Resident microbial communities inhibit growth and antibiotic resistance evolution of *Escherichia coli* in human gut microbiome samples

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Abstract

Countering the rise of antibiotic resistant pathogens requires improved understanding of how resistance emerges and spreads in individual species, which are often embedded in complex microbial communities such as the human gut microbiome. Interactions with other microorganisms in such communities might suppress growth and resistance evolution of individual species (e.g. via resource competition), but could also potentially accelerate resistance evolution via horizontal transfer of resistance genes. It remains unclear how these different effects balance out, partly because it is difficult to observe them directly. Here, we used a gut microcosm approach to quantify the effect of three human gut microbiome communities on growth and resistance evolution of a focal strain of *Escherichia coli*. We found the resident microbial communities not only suppressed growth and colonization by focal *E. coli*, they also prevented it from evolving antibiotic resistance upon exposure to a beta-lactam antibiotic. With samples from all three human donors, our focal *E. coli* strain only evolved antibiotic resistance in the absence of the resident microbial community, even though we found resistance genes, including a highly effective resistance plasmid, in resident microbial communities. We identified physical constraints on plasmid transfer that can explain why our focal strain failed to acquire some of these beneficial resistance genes, and we found some chromosomal resistance mutations were only beneficial in the absence of the resident microbiota. This suggests, depending on *in situ* gene transfer dynamics, interactions with resident microbiota can inhibit antibiotic resistance evolution of individual species.

Introduction

The over- and inappropriate use of antibiotics has promoted the evolution of resistance in pathogens, resulting in a crisis for human health care [1]. To combat this problem, it is important to understand the underlying mechanisms of how resistance is acquired by bacteria and spreads within bacterial populations and communities [2,3]. A large body of research has used direct observations of resistance evolution in simplified laboratory conditions to understand how antibiotics drive the spread of resistance [4,5]. A key limitation of this approach is that it excludes interactions with other microorganisms, which we can expect to be important for bacteria evolving in natural or clinical settings because they spend most of their time in dense and diverse microbial communities. Interactions in species-rich microbial communities might negatively affect growth of individual species via, for example, competition for resources or niche space [6]. This may in turn inhibit antibiotic resistance evolution of individual species, because reduced population growth should reduce the supply of new genetic variation. On the other hand, interspecific interactions also potentially have positive effects on growth and evolution of individual species via, for example, exchange of genetic material [7], cross-feeding or public goods sharing [9–11]. Community-level interactions can also alter the strength of selection for resistant variants in the population [12,13]. In support of a key role for interspecific interactions in resistance evolution, observations of bacteria isolated from natural and clinical settings indicate genes involved in antibiotic resistance evolution are often horizontally transferable [14–17]. Despite this, direct observations of how these different types of effects balance out are lacking. Consequently, it remains unclear how interactions with species-rich microbial communities affect growth and antibiotic resistance evolution of individual species or strains of bacteria.

61 The impact of interactions with other microorganisms for antibiotic resistance evolution is
62 likely to be particularly important in the human gastrointestinal tract. This is one of the most
63 densely inhabited environments in the world, colonized by a rich diversity of bacteria, viruses
64 and eukarya, which are embedded in a network of biotic interactions [18,19]. Interactions
65 among microorganisms in the gut microbiome (which we take here to mean the resident
66 microorganisms, their genes and the local abiotic environment, following Marchesi & Ravel
67 [20] and Foster et al. [19]) play an important role for human health [21]. For example, the
68 microbiome minimizes potential niche space for invading species, making it harder for them
69 to establish in the community, thereby contributing to colonization resistance against pathogens
70 [22,23]. This suggests competitive interactions with other microorganisms are common, which
71 we would expect to inhibit population growth of individual taxa and in turn constrain their
72 ability to evolve antibiotic resistance. On the other hand, some interactions in the gut may be
73 mutualistic [10] or modify the effects of antibiotics on individual species [24], potentially
74 resulting in a net positive effect on growth. Moreover, recent metagenomic studies [25–27]
75 showed the gut microbiome harbours a variety of mobile genetic elements, often carrying
76 resistance and virulence genes, that are shared by community members. Consistent with this,
77 horizontal transfer of resistance genes within individual hosts is central to resistance evolution
78 in several key pathogens found in the gastrointestinal tract [28–31]. This suggests interactions
79 with other microorganisms in the gut microbiome can also promote growth and resistance of
80 individual taxa. We aimed to quantify the net effect of interactions with species-rich
81 communities of other microorganisms, in particular those found in the human gastrointestinal
82 tract, for growth and resistance evolution of a given strain that newly arrives in the community.
83
84 We approached this question using a human gut microcosm system consisting of anaerobic
85 fermentors filled with human faecal slurry, including the resident microbial community and

86 the beta-lactam antibiotic ampicillin, to which bacteria can evolve resistance by chromosomal
87 mutations [32] or horizontal acquisition of beta-lactamase genes [33]. We used ampicillin
88 because beta-lactam antibiotics are very widely used in human healthcare [34] , resistance is a
89 major problem [35], and key mechanisms by which bacteria evolve resistance to ampicillin
90 overlap with resistance mechanisms against other antibiotics [36]. Because the microbiota in
91 faecal samples reflects the diversity of the distant human gastrointestinal tract [37], this
92 approach allowed us to produce microcosms containing species-rich communities sampled
93 from human gut microbiomes. We aimed to determine how interactions with this resident
94 microbial community affected growth and resistance evolution of *Escherichia coli*. We focused
95 on *E. coli* because it is a ubiquitous gut commensal [38] and key opportunistic pathogen [39]
96 for which antibiotic resistance is an increasing problem [40]. We inoculated each microcosm
97 with a tagged, focal *E. coli* strain, before tracking its growth and resistance evolution in the
98 presence and absence of ampicillin. By also including microcosms containing sterilized
99 versions of the same faecal slurry (where the resident microbial community had been
100 deactivated), we quantified the net effect of interactions with the resident microbial
101 community. This approach allowed us to (i) track growth and resistance evolution of the focal
102 strain in the presence and absence of resident microbial communities sampled from several
103 human donors; (ii) isolate plasmid-carrying *E. coli* strains from the resident microbial
104 community and identify constraints on horizontal transfer of resistance genes; (iii) characterize
105 the resident microbial communities and how they changed over time. Our results show the
106 resident microbial community inhibits both growth and resistance evolution of *E. coli*, despite
107 the presence of resistance plasmids that can be conjugatively transferred to our focal strain in
108 certain physical conditions.
109

110 **Results**

111

112 *Resident microbial communities suppressed growth of a focal* E. coli *strain*

113 We cultivated our focal *E. coli* strain in anaerobic microcosms in the presence and absence of
114 an antibiotic and three different samples of gastrointestinal microbiomes, each from a different
115 human donor, for seven days (S1 Fig). On average, antibiotic treatment (ampicillin) decreased
116 focal strain abundance (effect of antibiotic in a generalized linear mixed model with zero
117 inflation, glmmadmb, $\chi^2 = 14.14$, d.f. = 1, $P < 0.001$; Fig 1). For example, after 24h focal strain
118 abundance was reduced compared to ampicillin-free treatments by 69% (s.d.=6.02) in the basal
119 medium treatment, 78-90% (depending on human donor) in the sterilized slurry treatments,
120 and 84-99.9% (depending on human donor) in the "live" slurry treatments (Fig 1; S1 Table).
121 Inclusion of the resident microbial community from human faecal samples also reduced focal
122 strain abundance on average, which we inferred by comparing the community treatments
123 ("live" faecal slurries, including the resident microbial community) to the community-free
124 treatments (sterilized versions of the same faecal slurries; effect of community in glmmadmb,
125 $\chi^2 = 6.65$, d.f. = 1, $P = 0.01$; Fig 1).

126

127 **Fig 1. Resident microbial communities suppressed growth and resistance evolution of**
128 **focal *E. coli*.** Each panel shows abundance of the focal *E. coli* strain (in colony-forming units
129 per ml) over seven days for either basal medium (top row) or with faecal slurry from one of
130 three human donors, in the absence (left panels) or presence (right panels) of ampicillin, which
131 was applied at the sampling time points after 2h and thereafter at each daily transfer. Empty
132 symbols show community-free treatments; filled symbols show treatments with the resident
133 microbial community; red symbols show microcosms where we detected ampicillin-resistant
134 colony isolates of the focal strain. The three lines, each with different symbols, in each

135 treatment show three replicate microcosms. Microcosms where we detected no focal strain
136 colonies are shown at 10^0.
137
138 The suppressive effect of resident microbial communities depended on both which human
139 donor sample was used to prepare the microcosms (donor×community interaction in
140 glmmadmb, χ^2 = 10.23, d.f. = 2, P = 0.006) and the presence of ampicillin
141 (antibiotic×community interaction in glmmadmb, χ^2 = 5.2, d.f. = 1, P = 0.02), being strongest
142 for populations exposed to both resident microbiota and the antibiotic (Fig 1). This resulted in
143 extinction of the focal strain (below our detection limit) in populations exposed to ampicillin
144 and the resident microbial communities from human donors 1 and 3. That is, in these treatments
145 the focal strain failed to colonize the community. For the resident community from human
146 donor 2, the focal strain was driven to very low abundance in the presence of the community
147 and ampicillin together, but did not disappear completely (Fig 1). In the absence of ampicillin,
148 resident communities still suppressed the focal strain on average (effect of community in
149 glmmadmb for ampicillin-free treatments only, χ^2 = 10.04, d.f. = 1, P = 0.002). As in the
150 presence of ampicillin, the strength of this effect varied depending on human donor, being
151 strongest for the resident microbial community from human donor 1 (effect of community for
152 this donor in the absence of ampicillin: glmmadmb, χ^2 = 11.28, d.f. = 1, P = 0.0002; Fig 1; S1
153 Table), resulting in exclusion of the focal strain. The resident community from human donor 3
154 suppressed average growth of the focal strain by ~54% across the entire experiment (effect of
155 community for human donor 3 in the absence of ampicillin: glmmadmb, χ^2 = 4.77, d.f. = 1, P
156 = 0.03; Fig 1; S1 Table). For the resident community from human donor 2, average focal strain
157 abundance was lower in the presence of the community (mean reduction of 24% compared to
158 community-free microcosms; Fig 1; S1 Table), although this was not statistically significant
159 (effect of community for human donor 2 in the absence of ampicillin: glmmadmb, χ^2 = 0.66,

160 d.f. = 1, $P = 0.41$). We found no evidence that abiotic factors in the sterile faecal slurry were
161 suppressive for the focal strain: there was no significant variation in average focal strain
162 abundance among the community-free treatments and the control treatment containing only the
163 basal growth medium that was used to prepare faecal slurries (linear mixed effects model,
164 glmer: $\chi^2 = 0.41$, d.f. = 3, $P = 0.94$). In summary, the resident microbial communities sampled
165 from three human donors each suppressed growth of a focal *E. coli* strain in anaerobic
166 fermentors filled with faecal slurry, but to varying degrees, and this effect was amplified by
167 adding ampicillin.
168

169 *Stable total bacterial abundance but variable community composition over time*

