

1 Simplified fecal community transplant restores *Clostridioides*
2 *difficile* colonization resistance to antibiotic perturbed
3 murine communities

4 Running title: Fecal community transplant restores *Clostridioides difficile* colonization
5 resistance

6 Nicholas A. Lesniak¹, Sarah Tomkovich¹, Andrew Henry¹, Ana Taylor¹, Joanna Colovas¹,
7 Lucas Bishop¹, Kathryn McBride¹, Patrick D. Schloss^{1,†}

8 † To whom correspondence should be addressed: pschloss@umich.edu

9 1. Department of Microbiology and Immunology, University of Michigan, Ann Arbor, MI
10 48109

11 **Abstract**

12 Fecal communities transplanted into individuals can eliminate recurrent *Clostridioides*
13 *difficile* infection (CDI) with high efficacy. However, this treatment is only used once CDI
14 becomes resistant to antibiotics or has recurred multiple times. We sought to investigate
15 whether a fecal community transplant (FCT) pre-treatment could be used to prevent CDI
16 altogether. We treated male C57BL/6 mice with either clindamycin, cefoperazone, or
17 streptomycin, and then inoculated them with the microbial community from untreated
18 mice before challenging with *C. difficile*. We measured colonization and sequenced
19 the V4 region of the 16S rRNA gene to understand the dynamics of the murine fecal
20 community in response to the FCT and *C. difficile* challenge. Clindamycin-treated mice
21 became colonized with *C. difficile* but cleared it naturally and did not benefit from the FCT.
22 Cefoperazone-treated mice became colonized by *C. difficile*, but the FCT enabled clearance
23 of *C. difficile*. In streptomycin-treated mice, the FCT was able to prevent *C. difficile* from
24 colonizing. Then we diluted the FCT and repeated the experiments. Cefoperazone-treated
25 mice no longer cleared *C. difficile*. However, streptomycin-treated mice colonized with
26 1:10² dilutions resisted *C. difficile* colonization. Streptomycin-treated mice that received a
27 FCT diluted 1:10³, *C. difficile* colonized but later was cleared. In streptomycin-treated mice,
28 inhibition of *C. difficile* was associated with increased relative abundance of a group of
29 bacteria related to *Porphyromonadaceae* and *Lachnospiraceae*. These data demonstrate
30 that *C. difficile* colonization resistance can be restored to a susceptible community with a
31 FCT as long as it complements the missing populations.

32 **Importance**

33 Antibiotic use, ubiquitous with the healthcare environment, is a major risk factor for
34 *Clostridioides difficile* infection (CDI), the most common nosocomial infection. When
35 *C. difficile* becomes resistant to antibiotics, a fecal microbiota transplant from a healthy

³⁶ individual can effectively restore the gut bacterial community and eliminate the infection.
³⁷ While this relationship between the gut bacteria and CDI is well established, there are
³⁸ no therapies to treat a perturbed gut community to prevent CDI. This study explored
³⁹ the potential of restoring colonization resistance to antibiotic-induced susceptible gut
⁴⁰ communities. We described the effect gut bacteria community variation has on the
⁴¹ effectiveness of a fecal community transplant for inhibiting CDI. These data demonstrated
⁴² that communities susceptible to CDI can be supplemented with fecal communities but the
⁴³ effectiveness depended on the structure of the community following the perturbation. Thus,
⁴⁴ a simplified bacterial community may be able to recover colonization resistance to patients
⁴⁵ treated with antibiotics.

46 **Introduction**

47 The process by which gut bacteria prevent *Clostridioides difficile* and other pathogens
48 from infecting and persisting in the intestine is known as colonization resistance (1).
49 Antibiotic-induced disruption of the gut bacterial community breaks down colonization
50 resistance and is a major risk factor for *C. difficile* infection (CDI) (2). Gut bacteria
51 inhibit *C. difficile* through the production of bacteriocins, modulation of available bile acids,
52 competition for nutrients, production of short-chain fatty acids, and altering the integrity of
53 the mucus layer (1). After the initial CDI is cleared via antibiotics, patients can become
54 reinfected. When CDI recurs more than once, the gut bacterial community from a healthy
55 person typically is used to restore the gut community in the patient with recurrent CDI
56 (3). Fecal microbiota transplant (FMT) is effective, but 10 - 20% of people that receive
57 a FMT will still have another CDI (4). Additionally, transfer of a whole fecal community
58 can incidentally transfer pathogens and cause adverse outcomes (5). While the FMT is
59 effective at curing recurrent CDI, it also has risks that must be considered.

60 The benefits and risks of the FMT has led to the development of simplified bacterial
61 communities to treat CDI. Synthetic communities are more defined than an FMT, making
62 them easier to regulate as a drug. Tvede and Rask-Madsen were the first to successfully
63 treat CDI with a community of isolates cultured from human feces (6). More recently,
64 Lawley *et al.* analyzed murine experiments and the fecal communities from patients with
65 CDI to develop a synthetic community of six isolates to inhibit *C. difficile* colonization
66 (7). Simplified communities derived from human fecal communities, by methods such as
67 selective isolation of spores or culturing bacteria, have cured recurrent CDI in their initial
68 application (8–10). Although, a recent phase 2 trial of SER-109, a spore-based treatment,
69 failed its phase 2 clinical trial (11), these therapies have the potential to offer the benefits
70 of the FMT without the associated risks but are only used once a patient has had multiple
71 CDIs. For these to be successful, we need a better approach to identify candidate bacterial

72 populations. Recently, an autologous FMT was shown to be effective at restoring the gut
73 microbiota in allogeneic hematopoietic stem cell transplantation patients and prevented
74 future complications, such as systemic infections (12). It is unclear whether an treatment
75 similar to an autologous FMT or simplified bacterial communities could be used to restore
76 susceptible communities and prevent CDI (13).

