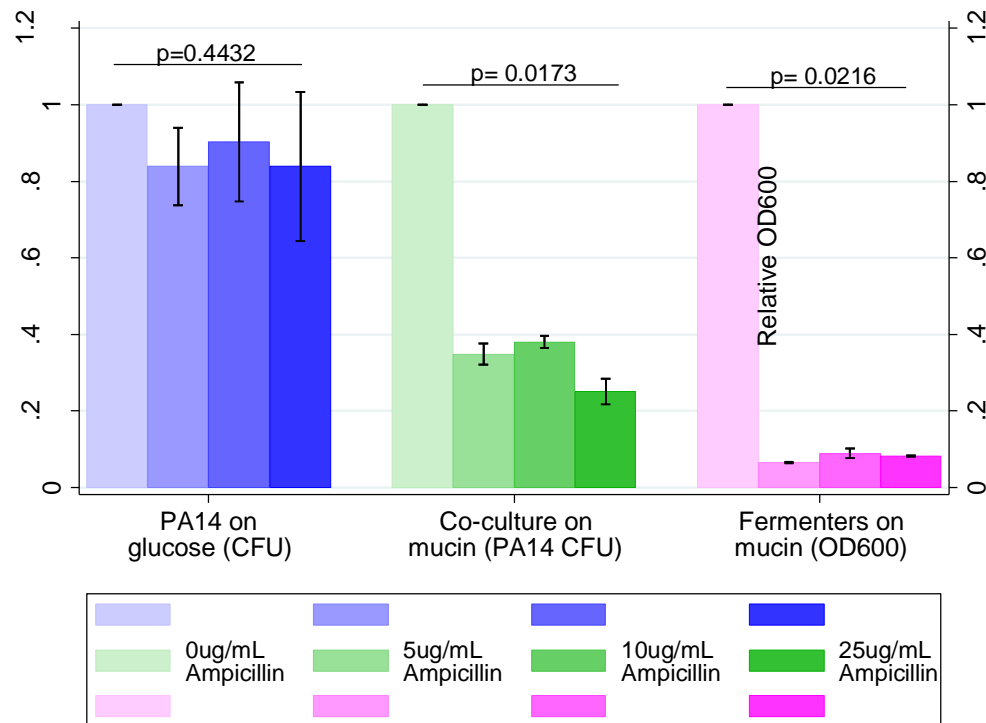
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Figure 7. *Pseudomonas aeruginosa* PA14 growth alone verses cross-feeding with a mucin-fermenting community across a range of ampicillin concentrations. Growth of PA14 was measued by colony forming units (CFU). Growth of fermenters was measued by OD600. In all cases growth was standardized against the relevant antibiotic-free control. Each point represents the mean and standard deviation of triplicate samples. P-values were calculated using a Kruskal-Wallis test across ampicillin concentrations using relative CFU (for PA14) or OD600 (for fermenters).