170 We used flow cytometry to estimate total bacterial abundance in microcosms containing
171 resident microbial communities. In antibiotic-free microcosms, total bacterial abundance was
172 approximately stable over time (>10^9 cells/ml; Fig 2), and was higher on average than in
173 microcosms exposed to ampicillin (effect of antibiotic in a linear mixed effects model, lmm:
174 $\chi^2 = 10.37$, d.f. = 1, $P = 0.001$). However, the suppressive effect of the antibiotic varied over
175 time (antibiotic×time interaction in lmm: $\chi^2 = 101.81$, d.f. = 7, $P < 0.001$), being strongest at
176 the beginning of the experiment. The effect of the antibiotic also varied across communities
177 from different human donors (antibiotic×donor interaction in lmm: $\chi^2 = 79.30$, d.f. = 2, $P <$
178 0.001), with those from human donors 1 and 2 showing a stronger recovery after the first
179 application of ampicillin (which resulted in a drop in abundance after 24h) than the community
180 from human donor 3. These results show our experimental set-up sustained high numbers of
181 microorganisms in the community treatments over time in both the presence and absence of
182 ampicillin.
183

184 **Fig 2. Total bacterial abundance over time in community treatments with and without**
185 **antibiotics.** Each row of panels shows data from one of the three human donors, and the
186 right/left panels show treatments with/without ampicillin. The three replicate communities in
187 each panel are shown by three different lines, each with a different symbol. Total bacterial
188 abundance was measured by flow cytometry (see Methods).
189
190 To investigate the community composition in these microcosms, we used amplicon sequencing
191 of the variable regions 3 and 4 of the 16S rRNA gene. This revealed similar levels of within-
192 sample diversity (Shannon's alpha diversity; Fig 3A) in microbiome samples from the three
193 human donors at the start of the experiment. Within-sample diversity then decreased slightly
194 over the first 24h of the experiment, and significantly between 24h and 168h (effect of time in
195 a linear mixed effects model including data from 24h and 168h, lme: $F_{1, 14} = 481.43$, $P < 0.001$).
196 This applied across the three human donors (effect of human donor, lme: $F_{2, 15} = 2.07$, $P =$
197 0.16), which also showed similar shifts in taxonomic composition over time (Fig 3B).
198 Communities at 0h were dominated by the families *Lachnospiraceae* and *Ruminococcaceae*,
199 plus *Prevotellaceae* for human donor 3. Over time these groups became less abundant relative
200 to *Enterobacteriaceae* and *Bacteroidaceae*. Analysis of the reads assigned to
201 *Enterobacteriaceae* indicated *E. coli* was always the most abundant member of this group (see
202 Methods), accounting for ~99% of the 16S rRNA amplicon reads assigned to
203 *Enterobacteriaceae* in all samples (S2 Table), with the remaining ~1% comprising other
204 species (e.g. *Enterobacter sp., Citrobacter sp.* and *Klebsiella sp.*). Compared to changes over
205 time, ampicillin had a weak effect on within-sample diversity (effect of antibiotic: $F_{1, 14} =$
206 11.37, $P = 0.006$). This interpretation was supported by an alternative analysis (Principal
207 Coordinate Analysis; S2 Fig) based on Bray-Curtis dissimilarities. Thus, despite approximately
208 stable total abundance, we saw changes in community composition over time that were more

209 pronounced than differences among communities from different human donors or antibiotic
210 treatments. Despite these changes in relative abundance of different taxa, the identities of the
211 top 5-6 families were stable over time and across human donors (Fig 3B).
212

213 **Fig 3. Within-sample diversity and changes in community composition over time. (A)**
214 Diversity is estimated here using Shannon's diversity index for samples from three timepoints
215 (0h, 24h, 168h, shown at top) and three human donors (see legend), in the presence and absence
216 of ampicillin (*x*-axis). Each box shows data from three replicate microcosms per treatment
217 group and time point (except for at 0h, which shows the single initial sample from each human
218 donor). **(B)** Relative abundance of the 15 most prevalent bacterial families in each microcosm
219 at three timepoints (0h, 24h and 168h, shown at top), for each human donor (rows of panels,
220 labelled at right) in the presence and absence of ampicillin (three replicates each treatment; *x*-
221 axis). Superscript " [a] " in the legend indicates obligately anaerobic families.

222

223 We used quantitative PCR to better understand the contribution of resident *E. coli* and the focal
224 strain to the total abundance of *E. coli* (see S1 Methods). Consistent with the amplicon data
225 above, this revealed increasing total abundance of *E. coli* sequences over time in both the
226 presence and absence of ampicillin (S3 Fig). The copy number of focal strain sequences relative
227 to total *E. coli* indicated the focal strain was rare relative to other *E. coli* after 24h (S3 Fig; S2
228 Table). At the end of the experiment, consistent with our estimates above from selective plating
229 and colony PCR, the focal strain was below the detection limit in treatments containing the
230 community from human donor 1, both with and without ampicillin, and the human donor 3
231 community with ampicillin; in the other treatments focal strain sequences were rare compared
232 to total *E. coli* (S2 Table).
233

234 *Antibiotic resistance evolved only in the absence of resident microbial*
235 *communities*

236 We screened for the emergence of antibiotic-resistant variants of the focal strain (that had
237 acquired resistance to ampicillin) after every growth cycle by plating each population onto
238 antibiotic-selective plates (8 μg/ml ampicillin; approximating the minimal inhibitory
239 concentration (MIC) of the focal strain). We never observed resistant variants of the focal strain
240 in any of the community treatments (populations exposed to the resident microbial
241 communities from human microbiome samples). By contrast, in community-free treatments
242 (basal growth medium and sterilized human faecal slurries) resistant variants appeared toward
243 the end of the experiment at 120h (slurry from human donor 1) and 144h (basal growth medium
244 and slurry from human donor 3), although not in sterilized samples from human donor 2 (Fig
245 1). Thus, the resident microbial community from human microbiome samples suppressed
246 antibiotic resistance evolution in our focal strain.

247

248 To investigate genetic mechanisms associated with resistance evolution and general adaptation
249 to our experimental conditions, we performed whole-genome sequencing for two sets of focal
250 strain isolates from the final time point: 8 ampicillin-resistant colony isolates from ampicillin
251 plates (one from each of the eight populations where we observed the emergence of antibiotic
252 resistance during the experiment) and 33 randomly selected colony isolates from ampicillin-
253 free plates (each from a different population and across all treatments). In the antibiotic-
254 resistant isolates, all SNPs and the deletion we found (Table 1) were in genes related to
255 membranes (*ompR, ftsI, opgB*), general stress response (*rpoC, relA*) or transcription regulation
256 (*rpoD*). Two genes were mutated independently in multiple colony isolates: *rpoC* and *rpoD*.
257 We also detected an insertion sequence (IS) movement between *perR* and *insN* in two colony
258 isolates. Of genes where we detected mutations in a single colony isolate, *ftsI* [41], *relA* [42]

259 and *ompR* [43] have each been previously annotated as being involved in resistance to beta-
260 lactam antibiotics. *ompR* was also mutated in three randomly selected colony isolates, all from
261 populations that had been exposed to ampicillin during the experiment (S3 Table). We found
262 six other genes mutated in parallel in between 2 and 5 randomly selected colony isolates (S3
263 Table). Five of these were mutated in isolates from both antibiotic and antibiotic-free
264 treatments. This included *insN* (involved in transposition) and *gtrS* (involved in aerobic
265 respiration), both mutated in five isolates. Across the two sets of colony isolates (randomly
266 selected and antibiotic-resistant), three other loci were mutated in both sets. These were *rpoD*
267 (only in isolates that had been exposed to antibiotics), *opgB* and *yaiO* (both in isolates from
268 antibiotic and antibiotic-free treatments). In summary, we found some parallel genetic changes
269 specific to antibiotic treatments and consistent with known resistance mechanisms, plus other
270 genetic changes that occurred across antibiotic and antibiotic-free treatments and are therefore
271 more likely involved in general adaptation.
272

Table 1: Genes mutated in ampicillin-resistant colony isolates of the focal strain from the end of the experiment. Asterisks indicate genes also mutated in randomly selected clones from ampicillin-free plates (see S3 Table).

Treatment group	Human donor	Replicate	*afuB*	*afuC*	*cspB <> cspF*	*cyoA*	*fecI*	*ftsI*	*insA <> uspC*	*insB1*	*insD1*	*ompR*	*opgB**	*perR*	*perR <> insN*	*relA*	*rpoC*	*rpoD*	*yaiO**	*yehE*	*yjhD <> insO*	*yjhD <> yjhE*
Basal medium with antibiotic	None	1			x			●							x							x
		2		x																x		
		3													x	●	●					
Community-free with antibiotic	1	1							x		x			x				●				
		2										●										
		3	x			x							●						x			
Community-free with antibiotic	3	1								x								●				
		2					x										▲				x	

▲ Deletion x Insertion ● SNP

278

279 *Plasmid acquisition was constrained by lack of transfer, not lack of fitness*
280 *benefits*

281 We next sought to explain why we never observed antibiotic resistance evolution of the focal
282 strain in the presence of resident microbial communities, which we had expected to harbour
283 beneficial resistance genes [26,27,44–46]. We hypothesized this could have been due to a lack
284 of horizontally transferable resistance genes in the resident microbial communities. However,
285 we detected ampicillin resistant *E. coli* in the resident microbial communities from human
286 donors 1 and 3 (by selective plating), and after sequencing their genomes we identified several
287 antibiotic resistance genes that were associated with plasmid genes (Fig 4; S4 Table). In the
288 hybrid assembly (using MinION and Illumina reads) of a representative isolate from human
289 donor 1, we identified two plasmids. The larger plasmid had four known resistance genes (Fig
290 4A), including one conferring resistance to beta-lactam antibiotics. We also identified three
291 IncF replicons on this plasmid and a complete set of *tra* genes, which are involved in
292 conjugative plasmid transfer. The second plasmid carried a known replicon (ColRNAI), and
293 mobilization genes (*mbeA* and *mbeC*), plus a complete colicin E1 operon (*cnl, imm, cea*). For
294 a representative isolate from human donor 3, we found the plasmid replicon and resistance
295 genes integrated on the chromosome (Fig 4B). This putative integrated plasmid from human
296 donor 3 also carried multiple resistance genes, including a beta-lactamase and an IncQ replicon,
297 which is a part of the *repA* gene [47], but we detected no *tra* genes. The other five genome
298 assemblies (Illumina reads for other isolates from the same human donor) contained the same
299 resistance genes and replicons across multiple smaller contigs (S4 Table). Mapping the
300 corresponding sequencing reads of all Illumina-sequenced isolates to the long-read data single
301 contig found in the isolates sequenced on the MinION platform revealed identical mapping in