77 Because FMT is often sufficient to restore colonization resistance to people with a
78 current infection, we hypothesized that a fecal community should be sufficient to restore
79 colonization resistance to an uninfected community. Therefore, we tested whether
80 a fecal community transplant (FCT) pre-treatment would prevent or clear *C. difficile*
81 colonization and how variation in susceptibility to *C. difficile* infection would affect the
82 effectiveness of FCT pre-treatment. After testing the same FCT pre-treatment across
83 different antibiotic-induced susceptibilities, we sought to determine whether diluted FCT
84 pre-treatment could maintain the inhibition of *C. difficile* colonization and identify the
85 bacterial populations associated with colonization resistance and clearance.

86 Results

87 **Effect of fecal transplant on *C. difficile* colonization was not consistent across**
88 **antibiotic treatments.** Our previous research demonstrated that when mice were
89 perturbed with different antibiotics, there were antibiotic-specific changes to the microbial
90 community that resulted in different levels of colonization and clearance of *Clostridioides*
91 *difficile* infection (14). Because each of these treatments opened different niche spaces
92 that *C. difficile* could fill, we hypothesized that the resulting community varied in the types
93 of bacteria required to recover colonization resistance. To test the ability of the murine
94 communities to recover colonization resistance, we treated conventionally raised SPF
95 C57BL/6 mice with either clindamycin, cefoperazone, or streptomycin. After a short
96 recovery period, the mice were given either phosphate-saline buffer (PBS) or a fecal

97 community transplant via oral gavage (Figure 1). The fecal community was obtained
98 from untreated mice. One day after receiving the FCT, the mice were challenged with
99 10^3 *C. difficile* 630 spores. One day after the challenge, mice that were treated with
100 either clindamycin or cefoperazone and received the FCT pre-treatment had similar
101 amounts of *C. difficile* colony forming units (CFU) as those which received PBS. Among
102 the clindamycin-treated mice, *C. difficile* colonization was cleared at similar rates
103 regardless of whether they received the FCT or PBS pre-treatments (Figure 2). For
104 cefoperazone-treated mice, *C. difficile* colonized all of the mice, but the mice that received
105 the FCT pre-treatment cleared the infection (Figure 2). For the streptomycin-treated mice,
106 the FCT pre-treatment resulted in either no detectable *C. difficile* colonization (8 of 14) or
107 an infection that the community cleared within 5 days (Figure 2). For mice that would have
108 normally had a persistent infection, the FCT enabled them to clear the infection and in the
109 streptomycin-treated mice it was able to prevent infection entirely for some mice.

110 **Diluted fecal communities prevented colonization and promoted clearance for**
111 **streptomycin-treated mice.** Next, we sought to test whether mice that received a diluted
112 FCT pre-treatment could still benefit. To identify the minimally effective dilution, we
113 repeated the same experimental design (Figure 1) with the FCT diluted serially down to
114 1:10⁵. Since the FCT pre-treatment had no detected effect in clindamycin-treated mice, we
115 did not study those mice further. Cefoperazone-treated mice pre-treated with diluted FCT,
116 1:10 and lower, were not affected and were colonized throughout the experiment (Figure
117 3). Streptomycin-treated mice pre-treated with diluted FCT either regained colonization
118 resistance or were enabled to clear *C. difficile*. The streptomycin-treated mice pre-treated
119 with FCT as dilute as 1:10³ cleared *C. difficile*. The streptomycin-treated mice pre-treated
120 with FCT as dilute as 1:10² had no *C. difficile* CFU detected throughout the length of the
121 experiment. While more mice pre-treated with lower FCT dilutions were colonized (FCT 6
122 of 14 were colonized, 1:10 10 of 12 were colonized, 1:10² 10 of 14 were colonized), the
123 colonized mice that received the lower dilutions were still able to clear *C. difficile* (Figure

¹²⁴ S1). Thus, the simplified fecal communities from the diluted FCT were able to restore
¹²⁵ colonization resistance and promote clearance of *C. difficile* in streptomycin-treated mice.

¹²⁶ The simplified fecal communities of the diluted FCT may have reduced abundance and
¹²⁷ membership. We compared the FCT communities to determine the differences between
¹²⁸ the dilutions. The most significant difference between the communities of the FCT and its
¹²⁹ dilutions was the quantity of the 16S rRNA gene, which decreased monotonically (Figure
¹³⁰ S2D). The FCT dilutions of 1:10³ to 1:10⁵ of the had few samples with sufficient sequencing
¹³¹ depth to provide bacterial community information. The FCT and its dilutions were not
¹³² significantly different in either α -diversity (number of operational taxonomic units (OTUs)
¹³³ (S_{obs}) or Inverse Simpson) or β -diversity (θ_{YC}) (Figure S2A-C). Populations of *Acetatifactor*,
¹³⁴ *Enterobacteriaceae*, *Lactobacillus*, *Ruminococcaceae*, and *Turicibacter* correlated with
¹³⁵ the FCT dilution factor (Figure S2E). Overall, the abundance of the bacteria appeared to
¹³⁶ be largest difference between FCT and its dilutions.