302 all ten cases, consistent with these genomes having the same structure across each of the
303 isolates (S4 Table).
304
305 **Fig 4. Schematic maps of plasmids and chromosomes for representative resident *E. coli***
306 **isolates from (A) human donor 1 and (B) human donor 3.** Sequences are annotated with
307 known plasmid replicon sequences (IncFIA, IncFIB, IncFIC on plasmid 1 and ColRNAI on
308 plasmid 2 in (A); IncQ on the chromosome in (B)), genes involved in horizontal transfer (*tra*
309 and *trb*), known resistance genes (bla_{TEM}, *sul2*, *aph(3), aph(6), mdf*), genes involved in Type 6
310 secretion systems (*tss, vgr*), genes involved in colicin production and immunity (*cea, cnl, imm*)
311 and mobilization (*mbeA* and *mbeC*). The genome of the isolate from human donor 3 (B) is not
312 closed, as indicated with a gap. Colours indicate coding (black) and non-coding regions
313 (green); note the scale varies among chromosomes and plasmids.
314
315 We hypothesized that the lack of plasmid-driven resistance evolution in our focal strain might
316 have been caused by constraints on conjugative transfer that made these plasmids inaccessible.
317 Using a conjugative mating assay on agar, we never found transconjugants of our focal strain
318 when it was mixed with an isolate from human donor 3 (identified above as carrying a putative
319 integrated plasmid). This is consistent with the lack of *tra* genes on this plasmid and suggests
320 it could not be transferred into our focal strain by conjugation in the absence of other drivers
321 of horizontal gene transfer (e.g. phages or other plasmids). This is also consistent with past
322 work suggesting IncQ plasmids are mobilizable rather than conjugative [48,49], and that we
323 did not detect any other plasmid replicons in the same isolates. However, for the plasmid from
324 human donor 1 we found transconjugants of our focal strain at the end of the mating assay,
325 which we confirmed by colony PCR (S4 Fig). This suggests this plasmid was conjugative and

326 could be transferred to our focal strain, consistent with the presence of *tra* genes on this plasmid
327 (Fig 4A).
328
329 Given that the resistance plasmid from human donor 1 was transferable into our focal strain,
330 why did it not spread in the main experiment above? We hypothesized this could result from
331 plasmid-borne resistance being less beneficial than resistance acquired by chromosomal
332 mutation (as we observed in community-free treatments in the main experiment). We would
333 expect a net benefit of resistance to result in increased population growth at the antibiotic
334 concentration applied during the experiment (7.2 μg/ml). We found acquisition of the plasmid
335 conferred a much larger increase in population growth across all non-zero antibiotic
336 concentrations than that observed for evolved colony isolates that had acquired resistance via
337 chromosomal mutation during the main experiment (Fig 5A; S5 Table). Furthermore, in
338 pairwise competition experiments, the transconjugant carrying this plasmid had a strong
339 competitive advantage relative to the wild type in the presence of the resident microbial
340 community from human donor 1 (S5 Fig A). This fitness advantage was increased by adding
341 ampicillin at the concentration we used in the main experiment, and even further by adding
342 ampicillin at three times the IC90 of the ancestral focal strain (community × ampicillin
343 interaction by permutation test; $P = 0.029$; S5 Fig B). This shows it would have been highly
344 beneficial for the focal strain to acquire the plasmid in our experiment, particularly in the
345 presence of ampicillin.
346
347 **Fig 5. Transfer of a resistance plasmid from the resident microbial community is sensitive**
348 **to abiotic conditions, and it confers a large increase in resistance. (A)** Antibiotic
349 susceptibility of the ancestral focal strain, the version of the focal strain used to isolate
350 transconjugants (see Methods), the focal strain with the plasmid (transconjugant), the resident

351 *E. coli* isolate used as the plasmid donor, and eight evolved focal strain colony isolates that we
352 isolated on ampicillin plates and that had chromosomal mutations (S2 Table). Average optical
353 density (OD) values ±s.e. are shown after 24h growth at each ampicillin concentration. **(B)**
354 Transconjugant frequency (as a percentage of the total recipient population) after mating
355 experiments in various conditions. The recipient strain was a tagged version of the ancestral
356 focal strain and the plasmid donor was a resident *E. coli* isolate from human donor 1. For the
357 faecal slurry treatment we used sterilized faecal slurry from human donor 1.
358
359 Unlike transconjugants carrying the plasmid from resident *E. coli*, two evolved colony isolates
360 of the focal strain carrying chromosomal resistance mutations had a fitness advantage relative
361 to the wild type only in the absence of the community; in the presence of the resident microbial
362 community they had a fitness disadvantage (effect of community by permutation test: $P <$
363 0.001 for isolates from human donors 1 and 3; S5 Fig B). This suggests the genetic changes
364 associated with increased resistance in these isolates in the absence of resident microbiota
365 would not have been beneficial in the Community+Ampicillin treatments of the main
366 experiment, unlike the plasmid we isolated from resident *E. coli*. This conclusion was
367 supported by direct competitions between transconjugants carrying the resistance plasmid and
368 evolved colony isolates from human donors 1 and 3 (S6 Fig). Further competition experiments
369 showed the competitive advantage of resident *E. coli* from human donors 1 and 3 carrying
370 resistance genes was also present in the absence of other resident microbiota (S7 Fig).
371
372 Another possible explanation for the lack of transfer of the resistance plasmid from human
373 donor 1 in the main experiment is that conjugative transfer might be specific to particular
374 environmental conditions. This has been observed for other plasmids across various
375 experimental conditions [50,51]. We tested this by mating assays as above, but in a range of

376 different experimental conditions. We detected transconjugants that had acquired the plasmid
377 at a final frequency of ~0.2% (as a fraction of the total recipient population) after mixing the
378 plasmid-carrying isolate from human donor 1 and the focal strain on an agar surface, but we
379 found no transconjugants after doing the same experiment in three different types of liquid
380 growth medium (lysogeny broth (LB), anaerobic LB, and anaerobic community-free faecal
381 slurry; Fig 5B). This shows transfer of the conjugative plasmid we isolated from human donor
382 1 requires particular abiotic conditions, which may explain why our focal strain failed to evolve
383 resistance via horizontal gene transfer in the presence of resident microbial communities. This
384 was supported by simulations of a hypothetical plasmid with similar properties but that is
385 transferable in our gut microcosm system (S1 Model). This indicated that, if the plasmid from
386 human donor 1 had been conjugatively transferable in our gut microcosm system, we would
387 have detected transconjugants in our main experiment (although only with relatively high
388 transfer rates; S1 Model). The same model also showed growth suppression of invading
389 lineages by resident microbiota can reduce transconjugant abundance, suggesting even when
390 horizontal acquisition of beneficial resistance genes is common, interactions with resident
391 microbiota can impede their spread.
392

393 # Discussion

394 We found resistance via chromosomal mutation to an important class of antibiotics (beta-
395 lactams) evolved in a focal *E. coli* strain in our experiment only in the absence of resident
396 microbial communities sampled from healthy human volunteers. The suppressive effect of
397 these resident microbial communities was strong enough that the focal *E. coli* strain was driven
398 towards extinction (below our detection limit) when it was exposed to both ampicillin and the
399 community simultaneously (with communities from two of the three human donors we tested).
400 Consequently, the net effect of the resident microbial communities here was to confer a form

401 of colonization resistance against a non-resident strain, and to prevent that strain from evolving
402 antibiotic resistance. Our analysis of resident *E. coli* isolates (not the focal strain) from the
403 microbial communities showed this occurred despite the presence of beneficial, potentially
404 horizontally transferable resistance genes. Genomic analyses and conjugation experiments
405 with these resident *E. coli* isolates showed the *in situ* transfer dynamics depend critically on
406 genetic (the presence of genes encoding the machinery for conjugative transfer) and abiotic
407 (physical structure of the environment) factors. This is important because ultimately it is the *in*
408 *situ* transfer dynamics that will determine whether or not horizontal transfer of beneficial
409 resistance genes is sufficient to counteract the growth-suppressive effects of interactions with
410 the community and confer a net benefit to invading lineages. Community-level interactions
411 also modified selection for resistance, amplifying growth inhibition by ampicillin and altering
412 the relative advantages/disadvantages of individual resistant genotypes (evolved isolates with
413 chromosomal mutations conferring relatively weak increases in ampicillin resistance had a
414 reduced advantage, but plasmids from resident *E. coli* that conferred relatively large increases
415 in ampicillin resistance were more beneficial in the presence of resident microbiota). Overall,
416 this indicates resident microbiota influence resistance evolution of invading strains via effects
417 on both population dynamics and the strength of selection for resistance.
418
419 The first key implication of our work is that as well as suppressing growth and colonization by
420 invading strains [23,52], the gastrointestinal microbiota can inhibit antibiotic resistance
421 evolution. There are several possible ecological mechanisms by which the microbiota may
422 suppress growth of invading lineages [53], such as niche and nutrient competition [54], direct
423 killing via bacteriocins [22,55], phage production [56] or by changing the concentration of
424 compounds such as primary and secondary bile acids [57,58]. It was not our aim to pull apart
425 the mechanisms by which resident microbiota suppress invading bacteria (studied in more

426 detail elsewhere; [55,59]). Nevertheless, our data on community structure indicate resident
427 *Enterobacteriaceae* including *E. coli* had a competitive advantage in our system, potentially
428 explaining suppression of the focal strain. This was further evidenced by the advantage of
429 transconjugants carrying plasmids from resident *E. coli* (S5 Fig) and resident *E. coli* over our
430 ancestral focal strain in competition experiments (S7 Fig). The competitive advantage of
431 resident *E. coli* extended to ampicillin-free conditions in pure culture. Possible contributors to
432 this include a type VI secretion system with various effectors we detected in the genomes of
433 the human donor 1 and 3 *E. coli* isolates, and the a colicin plasmid present in human donor 1
434 *E. coli* isolates (Fig 4). In supernatant experiments, we did not find evidence of direct inhibition
435 via phages in the community samples (see S1 Methods). Crucially, no matter how interactions
436 with the microbiota suppress growth of an invading lineage, we expect the reduced population
437 size, growth and replication to in turn reduce the supply of new genetic variation. This is
438 consistent with *in vitro* work with malaria parasites showing competition between two species
439 under resource limitation impeded drug resistance evolution [60] and previous studies with
440 *Pseudomonas fluorescens* showing that a eukaryotic predator [61] or a bacteriophage [62] can
441 suppress the emergence of antibiotic resistance. Thus, suppression of an invading lineage via
442 interactions with resident microbiota may frequently have a knock-on effect on resistance
443 evolution.
444
445 A second key implication is that resident microbiota modified selection on antibiotic resistance
446 in our focal strain. The stronger effect of ampicillin on focal strain growth in the presence of
447 resident microbiota (Fig 1; S1 Table) indicates resistance would have been more beneficial
448 here. In support, the benefits of resistance plasmid acquisition were greatest in the presence of
449 resident microbiota (S5 Fig). This is counter to the expectation that antibiotics may be less
450 effective in more dense communities due to an 'inoculum effect' [63]. We found inhibition of

451 our focal strain was indeed altered by very high *E. coli* abundance in pure cultures (S8 Fig),
452 although there was still considerable inhibition even at the highest densities. This indicates
453 such inoculum effects were weaker in the presence of species-rich communities than in pure
454 cultures of *E. coli*, and/or were counterbalanced by opposing effects of community-level
455 interactions on ampicillin inhibition of *E. coli*. By contrast, chromosomal resistance mutations
456 that emerged in the absence of resident microbiota (and which conferred relatively small
457 increases in resistance in pure culture) were no longer beneficial in the presence of resident
458 microbiota, indicating a larger change in resistance is needed to overcome the relatively strong
459 effect of ampicillin here. This complements recent work showing natural communities from
460 pig faeces can increase costs of antibiotic resistance for individual species [12], and that costs
461 of CRISPR-based phage resistance can be amplified by interactions with other bacterial species
462 [64]. More generally, this supports the notion that community-level interactions modulate the
463 costs and benefits of antibiotic resistance via mechanisms that are only just beginning to be
464 understood [65].
465
466 A third key implication of our data concerns the genetic and environmental constraints on
467 horizontal gene transfer that determine whether or not the growth-suppressive effects of the
468 microbiota are counter-balanced by horizontal transfer of beneficial genes. The unavailability
469 of known resistance genes to the invading focal strain in the community from human donor 3
470 was because they were integrated in the chromosome. The plasmid we isolated from the human
471 donor 1 community was conjugative, but transfer depended on the abiotic conditions. This
472 suggests the potential for plasmid transfer to allow invading lineages to overcome the
473 suppressive effects of the microbiota depends critically on whether they are conjugative (which
474 can be predicted from sequence data) and on the sensitivity of conjugative transfer to local
475 physical conditions (which is harder to predict from sequence data). Consistent with this,

476 previous research has shown conjugative transfer in *E. coli* and other species is sensitive to the
477 physical experimental conditions [51,66]. Furthermore, mating pair formation machinery,
478 usually encoded by the plasmid, in some cases promotes biofilm formation, which can in turn
479 promote the spread of plasmids [67]. This raises the question of whether some plasmids have
480 evolved to manipulate the physical structure of bacterial populations to promote transfer.
481 Despite these constraints, plasmids are clearly sometimes transferred *in vivo*, as has been
482 observed in animal models [68,69] and human gut microbiomes [33,44,45]. In line with
483 plasmids being key vectors of beta-lactamases [70], the conjugative plasmid we identified was
484 highly effective in terms of resistance. This suggests plasmid-borne resistance will be under
485 strong positive selection once established and can spread rapidly via clonal expansion.
486 However, our experiment showed the initial horizontal transfer required for such spread is
487 sensitive to genetic and abiotic constraints.
488
489 Our approach allowed us to isolate the effect of interactions between diverse microbial
490 communities and a focal *E. coli* strain. Our amplicon sequence data showed the communities
491 had a representative taxonomic composition for healthy human donors. There were still diverse
492 communities present at the end of the seven-day experiment, albeit with a change in the relative
493 abundance of different taxa. The observed rise of *Enterobacteriaceae* and *Bacteroidaceae* has
494 been seen in other experiments with gastrointestinal communities (e.g. [71]) and might be
495 explained by the nutrient content of the medium, micromolar oxygen levels or such *in vitro*
496 systems favouring faster population growth [72]. In conditions where antibiotics are applied at
497 higher concentrations or affect a greater fraction of extant taxa, we can expect stronger shifts
498 in community composition in response to antibiotic treatment. We used a sub-lethal
499 concentration in our main experiment to allow us to track invasion and growth by the focal
500 strain. Nevertheless, in competition experiments at higher antibiotic concentrations we saw

501 similar outcomes in that plasmids from the resident microbiota were highly beneficial whereas
502 chromosomal mutations were not (S5 Fig). More importantly, the shift in community
503 composition over the seven-day experiment does not explain the observed suppression of the
504 focal strain, because this was already visible after one day.
505
506 Although our experimental system likely differs from the gastrointestinal tracts these bacteria
507 were isolated from in ways that affect community composition, cultivating them *in vitro*
508 allowed us to quantify the effect of species-rich communities sampled from gastrointestinal
509 tracts on resistance evolution of a relevant opportunistic human pathogen. A key limitation of
510 our study is the sample size (three human donors, one focal strain, one antibiotic). Some
511 outcomes might change with different types of resident microbiota or different types of
512 plasmids (explored in the Supplementary Model). Nevertheless, we observed a qualitatively
513 consistent suppression of the focal strain across the three human donors, which was always
514 stronger in the presence of ampicillin, and in some cases was associated with colonization
515 resistance (extinction of the focal strain). Additionally, we chose *E. coli* and ampicillin because
516 they are both important for understanding resistance evolution in nature and share some
517 important properties in this respect with other bacteria and antibiotics (our rationale is
518 explained further in the introduction). Despite the low sample size, we observed a qualitatively
519 consistent suppression of the focal strain across the three human donors, which was always
520 stronger in the presence of ampicillin, and in some cases was associated with colonization
521 resistance (extinction of the focal strain). A key challenge for future work will be to determine
522 how such effects are modified *in vivo* by local spatial structure [73] and immune responses
523 [74]. Indeed, interactions mediated via the host immune system are another possible
524 mechanism of colonization resistance [75–77].
525

526 In conclusion, we showed species-rich microbial communities sampled from human
527 gastrointestinal tracts can suppress growth and resistance evolution of an invading lineage.
528 Given the variety and likely common occurrence of mechanisms that can generate such
529 suppression of invaders (e.g. resource competition), these types of effects are probably
530 common in species-rich communities such as the mammalian gastrointestinal tract. Crucially,
531 resident microbiota also altered the strength of selection for resistance (ampicillin was more
532 suppressive for the focal strain in community treatments) and the fitness effects of individual
533 genetic changes (high-level resistance plasmids became more beneficial in the community
534 treatments, but low-level resistance mutations became less beneficial). Our other data and
535 simulations showed whether or not the growth-suppressive effects of resident microbiota are
536 counter-balanced by beneficial horizontal gene transfer depends on genetic and environmental
537 constraints that can impede the spread of resistance plasmids. This has important implications
538 for the prediction of resistance evolution from genetic and metagenomic data, such as those
539 widely collected through surveillance efforts [78,79]: identifying mobile resistance genes in a
540 diverse community is not enough to predict resistance evolution, requiring in addition
541 information about genetic and environmental constraints on *in situ* transfer dynamics.
542

543 # Material and Methods

544

545 *Bacterial strains*

546 We used *Escherichia coli* K12 MG1655 carrying a Streptomycin resistance mutation (*rpsL*
547 K43R) as the focal strain. Two days prior to the experiment we streaked the focal strain on LB-
548 agar (Sigma-Aldrich, Buchs, Switzerland) and incubated overnight at 37 °C. To incubate the
549 focal strain cultures anaerobically prior to the microcosm experiment, we prepared 42 Hungate
550 tubes (VWR, Schlieren, Switzerland) with LB (Sigma-Aldrich), which was supplemented with

551 0.5 g/l L-Cysteine and 0.001 g/l Resazurin (reducing agent and anaerobic indicator
552 respectively), flushed the headspace with nitrogen, sealed the tubes with a rubber stopper and
553 autoclaved them. One day before the experiment, we randomly picked 42 colonies and
554 inoculated them in the 42 Hungate tubes containing anaerobic LB, and incubated at 37 °C
555 overnight with 220 rpm shaking. We then used these 42 independent cultures of the focal strain
556 to inoculate the main experiment described below.
557
558 *Human microbiome samples*
559 We used three stool samples from anonymous, consenting human donors. All samples were
560 collected at the Department of Environmental Systems Science, ETH Zürich on the 15th of May
561 2018. Inclusion criteria were: older than 18 years, not obese, not recovering from surgery and
562 no antibiotics in the last six months. The sampling protocol was approved by the ETHZ Ethics
563 Commission (EK 2016-N-55). Each sample was collected in a 500 ml plastic specimen
564 container (Sigma-Aldrich) and kept anaerobic using one AnaeroGen anaerobic sachet (Thermo
565 Scientific, Basel, Switzerland). The three samples used for the experiment were randomly
566 selected from a larger number of donated samples. We collected the samples in the morning
567 before the experiment and kept them for maximum one hour before processing. To prepare
568 faecal slurry from each sample, we re-suspended 20 g of sample in 200 ml anaerobic peptone
569 wash (1 g/l peptone, 0.5 g/l L-Cysteine, 0.5 g/l bile salts and 0.001 g/l Resazurin; Sigma-
570 Aldrich) to prepare a 10% (w/v) faecal slurry. We then stirred the slurry for 15 min on a
571 magnetic stirrer to homogenize, followed by 10 min of resting to sediment. At this point we
572 removed 100 ml of each faecal slurry ('fresh slurry'), which we used later to re-introduce the
573 resident microbial community to sterilized slurry (for the community treatments). To sterilize
574 the faecal slurries, we transferred 100 ml to a 250 ml Schott bottle, flushed the headspace with
575 Nitrogen gas, sealed them with rubber stoppers and autoclaved for 20 min at 121 °C.

576

577 *Inoculating anaerobic gut microcosms, sampling and bacterial enumeration*

578 For the start of the experiment (S1 Fig), we filled 42 Hungate tubes with 7 ml of basal medium,
579 which was based on earlier studies [80,81] with some modifications (2 g/l Peptone, 2 g/l
580 Tryptone, 2 g/l Yeast extract, 0.1 g/l NaCl, 0.04g K_2HPO_4, 0.04 g/l KH_2PO_4, 0.01 g/l
581 $MgSO_4x7H_2O$, 0.01 g/l $CaCl_2x6H_2O$, 2g/l $NaHCO_3$, 2 ml Tween 80, 0.005 g/l Hemin, 0.5 g/l
582 L-Cysteine, 0.5 g/l bile salts, 2g/l Starch, 1.5 g/l casein, 0.001g/l Resazurin, pH adjusted to 7,
583 addition of 0.001g/l Menadion after autoclaving; Sigma-Aldrich), and for the subsequent re-
584 inoculation cycles with 6.5 ml of basal medium. We flushed the head space of each tube with
585 nitrogen gas, sealed it with a rubber septum and autoclaved to produce anaerobic microcosms
586 containing only basal medium.