¹³⁷ **Murine gut bacterial communities had not recovered their diversity by the time of**
¹³⁸ ***C. difficile* challenge.** To elucidate the effects of the fecal community dilution on the
¹³⁹ murine gut bacterial community and *C. difficile* infection, we sequenced the V4 region of
¹⁴⁰ the 16S rRNA gene from the fecal community. For the gut communities, in comparison to
¹⁴¹ the initial community (day -9), FCT pre-treatment did not result in a significant recovery of
¹⁴² diversity at the time of *C. difficile* challenge (day 0) for cefoperazone-treated mice (Figure
¹⁴³ S3) or streptomycin-treated mice (Figure 4). At the end of the experiment (day 10), the gut
¹⁴⁴ bacterial communities were more similar to their initial community in α -diversity (number
¹⁴⁵ of OTUs (S_{obs}) and Inverse Simpson diversity index) and β -diversity (θ_{YC}) diversity. The
¹⁴⁶ mice pre-treated with less dilute FCT were most similar to the initial community structure,
¹⁴⁷ whereas, the mice pre-treated with more dilute FCT resulted in little recovery of diversity,
¹⁴⁸ similar to the mice given PBS. Thus, the effect of FCT was not large enough at the time
¹⁴⁹ of *C. difficile* challenge to be detected in the community diversity but sufficient to affect

150 *C. difficile* colonization. This would suggest the effect was driven by the most abundant
151 populations.

152 **Gut bacterial community members are differentially abundant in streptomycin-treated**
153 **mice resistant to colonization.** Although there were no significant differences in diversity
154 at the time of challenge, we next investigated how the individual bacterial populations were
155 different in the uncolonized streptomycin-treated mice pre-treated with FCT. We used
156 linear discriminant analysis (LDA) effect size (LEfSe) analysis to identify OTUs within the
157 fecal bacterial communities from the streptomycin-treated mice that were differentially
158 abundant between uncolonized and colonized mice. The antibiotic treatment significantly
159 altered 99 OTUs (Figure S5), but on the day of *C. difficile* challenge only 7 OTUs were
160 differentially abundant between colonized and uncolonized communities (Figure 5A).
161 Communities resistant to *C. difficile* colonization had more abundant populations of OTUs
162 related to *Akkermansia*, *Clostridiales*, *Olsenella*, and *Porphyromonadaceae* and less
163 abundant populations of an OTU related to *Enterobacteriaceae*. Thus, a small portion of
164 OTUs, relative to the changes due to streptomycin treatment, were differentially abundant
165 in mice that resisted *C. difficile* colonization compared to those that were colonized.

166 **Murine gut bacterial communities that cleared *C. difficile* colonization were**
167 **more similar to the initial community.** To better understand the differences in
168 streptomycin-treated murine fecal community that contributed to *C. difficile* clearance, we
169 compared the communities that cleared *C. difficile* to those that did not at the time of
170 challenge and 10 days post infection. Communities from mice that cleared colonization
171 were more similar to their initial community at the end of the experiment than the mice
172 that remained colonized (Figure S4). At the time of *C. difficile* challenge, 9 OTUs were
173 differentially abundant between communities that remained colonized to those that cleared
174 colonization (Figure 5B). Communities that cleared *C. difficile* colonization had more
175 abundant populations of OTUs related to *Porphyromonadaceae* and *Lachnospiraceae* and

176 less abundant populations of OTUs related to *Acetatifactor*, *Lachnospiraceae*, *Olsenella*,
177 *Porphyromonadaceae*, and *Salmonella*. At the end of the experiment, 29 of the 34
178 differentially abundant OTUs were more abundant in the mice that were able to clear
179 the colonization (Figure 5C). The relative abundance of OTUs related to *Acetatifactor*,
180 *Anaeroplasma*, *Enterococcus*, *Lachnospiraceae*, *Lactobacillus*, *Porphyromonadaceae*,
181 and *Ruminococcaceae* were higher in communities that cleared, recovering in abundance
182 from the streptomycin treatment. Multiple OTUs related to *Lachnospiraceae* and
183 *Porphyromonadaceae* ($N = 14$ and $N = 5$, respectively) were significant and accounted for
184 greater portions of the community (more than 10%). However one *Porphyromonadaceae*
185 population (OTU 5) and two *Lachnospiraceae* related populations (OTU 40 and 95) were
186 more abundant in the mice that remain colonized. Thus, the more the gut bacterial
187 members returned to their initial abundance, there was a greater likelihood of clearing *C.*
188 *difficile*.