587

588 On day one of the experiment, we introduced faecal slurry and antibiotics to each tube
589 according to a fully factorial design (S1 Fig), with three replicate microcosms in each
590 combination of Human Donor (1, 2 or 3), Community (present or absent) and Antibiotic (with
591 or without). In the community-free treatments, we added 850 µl of sterile slurry. In the
592 community treatments, we added 350 µl of fresh slurry and 500 µl of sterilized slurry. In the
593 antibiotic treatment, we added ampicillin to a final concentration of 7.2 µg/ml, approximating
594 the IC90 (concentration required to reduce growth by 90%) for the focal strain; this was
595 introduced 2h after the focal strain had been inoculated (8 µl of focal *E. coli* from one of the
596 42 overnight cultures introduced at 0h; approximately 1:1000 dilution). As a control treatment
597 testing for the effect of sterilized slurry, we also inoculated the focal strain into three replicate
598 microcosms containing only the basal medium (supplemented with 850 µl of peptone wash to
599 equalize the volume with the slurry treatments), and we did this with and without antibiotic
600 treatment. We incubated all microcosms at 37 °C in a static incubator. After 24h we transferred

601 a sample of 800 µl from each microcosm to a new microcosm (containing basal medium plus
602 500 µl of sterile slurry from the corresponding human donor in the community and community-
603 free treatments, or basal medium plus peptone wash for the basal medium treatment,
604 supplemented with ampicillin at each transfer in the antibiotic treatments), and we repeated
605 this for seven days.
606
607 To estimate the abundance of the focal strain during the experiment, we used a combination of
608 selective plating and colony PCR. For selective plating, we serially diluted the samples and
609 plated them on Chromatic MH agar (Liofilchem, Roseto degli Abruzzi, Italy), which allowed
610 us in a first step to discriminate *E. coli* from other species based on colony colour. By
611 supplementing these agar plates with Streptomycin (200 µg/ml), to which our focal strain is
612 resistant, we selected against other *E. coli* that were not resistant to Streptomycin. To screen
613 for variants of our focal strain that acquired resistance to Ampicillin during the experiment, we
614 additionally plated each sample onto the same agar supplemented with both Streptomycin (200
615 µg/ml, Sigma-Aldrich) and Ampicillin (8 µg/ml, Sigma-Aldrich). We did this after every
616 growth cycle. Despite initial screening of microbiome samples revealing no resident *E. coli*
617 that could grow on our selective plates, later in the experiment we found such bacteria to be
618 present in some samples (that is, non-focal strain *E. coli* that could grow on our plates and were
619 presumably very rare at the beginning of the experiment). To discriminate between these *E.*
620 *coli* and our focal strain, we used colony PCR with specific primers (forward (5'- AGA CGA
621 CCA ATA GCC GCT TT-3'); reverse (5'- TTG ATG TTC CGC TGA CGT CT-3')). For
622 colony PCR, we picked 10 colonies randomly for each time point and treatment. The PCR
623 reaction mix consisted of 2x GoTaq green master mix, 2.5 µM of each primer and nuclease
624 free water. The thermal cycle program ran on a labcycler (Sensoquest, Göttingen, Germany)
625 with 6 min 95°C initial denaturation and the 30 cycles of 95°C for 1 min, 58°C for 30s, 72°C

626 for 35s and a final elongation step of 72°C for 5 min. For gel electrophoresis we transferred 5
627 µl of the PCR reaction to a 1.5% agarose gel stained with SYBR Safe (Invitrogen, Thermo F.
628 Scientific) and visualized by UV illumination. Focal strain abundance was then estimated by
629 multiplying the frequency of the focal strain determined by colony PCR with the total colony
630 count for each plate. To account for the possibility that the focal strain was still abundant in
631 populations where we found 0/10 colonies (that is, where it could have been rare relative to
632 resident *E. coli* but still present), we additionally screened the DNA extracted from the
633 community (described below) of the final time point by PCR (as described above for the colony
634 PCR, but using 30 ng of DNA as template). We did this for all microcosms from the community
635 treatment and detected PCR products in all cases where we detected focal strain by plating and
636 colony PCR, and none of the cases where we did not, consistent with our analysis of individual
637 colonies and suggesting that in those microcosms the focal strain had been completely excluded
638 during the experiment.
639
640 To estimate total microbial abundance in each microcosm supplemented with the microbiome
641 we used flow cytometry. We diluted samples by 1:10000 with phosphate buffered saline
642 (PBS) and stained them with SYBR Green (Invitrogen, Thermo F. Scientific). We used a
643 Novocyte 2000R (ACEA Biosciences Inc. , San Diego, CA, USA), equipped with a 488 nm
644 laser and the standard filter setup for the flow cytometric measurements.
645
646 We froze samples after every transfer from each microcosm at -80 °C, and at the end of the
647 experiment we isolated two sets of focal strain colony isolates for sequencing. First, we
648 randomly picked a single focal strain colony isolate from each microcosm where the focal
649 strain was detected at the end of the experiment (from Streptomycin plates; n=33). Second, we
650 randomly picked a single ampicillin-resistant colony isolate of the focal strain from each of the

651 8 populations at the end of the experiment where they were detected (from Streptomycin +
652 Ampicillin plates; *n*=8). We grew each colony isolate overnight in LB (with Ampicillin for the
653 ampicillin-resistant isolates), mixed 1:1 with 50% glycerol and stored at -80 °C.
654
655 *Whole genome sequencing and Bioinformatics*
656 We sequenced all of the randomly selected (*n*=33) and ampicillin-resistant (*n*=8) focal strain
657 colony isolates (S7 Table). Prior to DNA extraction, we grew each isolate overnight in LB,
658 then concentrated the liquid cultures by centrifugation and extracted DNA with the Magattract
659 kit (Qiagen, Hilden;Germany) according to the manufacturer's protocol. Quality and quantity
660 of DNA was assessed with Nanodrop (Thermo Fisher) and Qubit (Thermo Fisher). At the
661 Functional Genomics Center (ETH Zürich / University of Zürich) DNA was processed with
662 Nextera library prep kit and sequenced on the Illumina Hiseq 4000 platform. We filtered raw
663 sequencing reads with trimmomatic version 0.38 [82] and mapped the reads to the reference
664 with snippy version 0.9.0 (https://github.com/tseemann/snippy) to detect variants. Deletions
665 were identified by using the coverage analysis tool of CLC Genomics Workbench 11 (Qiagen)
666 with a read threshold value of 0 and a window size of 1. We identified IS elements with
667 ISfinder web server (database from July 2018) (Siguier 2006) in the ancestor genome and used
668 these sequences to detect IS movements in the evolved strains with ISMapper version 2.0 [84].
669
670 We additionally sequenced 12 ampicillin-resistant resident *E. coli* colony isolates (not the focal
671 strain, S7 Table). We isolated these colony isolates from microcosms filled with faecal slurry
672 from human donors 1 and 3 at the final time point of the experiment (6 for each donor, each
673 from a different microcosm). We picked, grew and sequenced these colony isolates as
674 described above for focal strain isolates. We then made de Novo assemblies of the resulting
675 sequences with spades version 3.13.0 [85] and annotated them with prokka version 1.13.7 [86].

676 Additionally, we sequenced one of these resident, resistant *E. coli* isolates from both human
677 donors (1 & 3) with the Oxford nanopore long-read sequencing platform MinION at University
678 Hospital Basel, Switzerland. These genomes were assembled using Unicycler v0.4.8 with a
679 hybrid assembly approach combining MinION and Illumina reads. Assembly statistics can be
680 found in S6 Table. We screened for known antibiotic resistance genes and the presence of
681 plasmid replicons by a local blast query against the resfinder (downloaded Oct. 2018) [87] and
682 plasmidfinder (downloaded Oct. 2018) [47], which is a repository of whole-plasmid sequences
683 from members of the *Enterobacteriaceae*. To identify genes that are involved in mating pair
684 formation or mobilization of the plasmid we screened the genome annotation files and blasted
685 potential candidate genes against the NCBI nucleotide database to verify them.
686
687 *Mating experiments with plasmids from resident* E. coli
688 We aimed to determine whether the resident *E. coli* colony isolates could act as plasmid donors
689 for our focal strain (transferring antibiotic resistance plasmids via conjugation). Because the
690 replicate *E. coli* colony isolates that we sequenced from each human donor (1 and 3) were
691 almost identical on the DNA sequence level (S2 Table), we randomly chose one colony isolate
692 from each human donor as a potential plasmid donor strain. We used a focal strain as the
693 potential plasmid recipient in these experiments, which was only different from the focal strain
694 used in the main experiment by addition of a dTomato marker and a Chloramphenicol
695 resistance cassette (enabling us to detect transconjugants by selective plating). In the first set
696 of mating experiments, we grew overnight cultures of the potential plasmid donor strains and
697 the potential plasmid recipient strain in LB at 37 °C with shaking. We then pelleted the
698 overnight cultures by centrifugation (3000 rpm, 5 min), washed them twice with PBS and
699 resuspended them in 300 µl PBS. We then mixed the donor and recipient strains 1:1 (v:v), and
700 transferred 100 µl of this mixture to each of three replicate LB plates, and incubated the plates

701 for 6h at 37 °C. After that, we washed the cells off the plate with 500 µl PBS and streaked out
702 50 µl of this plate wash on LB plates supplemented with Ampicillin and Chloramphenicol. To
703 verify plasmid uptake by the transconjugants, we used a colony-PCR screen with primers
704 targeting the recipient strain (same primer set we used above to identify the focal strain) and
705 three additional primer sets targeting the beta-lactamase gene bla_{Tem}-*1b* (blaFW: 5'-
706 TGCAACTTTATCCGCCTCCA-3'; blaRV 5'-TTGAGAGTTTTCGCCCCGAA-3'), the
707 *traC* gene (traFW 5'- TCGATAAACGCCAGCTGGTT-3'; traRV 5'-
708 AGGTGAAAACCCACAGCGAA-3') and the replicon *IncFIC (FII)* (IncFW 5'-
709 CACCATCCTGCACTTACAATGC-3'; IncRV 5'- TCAGGCCCGGTTAAAAGACA-3')
710 with the same PCR reaction mix and settings as described above.
711
712 To test whether environmental conditions affected conjugation efficiency, we performed a
713 second set of mating experiments in four different conditions: solid LB agar, liquid LB, liquid
714 anaerobic LB and anaerobic sterile faecal slurry. We prepared the liquid LB (Sigma-Aldrich)
715 and LB agar (Sigma-Aldrich) for all treatments in the same way (independent of whether they
716 were aerobic or anaerobic), supplementing them with 0.5 g/l L-Cysteine and 0.001 g/l
717 Resazurin. For the anaerobic LB treatment, we transferred 0.9 ml LB to each Hungate tube,
718 flushed the headspace with nitrogen and sealed it with a rubber stopper before autoclaving. For
719 the anaerobic sterile faecal slurry treatment, we added 0.45 ml LB to each Hungate tube and
720 0.45ml thawed slurry under anaerobic conditions, before flushing the headspace with Nitrogen,
721 sealing and autoclaving. Prior to each mating assay, recipient and donor strains were
722 inoculated, washed, concentrated and mixed exactly the same way as described above for the
723 first set of mating experiments. For each solid and liquid treatment four replicates were
724 inoculated with 100 µl of the 1:1 donor recipient mix, either under aerobic or anaerobic
725 conditions according to the respective treatment and all tubes and plates were incubated for 6h

726 under static conditions at 37 °C. We stopped the mating assay by either vortexing the liquid
727 cultures or washing off the cells from the plates with 1 ml of PBS. 100 µl of each bacterial
728 suspension was then plated on selective agar plates containing either Chloramphenicol to count
729 total number of recipient cells or a mix of Chloramphenicol and Ampicillin to count the number
730 of transconjugants. After 24h we calculated transconjugant frequencies by dividing colony-
731 forming unit (C.F.U.) counts of plasmid-positive colonies by the total C.F.U. count of recipient
732 cells [69].
733
734 We measured susceptibility to ampicillin for the ancestral focal strain from the main
735 experiment, the focal strain used in the conjugation experiment (with the dTomato tag), one
736 focal strain transconjugant (with the plasmid from the resident *E. coli* isolate of human donor
737 1), the resident *E. coli* isolate of human donor 1 (carrying the same plasmid), and all eight
738 ampicillin resistant focal strain isolates from the main experiment. We did this by measuring
739 OD600 after 24h incubation at various concentrations of ampicillin. We prepared overnight
740 cultures of each isolate in a randomly organized master plate, and then inoculated the
741 susceptibility assay using a pin replicator to transfer approximately 1 µl of the overnight
742 cultures to assay plates filled with 100 µl of 0-60 µg/ml ampicillin per well. We measured OD
743 at 0h and after 24h with a NanoQuant infinite M200Pro platereader (Tecan).
744
745 *Amplicon sequencing*
746 We thawed samples of fresh faecal slurry from 0h and samples from each microcosm in the
747 community treatments after 24h and 168h on ice and homogenised them by vortexing. We
748 concentrated each slurry sample by centrifuging 1.5 ml of each sample at 3000 rpm directly in
749 the bead beating extraction tube, before removing the supernatant and repeating this step,
750 resulting in a total volume of 3 ml of each slurry sample. We then extracted the DNA from this

751 concentrate following the protocol of the powerlyzer power soil kit (Qiagen). DNA yield and
752 quality were checked by Qubit and Nanodrop.
753
754
755 We amplified the V3 and V4 region of the 16S rRNA gene with three slightly modified
756 universal primers [88] with an increment of a 1-nt frame shift in each primer [89] to increase
757 MiSeq sequencing output performance between target region and Illumina adapter. The target
758 region was amplified by limited 17 cycle PCR with all three primer sets in one reaction for
759 each sample. We cleaned up PCR products and in a second PCR adapters with the Illumina
760 barcodes of the Nextera XT index Kit v2 were attached. We checked 10 randomly selected
761 samples on the Tapestation (Agilent, Basel, Switzerland) for the proper fragment size. We
762 quantified library by qPCR with the KAPA library quantification Kit (KAPA Biosystems,
763 Wilmington, MA, USA) on the LightCycler 480 (Roche, Basel, Switzerland). We normalized
764 quantified samples, pooled them in equimolar amounts and loaded the library with 10% PhiX
765 on the Illumnina MiSeq platform with the Reagent Kit V3 at the Genetic Diversity Center
766 (ETHZ).
767
768 Sequencing reads were trimmed on both ends with seqtk version 1.3
769 (https://github.com/lh3/seqtk) and amplicons were merged into pairs with flash version 1.2.11
770 [90]. USEARCH version 11.0.667 [91] was then used to trim primer sites and amplicons were
771 quality filtered using prinseq version 0.20.4 [92]. We clustered the quality filtered sequences
772 into zero-radius operational taxonomic units (ZOTU) using USEARCH. We used Sintax
773 implemented in the USEARCH pipeline with the SILVA database [93] for the taxonomic
774 assignment of the ZOTU sequences. For the analysis of taxonomic data including plotting
775 Shannnon diversity, relative proportions of taxa and to generate the PCoA plots, we used the

776 Phyloseq package in R [94]. To estimate the frequency of *E. coli* relative to total
777 *Enterobacteriaceae*, we first isolated all ZOTUs assigned to *Enterobacteriaceae* and divided
778 these reads into *Escherichia coli* and other bacteria. We blasted both groups against the SILVA
779 database to check if the reads were assigned to the right group.
780
781 *Statistical analyses*
782 We used R 3.5.1 [95] for all analyses. To test whether focal strain abundance differed between
783 treatments, we used a generalized linear mixed effects model with the glmmadmb function of
784 the glmmADMB package, with zero inflation and a Poisson error distribution [96]. For this
785 analysis we excluded the basal medium treatment and used time, antibiotic (with / without),
786 resident microbial community (with / without) and human donor (1 / 2 / 3) as fixed effects and
787 replicate population as a random effect. The model was reduced by removing non-significant
788 ($P>0.05$) interactions using F-tests. P values for interaction terms in the reduced model were
789 obtained with type II Wald chisquare tests. To test whether there was an inhibitory effect of
790 sterilized slurry on the focal strain, we used the glmer function of the lme4 [97] R package. For
791 this analysis we included only community-free and antibiotic-free treatments, with focal strain
792 abundance as the response variable, donor as a fixed effect, replicate population as a random
793 effect, and a Poisson error distribution. After finding interactions between the effects of
794 resident microbiota depending on human donor and antibiotic, we analyzed subsets of the
795 dataset to look at ampicillin-free treatments only and individual human donors, using the same
796 approach as for the main model.
797
798 To analyse the effects of ampicillin and community presence/absence on the competitive
799 fitness of mutants and transconjugants (see S1 Methods), we used a linear model with the lmp
800 function of the lmperm package [98]. Here we took relative fitness for the respective mutant

801 or transconjugant as the response variable and antibiotic concentration and community
802 presence/absence as fixed effects, testing factor effects by permutation test (accounting for the
803 non-normal distribution of our fitness data).
804
805 To analyse differences in total bacterial abundance in the community treatments, we used a
806 linear mixed effects model with the lmer function of lme4 [97], with a Poisson error
807 distribution. Time, donor and antibiotic were fixed effects and replicate population a random
808 affect. To analyse variation of Shannon diversity, we used a linear mixed effects model with
809 the lme function of nlme [99]. We excluded time point 0h from the analysis, and included
810 Time, Donor and Antibiotic as fixed effects and replicate population as a random effect. To
811 analyze similarities of microbiome samples based on the 16S rRNA data, we applied the Bray-
812 Curtis distance metric with the ordinate function of the Phyloseq R package to get coordinates
813 for the principal coordinate analysis (PCoA). On this dataset we ran a permutational
814 multivariate analysis of variance (PERMANOVA) with the adonis function of vegan [100],
815 using the distance matrix obtained from the PCoA analysis but omitting time point 0h. We did
816 this separately for time points 24h and 168h.
817
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1126

1127 **Supplementary Figure legends**

1128

1129 **S1 Fig. Summary of experimental evolution in faecal slurry**. Treatments consisted of basal
1130 medium only, basal medium supplemented with sterilized faecal slurry (without the resident
1131 microbial community) from one of three human donors, or basal medium supplemented with
1132 sterilized faecal slurry to which the resident microbial community had been reintroduced (with
1133 community). After inoculation, all treatments were incubated for 2h at 37 °C, before 7 µg/ml
1134 ampicillin was added in the antibiotic treatment. Every 24h, we sampled each microcosm and
1135 transferred an aliquot to fresh medium (either basal medium or sterilized faecal slurry) with or
1136 without antibiotics. We serially diluted each sample and spread it on chromatic agar plates with
1137 or without antibiotics to quantify focal strain abundance (verified by colony PCR) and to screen
1138 for resistance. We sequenced focal strain isolates from the final time point and investigated
1139 community composition by 16S rRNA gene amplicon sequencing. We monitored total
1140 bacterial abundance in the community treatments by flow cytometry.

1141

1142 **S2 Fig. Variable similarity of microbial communities across time and treatment groups**.
1143 Each panel shows samples from a single human donor, with the same axes used in each panel.
1144 Points show the initial sample (0h) and microcosms from 24h and 168h with and without
1145 antibiotics (legend at right). Similarities between communities were calculated by Bray-Curtis
1146 distance and plotted using Principal Coordinate Analysis (see Methods).

1147

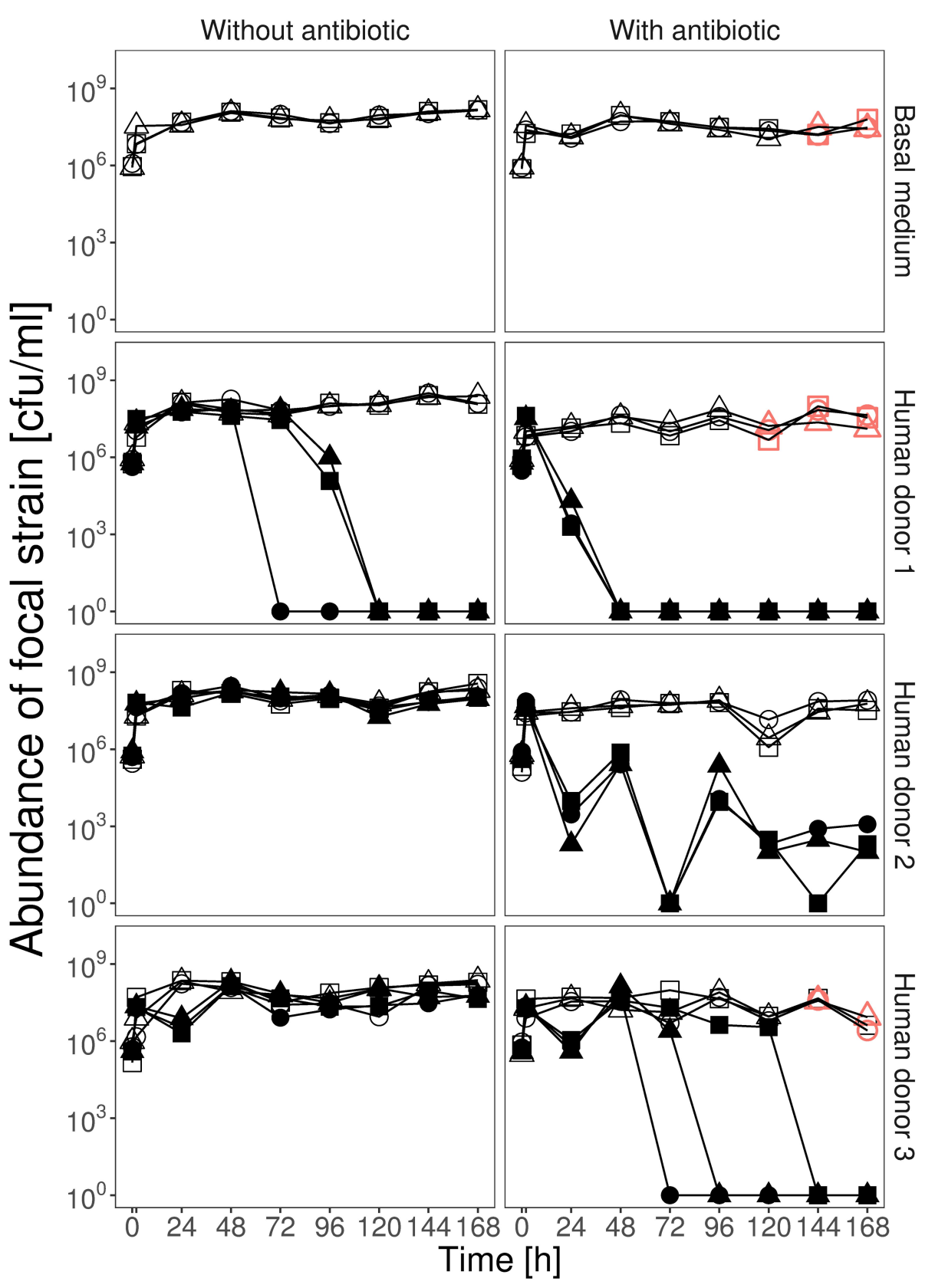
1148 **S3 Fig. Abundance of sequences associated with the focal strain, total *E. coli* and the**
1149 **resistance plasmid from the microbiota of human donor 1, inferred with qPCR**. Each
1150 panel shows the copy number of sequences detected with primers specific for the focal strain,
1151 total *E. coli* and the resistance plasmid (see legend; further details of primers in S1 Methods)