189 **Negative associations dominated the interactions between the gut bacterial**
190 **community and *C. difficile* in streptomycin-treated mice.** In streptomycin-treated
191 mice, pre-treatment with FCT and its dilutions had different effects on the bacterial
192 community members which resulted in different community relative abundance and *C.*
193 *difficile* colonization dynamics. We quantified the relationships occurring throughout
194 this experiment using SPIEC-EASI (sparse inverse covariance estimation for ecological
195 association inference) to construct a conditional independence network. Here, we
196 focused on the associations of the *C. difficile* subnetwork (Figure 6). *C. difficile* CFU
197 had positive associations with populations of OTUs related to *Enterobacteriaceae*
198 (OTU 4) and *Peptostreptococcaceae* (OTU 19). OTUs related to *Clostridiales* (OTU
199 27), *Lachnospiraceae* (OTUs 15, 51, and 83), and *Porphyromonadaceae* (OTUs 23,
200 25, and 29) had negative associations with *C. difficile*, as well as the OTUs related to
201 *Enterobacteriaceae*, and *Peptostreptococcaceae*. Overall, the majority of the associations
202 between *C. difficile* and the gut bacterial community in streptomycin-treated mice were

203 negative. This suggests this subset of the community may be driving the inhibition of *C.*
204 *difficile* in streptomycin-treated communities.

205 **Discussion**

206 Transplanting the fecal community from untreated mice to antibiotic-treated mice prior
207 to being challenged with *C. difficile* varied in effectiveness based on the antibiotic
208 treatment. This indicated that FCT pre-treatment can prevent *C. difficile* colonization in
209 an antibiotic-specific manner. Additionally, by diluting the FCT we were able to narrow
210 the community changes responsible for the effect to the most abundant OTUs. Overall,
211 these results show that a simplified fecal community can assist a perturbed microbiota
212 in preventing or resisting *C. difficile* colonization but the effect was dependent on the
213 antibiotic that was given.

214 By diluting the FCT we were able to narrow the definition of the minimal community features
215 that restored colonization resistance. Bacterial interactions with *C. difficile* were associated
216 with the identity, abundance, and functions of adjacent bacteria. Ghimire *et al.* recently
217 showed individual species that inhibited *C. difficile* in co-culture but when other inhibitory
218 species were added the overall effect on *C. difficile* was changed, in some cases to a
219 increased *C. difficile* growth (15). Based on these observations from their bottom-up
220 approach, it is unclear how more complex combinations would affect the inhibition of *C.*
221 *difficile*. So instead, we sought to find an inhibitory community using a top-down approach
222 and begin with an inhibitory community. In a recent top-down approach, Auchtung *et al.*
223 recently developed a set of simplified communities from human fecal communities that
224 were grown in minibioreactor arrays and tested for inhibition first *in vitro* then in a mouse
225 model (16). They found four simplified communities that were able to reduce *C. difficile*
226 colonization but with varied effect in a mouse model with the same gut microbiota. One
227 way they simplified the community was through diluting the initial fecal sample. In our

228 experiments, we began with a fresh whole fecal community to first determine if inhibition
229 was possible. In the conditions which *C. difficile* was inhibited, with cefoperazone and
230 streptomycin, we diluted the FCT to determine the minimal community which maintained
231 inhibition. Cefoperazone-treated mice were unable to maintain inhibition of *C. difficile* with
232 diluted FCT pre-treatments and *C. difficile* remained colonized. Streptomycin-treated mice
233 were able to maintain inhibition with diluted FCT pre-treatment. While the diluted FCTs had
234 similar diversity and bacterial abundances, the differences in effect on *C. difficile* revealed
235 the minimal changes associated with either colonization resistance or clearance.

236 We previously hypothesized that mice treated with either clindamycin, cefoperazone, or
237 streptomycin would not have the same bacterial community changes associated with *C.*
238 *difficile* clearance (14). In that set of experiments, the dose of the antibiotic was varied to
239 titrate changes to the community and determine what changes allow *C. difficile* to colonize
240 and then be spontaneously cleared. We observed antibiotic-specific changes associated
241 with *C. difficile* clearance. The data presented here complement those observations. For
242 clindamycin-treated mice, there was no difference in colonization, clearance or relative
243 abundance between PBS and FCT pre-treatment. *C. difficile* had similar colonization
244 dynamics. It is possible that there was insufficient time for the FCT to engraft. However,
245 when we added more time between clindamycin treatment and *C. difficile* challenge,
246 *C. difficile* was unable colonize (data not shown). Therefore, clindamycin-treated mice
247 appeared to have been naturally recovering inhibition to *C. difficile*, which was unaffected
248 by the FCT pre-treatment. For cefoperazone-treated mice, the FCT pre-treatment enabled
249 the gut microbiota to eliminate the colonization but only in its most concentrated dose.
250 This observation supports our previous report, indicating that the cefoperazone-treated
251 community is more sensitivity to the amount of FCT it receives since cefoperazone reduced
252 many bacterial groups and associations (Figure S6). As we previously hypothesized,
253 streptomycin-treated mice were enabled to clear with a subset of the community, with the
254 FCT pre-treatment diluted 1:10³. Since we titrated the FCT dilutions, we could compare the

255 bacterial communities of the mice which gained the ability to clear *C. difficile* to the mice
256 that received the next dilution which could not to elucidate the minimal relative abundance
257 differences. In agreement with previous studies, OTUs related to *Lachnospiraceae*,
258 *Porphyromonadaceae*, and *Ruminococcaceae* increased with the clearance of *C. difficile* in
259 the streptomycin-treated mice (14, 17–21). These data agree with our previous hypothesis
260 that a simplified fecal community would only be able to promote clearance of *C. difficile* in
261 streptomycin-treated mice.

262 In addition to clearing *C. difficile*, a simplified fecal community restored colonization
263 resistance to streptomycin-treated mice. Mice that received FCT pre-treatment as
264 dilute as 1:10² were not colonized to a detectable level. While restoring colonization
265 resistance is not novel (22), here we have shown that the restoration of colonization
266 resistance is dependent on the community perturbation and the fecal community being
267 transplanted. As we identified community members associated with clearance, OTUs
268 related to *Akkermansia*, *Olsenella*, and *Porphyromonadaceae* were more abundant and an
269 OTU related to *Enterobacteriaceae* was less abundant at the time of *C. difficile* challenge.
270 *Enterobacteriaceae* has been associated with *C. difficile* colonization and inflammation (14,
271 23, 24). Larger populations of *Akkermansia* were associated with preventing colonization,
272 which we had previously observed, potentially indicating the maintenance of a protective
273 mucus layer (14, 25–27). Increased populations of a select set of OTUs related to
274 *Porphyromonadaceae* were also more abundant in mice that were resistant to colonization.
275 *Porphyromonadaceae* may be inhibiting *C. difficile* via butyrate and acetate production,
276 which has been associated with successful FMT treatments (28–30). Different populations
277 of OTUs associated with *Porphyromonadaceae* were associated with colonization
278 resistance than with colonization clearance. These colonization resistance-associated
279 OTUs (OTUs 8, 25 and 29) may have OTU-specific functions or dependent abundances
280 of members of the community, such as *Akkermansia* and *Enterobacteriaceae*. With our
281 top-down approach, we reduced the number of gut bacterial community members that

282 were associated with colonization resistance in streptomycin-treated mice.

283 We have demonstrated that a simplified bacterial community can restore colonization
284 resistance but the effect of the community and the bacteria that colonized was dependent
285 on the specific changes to the community that were caused by each antibiotic. When
286 transplanting the fecal community into antibiotic-induced susceptible mice, only mice
287 treated with streptomycin were able to restore colonization resistance. Previous studies
288 that have identified simplified communities in a murine model using a homogeneous
289 gut microbiota with a bottom-up approach (7, 19). Treatments supplementing the gut
290 microbiota would benefit from being tested in different communities susceptible to CDI.
291 Further research is necessary to characterize the specific niche spaces *C. difficile* of
292 susceptibilities communities and the specific requirements fill those spaces. Then it may
293 be possible to identify people with gut microbiota that are susceptible to CDI and develop
294 targeted simplified bacterial communities to recover colonization resistance and reduce
295 the risk of CDI.

296 Materials and Methods

297 **Animal care.** Mice used in experiments were 6- to 13-week old conventionally reared SPF
298 male C57BL/6 mice obtained from a single breeding colony at the University of Michigan.
299 During the experiment, mice we housed with two or three mice per cage. All murine
300 experiments were approved by the University of Michigan Animal Care and Use Committee
301 (IACUC) under protocol number PRO00006983.

302 **Antibiotic administration.** Mice were given either cefoperazone, clindamycin, or
303 streptomycin. Cefoperazone (0.5 mg/ml) and streptomycin (5 mg/ml) were administered
304 via drinking water for 5 days, beginning 9 days prior to *C. difficile* challenge. Antibiotic
305 water was replaced every two days. Clindamycin (10 mg/kg) was injected into the
306 intraperitoneal space, 2 days prior to challenge with *C. difficile*. All antibiotics were filter

307 sterilized with a 0.22 μ m syringe filter prior to use.

308 **Fecal community transplants.** Fecal pellets were collected from similar aged C57BL/6
309 mice not being used in an experiment the day of the fecal community transplants. 15-20
310 pellets were collected and weighed. The fecal pellets were homogenized weight per weight
311 in phosphate-saline buffer (PBS) containing 15% glycerol (fecal community transplant,
312 FCT) in anaerobic conditions. The FCT was serially diluted in PBS containing 15% glycerol
313 down to 1:10⁵ fecal dilution and aliquoted into tubes for gavaging into mice. One set
314 of aliquots were frozen at -80°C to be used the following day for the cefoperazone and
315 streptomycin experiments. Frozen aliquots were thawed at 37°C for 5 minutes prior to
316 being used. All fecal community dilutions were centrifuged at 7500 RPM for 60 seconds.
317 Mice were inoculated with 100 uL of the fecal dilution oral via a 21 gauge gavage needle.
318 Fecal community transplants were administered from most dilute to least, which began
319 with mice receiving PBS and finished with mice receiving FCT. Aliquots were frozen at
320 -80°C after use for sequencing. These experiments were repeated 8 times with a different
321 starting source each time.

322 **16S rRNA quantitative real-time PCR.** Quantitative analysis of 16S rRNA in fecal
323 community dilutions used for FCT was carried out using quantitative real-time PCR using
324 primers and cycler conditions specified previously (31). Reaction volumes were prepared
325 using 6 uL of SYBRTM Green PCR Master Mix (Applied Biosciences Ref 4344463), 1 uL
326 each forward and reverse primer, and 2 uL sample DNA template. All qPCR reactions
327 were run on a LightCycler® 96 (Roche Ref 05815916001) using instrument-specific plates
328 and seals.