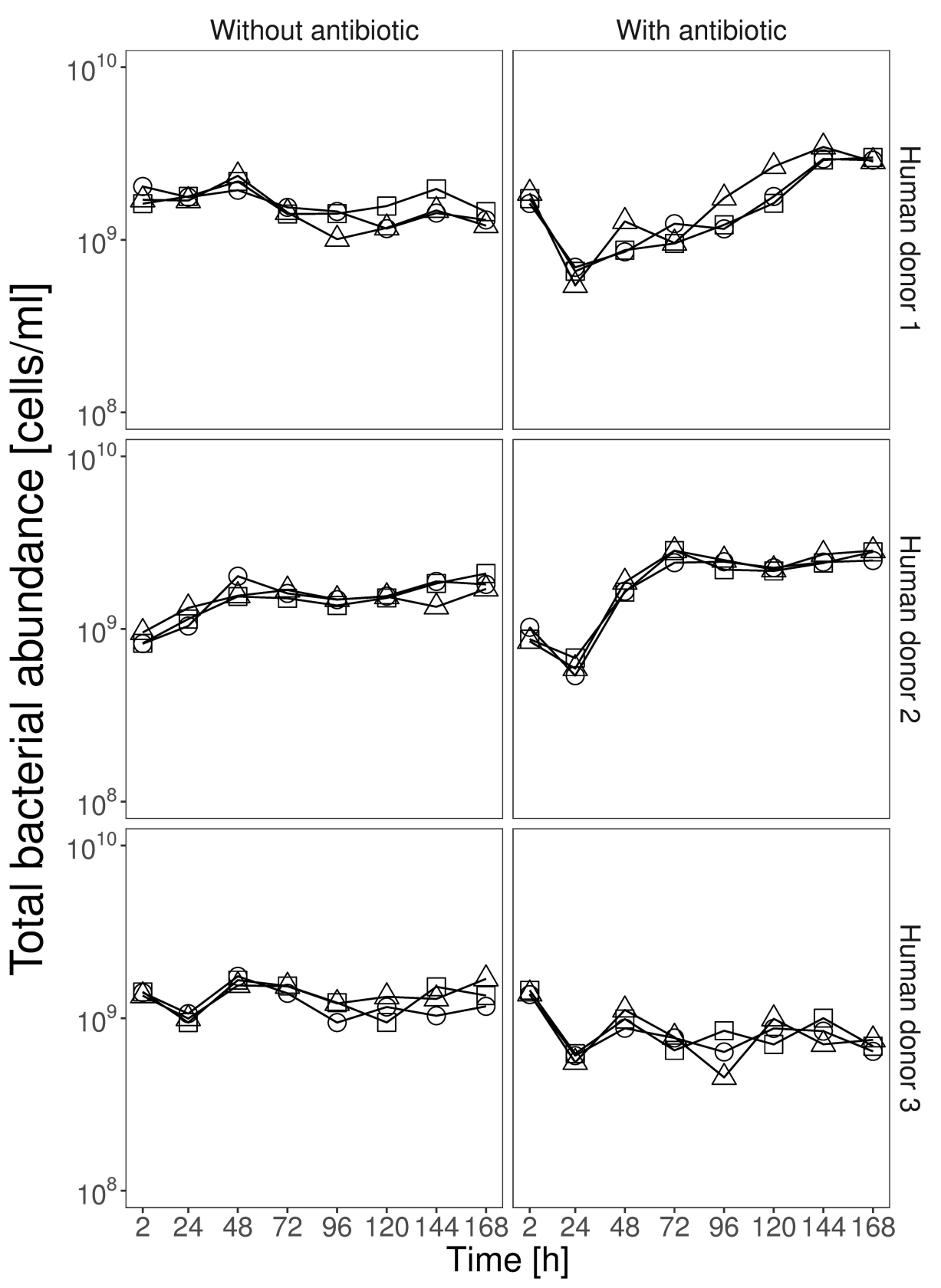
1152 at timepoint 0h (left panel), 24h (middle panel) and 168h (right panel). Each point shows the
1153 mean of three technical replicates. Reactions where no amplification was detected are shown
1154 at 10^0. We expect plasmid copy number to reflect the abundance of plasmid donor cells,
1155 because coverage analysis of whole genome sequencing data indicated a copy number per cell
1156 of ~1. For the focal strain and total *E. coli*, the copy number of sequences does not necessarily
1157 reflect the total number of cells of each type, but changes in strain abundance over time would
1158 nevertheless be expected to result in strongly correlated changes in sequence copy numbers
1159 over time.
1160

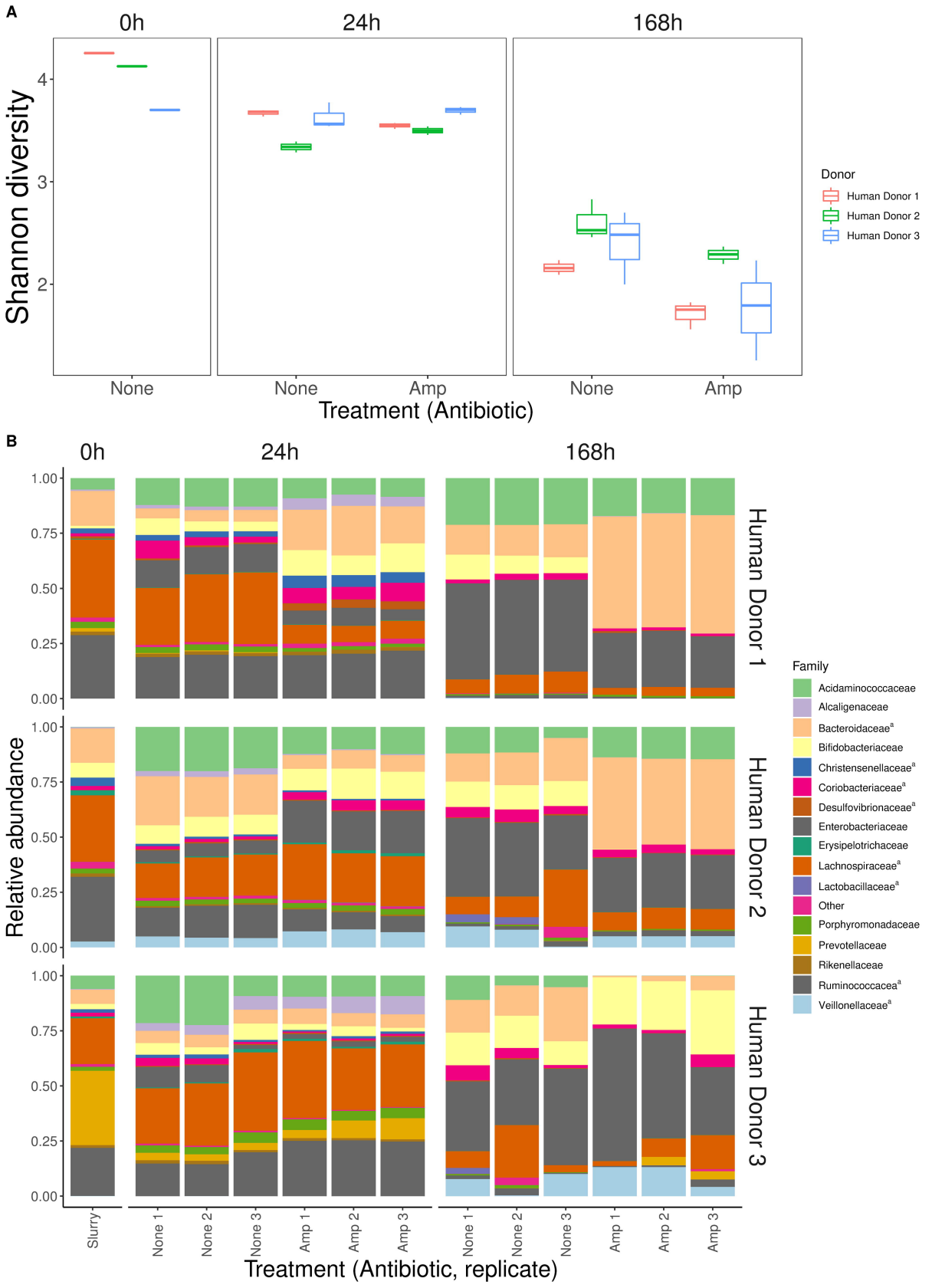
1161 **S4 Fig. Agarose gel electrophoresis picture of the PCR products specific for plasmid genes**
1162 **and a chromosomal marker of the focal strain.** We used these primer sets to verify plasmid
1163 uptake of the transconjugants. Primers are given in the main text in the methods section.
1164

1165 **S5 Fig. Competitive fitness of transconjugants and mutants relative to the ancestral focal**
1166 **strain in the presence and absence of resident microbial communities with no, sub-MIC**
1167 **and supra-MIC ampicillin**. (A) Final cell densities of competing strains (see legend;
1168 Transconjugant is a transconjugant of the focal strain carrying the plasmid from Human Donor
1169 1, in the left panel; Mutant is an evolved isolate with increased ampicillin-resistance from the
1170 community-free treatments with slurry from Human Donors 1, in the middle panel or Human
1171 Donor 3, in the right panel; Ancestor is the respective ancestral focal strain). Data are shown
1172 after 24h of competition in sterile slurry or community treatments, with and without low or
1173 high concentrations of ampicillin (*x*-axis). (B) Fitness of the transconjugant or mutant relative
1174 to the ancestor, calculated as the difference of their Matlhusian growth parameter in the same
1175 experiment. In both panels the three points show three replicates of the experiment.
1176
1177