329 **C. difficile challenge.** For experiments using streptomycin or cefoperazone, mice were
330 given untreated drinking water for 96 hours before challenging with *C. difficile* strain
331 630Δerm spores. For experiments using clindamycin, mice were given untreated drinking
332 water for 48 hours the time of the intraperitoneal injection and being challenged with *C.*

333 *C. difficile* strain 630Δerm spores. *C. difficile* spores were aliquoted from a single spore stock
334 stored at 4°C. Spore concentration was determined two days prior to the day of challenge
335 (32). Mice were inoculated with 10³ *C. difficile* spores via oral gavage. After inoculating
336 the mice, remaining spore solution was serially diluted and plated to confirm the spore
337 concentration.

338 **Sample collection.** Fecal samples were collected prior to administering antibiotics, after
339 antibiotics were removed, prior to *C. difficile* challenge and on each of the 10 days post
340 infection. Approximately 15 mg of each fecal sample was collected and weighed for plating
341 *C. difficile* colony forming units (CFU) and the remaining sample was frozen at -80°C for
342 later sequencing. The weighed fecal samples were anaerobically serially diluted in PBS,
343 plated on TCCFA plates, and incubated at 37°C for 24 hours. The resultant colonies were
344 enumerated to determine the *C. difficile* CFUs (33).

345 **DNA sequencing.** Total bacterial DNA was extracted from the frozen samples by the
346 MOBIO PowerSoil-htp 96-well soil DNA isolation kit. We amplified the 16S rRNA gene V4
347 region and the amplicons were sequenced on an Illumina MiSeq as described previously
348 (34).

349 **Sequence curation.** Sequences were processed using mothur (v.1.44.1) (34, 35). We
350 used a 3% dissimilarity cutoff to group sequences into operational taxonomic units (OTUs)
351 and a naive Bayesian classifier with the Ribosomal Database Project training set (version
352 16) to assign taxonomic classifications to OTUs (36). We sequenced a mock community of
353 a known 16S rRNA gene sequences and composition. We processed this mock community
354 in parallel with our samples to determine the error rate for our sequence curation, which
355 resulted in an error rate of 0.029%.

356 **Statistical analysis and modeling.** We calculated diversity metrics in mothur. For
357 α-diversity comparisons, we calculated the number of OTUs (S_{obs}) and the Inverse

358 Simpson diversity index. For β -diversity comparisons, we calculated dissimilarity matrices
359 based on metric of Yue and Clayton (θ_{YC}) (37). We rarefied samples to 2,480 sequences
360 per sample to limit uneven sampling biases. OTUs were subsampled to 2,480 counts
361 per sample. We tested for differences in relative abundance between outcomes with
362 LEfSe in mothur. All other statistical analyses and data visualization was completed in
363 R (v4.0.5) with the tidyverse package (v1.3.1). Pairwise comparisons of α -diversity (S_{obs}
364 and Inverse Simpson), β -diversity (θ_{YC}), were calculated by pairwise Wilcoxon rank sum
365 test. Correlations between bacterial genera and fecal community dilution were calculated
366 using the Spearman correlation. P values were corrected for multiple comparisons with a
367 Benjamini and Hochberg adjustment for a type I error rate of 0.05 (38). For streptomycin
368 experiments, conditional independence networks were calculated from the day 1 through 5
369 samples of all mice using SPIEC-EASI (sparse inverse covariance estimation for ecological
370 association inference) methods from the SpiecEasi R package after optimizing lambda to
371 0.001 with a network stability between 0.045 and 0.05 (v1.0.7) (39).

372 **Code availability.** Scripts necessary to reproduce our analysis and this paper are available
373 in an online repository (https://github.com/SchlossLab/Lesniak_restoreCR_XXXX_2022).

374 **Sequence data accession number.** All 16S rRNA gene sequence data and associated
375 metadata are available through the Sequence Read Archive via accession SRP373949.

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³⁸² **Author contributions**

³⁸³ Conceptualization: N.A.L., S.T., P.D.S.; Data curation: N.A.L., L.B., K.M.; Formal analysis:
³⁸⁴ N.A.L., ; Investigation: N.A.L., S.T., A.H., A.T., J.C., L.B., K.M., P.D.S.; Methodology: N.A.L.,
³⁸⁵ S.T., P.D.S.; Resources: N.A.L., S.T., L.B., K.M., P.D.S.; Software: N.A.L.; Visualization:
³⁸⁶ N.A.L., P.D.S.; Writing - original draft: NAL; Writing - review & editing: N.A.L., S.T., A.H.,
³⁸⁷ A.T., J.C., L.B., K.B., P.D.S.; Funding acquisition: P.D.S.; Project administration: P.D.S.;
³⁸⁸ Supervision: P.D.S.

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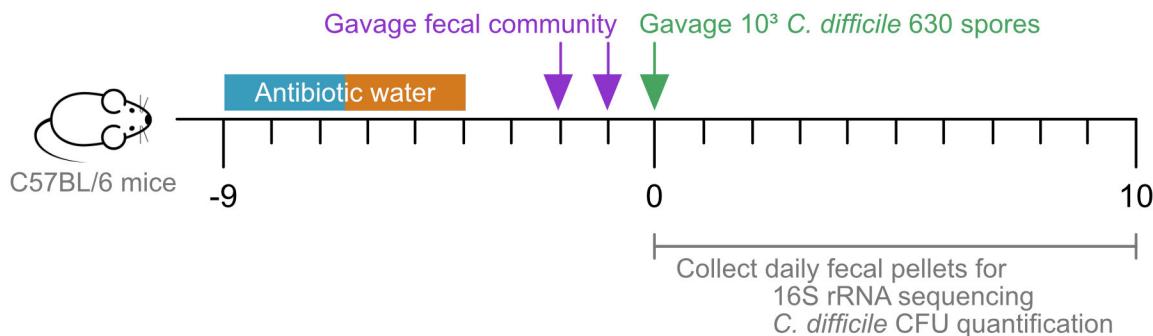
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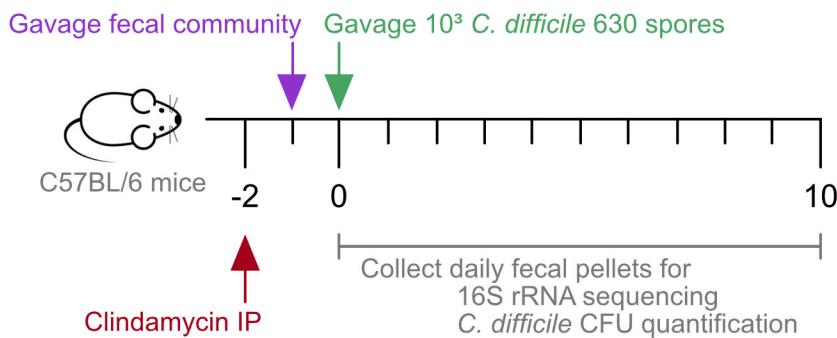
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Cefoperazone/Streptomycin