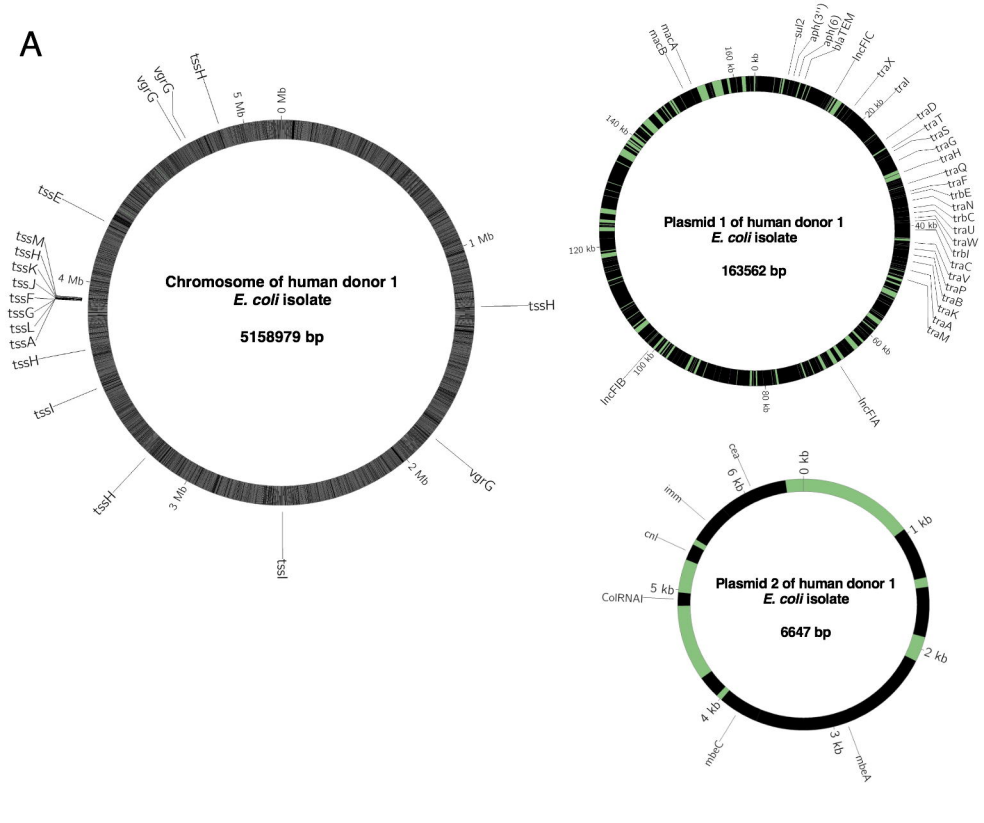
1178 **S6 Fig. Competitive fitness of transconjugants (carrying the plasmid from resident *E. coli***
1179 **of Human Donor 1) relative to evolved isolates (from community-free treatments with**
1180 **faecal slurry from Human Donor 1, left, and Human Donor 3, right).** In each panel relative
1181 fitness of the transconjugant strain is shown as the difference in malthusian growth parameters
1182 compared to the respective evolved isolate (see S1 Methods). Competitions were done in sterile
1183 faecal slurry or the presence of the resident microbial community, and with no, low or high
1184 ampicillin concentrations (*x*-axis). Each point shows a different replicate.
1185
1186 **S7 Fig. Abundance of the focal *E. coli* strain and resident *E. coli* strains isolated from**
1187 **Human Donors 1 and 3 (see legend) in monoculture (left) and in co-culture (right).** Each
1188 strain was grown in monoculture in the absence of antibiotics and each co-culture combination
1189 was grown in the presence and absence of ampicillin (*x*-axis). Each point shows a different
1190 replicate.
1191
1192
1193 **S8 Fig. Effect of starting bacterial density on growth inhibition by ampicillin ('inoculum**
1194 **effect'):** Changes in bacterial abundance over 24h are shown using three different
1195 quantification methods (optical density, top panel; plating and C.F.U. counting, middle panel;
1196 flow cytometry, bottom panel). In each panel the change in abundance is shown for four starting
1197 densities (see legend) and at four antibiotic concentrations. In each panel the change between
1198 0h and 24h is shown (in OD in the top panel, in C.F.U./ml in the middle panel, and in recorded
1199 events/ml in the bottom panel). Each point shows the mean of three replicates, error bars show
1200 1 s.d.
1201


Without antibiotic
With antibiotic
Basal medium
Human donor 1
Human donor 2
Human donor 3
Abundance of focal strain [cfu/ml]
10^9
10^6
10^3
10^0
0
24
48
72
96
120
144
168
Time [h]

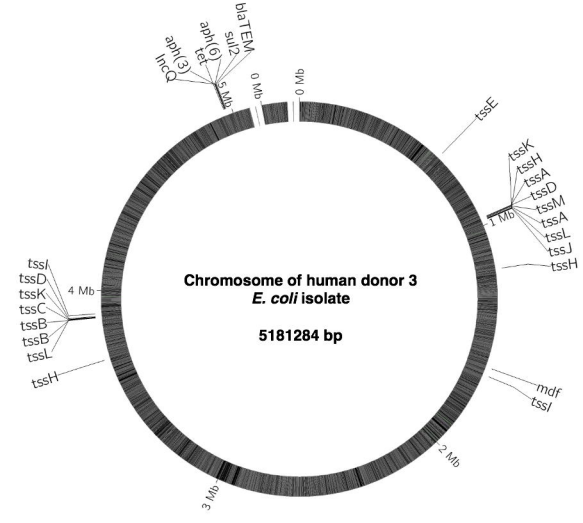

Without antibiotic
With antibiotic
Human donor 1
Human donor 2
Human donor 3
Total bacterial abundance [cells/ml]
10^{10}
10^{9}
10^{8}
2 24 48 72 96 120 144 168
Time [h]

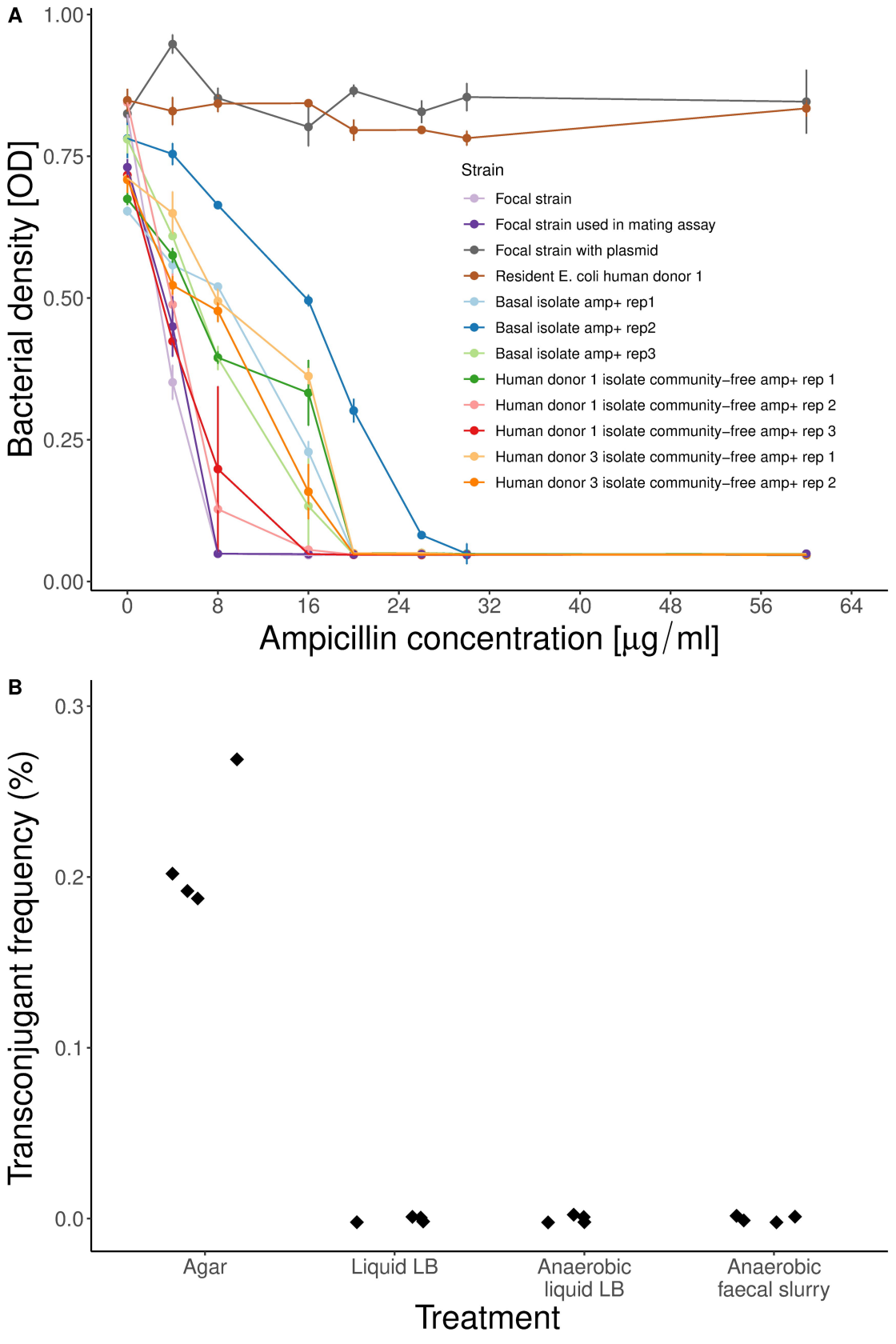

A
0h
24h
168h
Shannon diversity
4
3
2
None
None
Amp
None
Amp
Treatment (Antibiotic)
Donor
Human Donor 1
Human Donor 2
Human Donor 3
B
0h
24h
168h
Relative abundance
1.00
0.75
0.50
0.25
0.00
Human Donor 1
Human Donor 2
Human Donor 3
Slurry
None 1
None 2
None 3
Amp 1
Amp 2
Amp 3
Treatment (Antibiotic, replicate)
Family
Acidaminococcaceae
Alcaligenaceae
Bacteroidaceae[a]
Bifidobacteriaceae
Christensenellaceae[a]
Coriobacteriaceae[a]
Desulfovibrionaceae[a]
Enterobacteriaceae
Erysipelotrichaceae
Lachnospiraceae[a]
Lactobacillaceae[a]
Other
Porphyromonadaceae
Prevotellaceae
Rikenellaceae
Ruminococcaceae[a]
Veillonellaceae[a]

A


Chromosome of human donor 1
E. coli isolate
5158979 bp
0 Mb
1 Mb
2 Mb
3 Mb
4 Mb
5 Mb
tssH
vgrG
tssE
tssM
tssK
tssJ
tssF
tssG
tssL
tssA
tssI
Plasmid 1 of human donor 1
E. coli isolate
163562 bp
0 kb
20 kb
40 kb
60 kb
80 kb
100 kb
120 kb
140 kb
160 kb
macA
macB
sul2
aph(3'')
aph(6)
blaTEM
IncFIC
traX
traI
traD
traT
traS
traG
traH
traQ
traF
trbE
traN
trbC
traU
traW
trbI
traC
traV
traP
traB
traK
traA
traM
IncFIB
IncFIA
Plasmid 2 of human donor 1
E. coli isolate
6647 bp
0 kb
1 kb
2 kb
3 kb
4 kb
5 kb
6 kb
cea
imm
cnl
ColRNAI
mbeC
mbeA

B


Chromosome of human donor 3
E. coli isolate
5181284 bp
0 Mb
1 Mb
2 Mb
3 Mb
4 Mb
5 Mb
blaTEM
sul2
aph(6)
tet
aph(3'')
IncQ
tssE
tssK
tssH
tssA
tssD
tssM
tssA
tssL
tssJ
tssH
mdf
tssI
tssI
tssD
tssK
tssC
tssB
tssB
tssL
tssH


A
Bacterial density [OD]
1.00
0.75
0.50
0.25
0.00
0
8
16
24
32
40
48
56
64
Ampicillin concentration [µg/ml]
Strain
Focal strain
Focal strain used in mating assay
Focal strain with plasmid
Resident E. coli human donor 1
Basal isolate amp+ rep1
Basal isolate amp+ rep2
Basal isolate amp+ rep3
Human donor 1 isolate community−free amp+ rep 1
Human donor 1 isolate community−free amp+ rep 2
Human donor 1 isolate community−free amp+ rep 3
Human donor 3 isolate community−free amp+ rep 1
Human donor 3 isolate community−free amp+ rep 2
B
Transconjugant frequency (%)
0.3
0.2
0.1
0.0
Agar
Liquid LB
Anaerobic liquid LB
Anaerobic faecal slurry
Treatment