Clindamycin

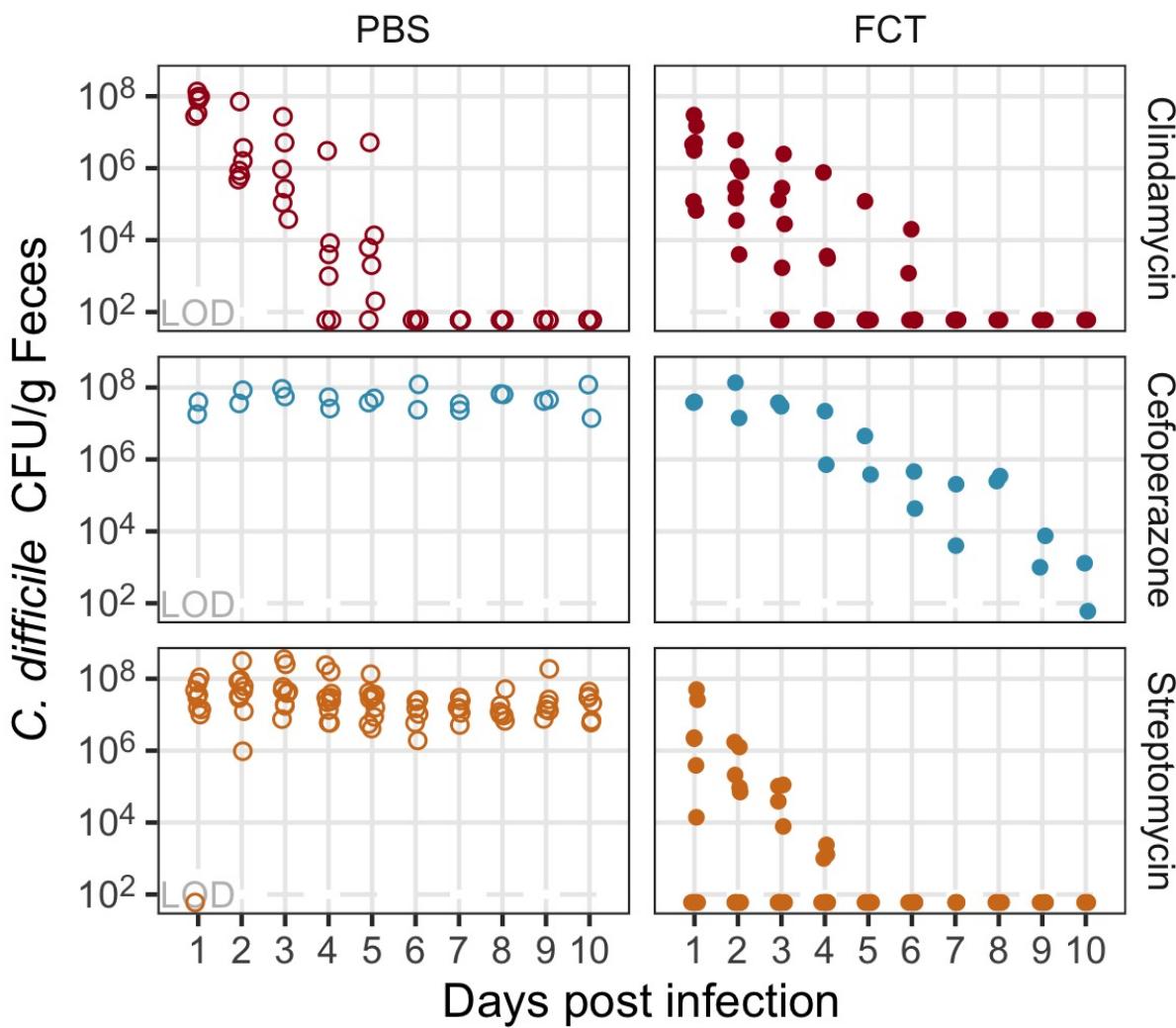


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538 **Figure 1. Mouse experiment timeline.** Mice were given water with cefoperazone (0.5
539 mg/ml) or streptomycin (5 mg/ml) for 5 days. The mice were returned to untreated water
540 for the remainder of the experiment. Two days after the antibiotic water was removed,
541 mice were orally gavaged 100 μ l of PBS or fecal community, once a day for two days.
542 The following day, the mice were challenged with 10^3 *C. difficile* 630 spores. Alternatively,
543 mice were given an intraperitoneal injection of clindamycin (10 mg/kg) 2 days prior to *C.*
544 *difficile* infection. 24 hours later, mice were orally gavaged with 100 μ l of PBS or fecal
545 community. The following day, the mice were challenged with 10^3 *C. difficile* 630 spores.
546 Fecal pellets were collected prior to treatment (day -9 for cefoperazone/streptomycin, day
547 -2 for clindamycin), cessation of antibiotics (day -2 for cefoperazone/streptomycin, day -1
548 for clindamycin), prior to *C. difficile* infection (day 0), and each of the following 10 days.

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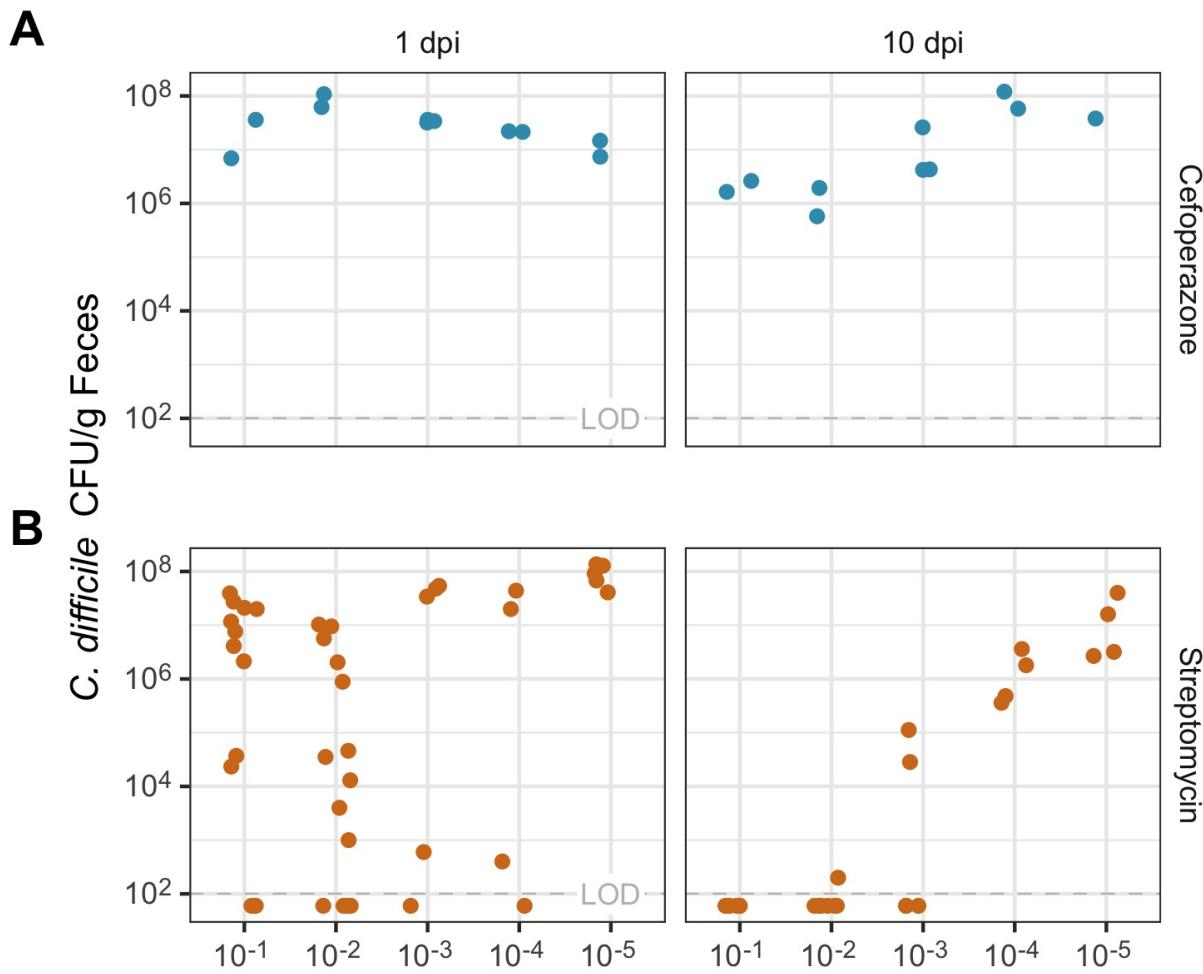


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Figure 2. Fecal community transplant inhibited *C. difficile* colonization for mice treated with cefoperazone or streptomycin. *C. difficile* CFU per gram of feces for mice treated with clindamycin (red points), cefoperazone (blue points), or streptomycin (orange points). Mice were orally gavaged either PBS (open circles) or FCT (fecal community transplant, filled circles) prior to the *C. difficile* infection. Each point represents an individual mouse. LOD = limit of detection.

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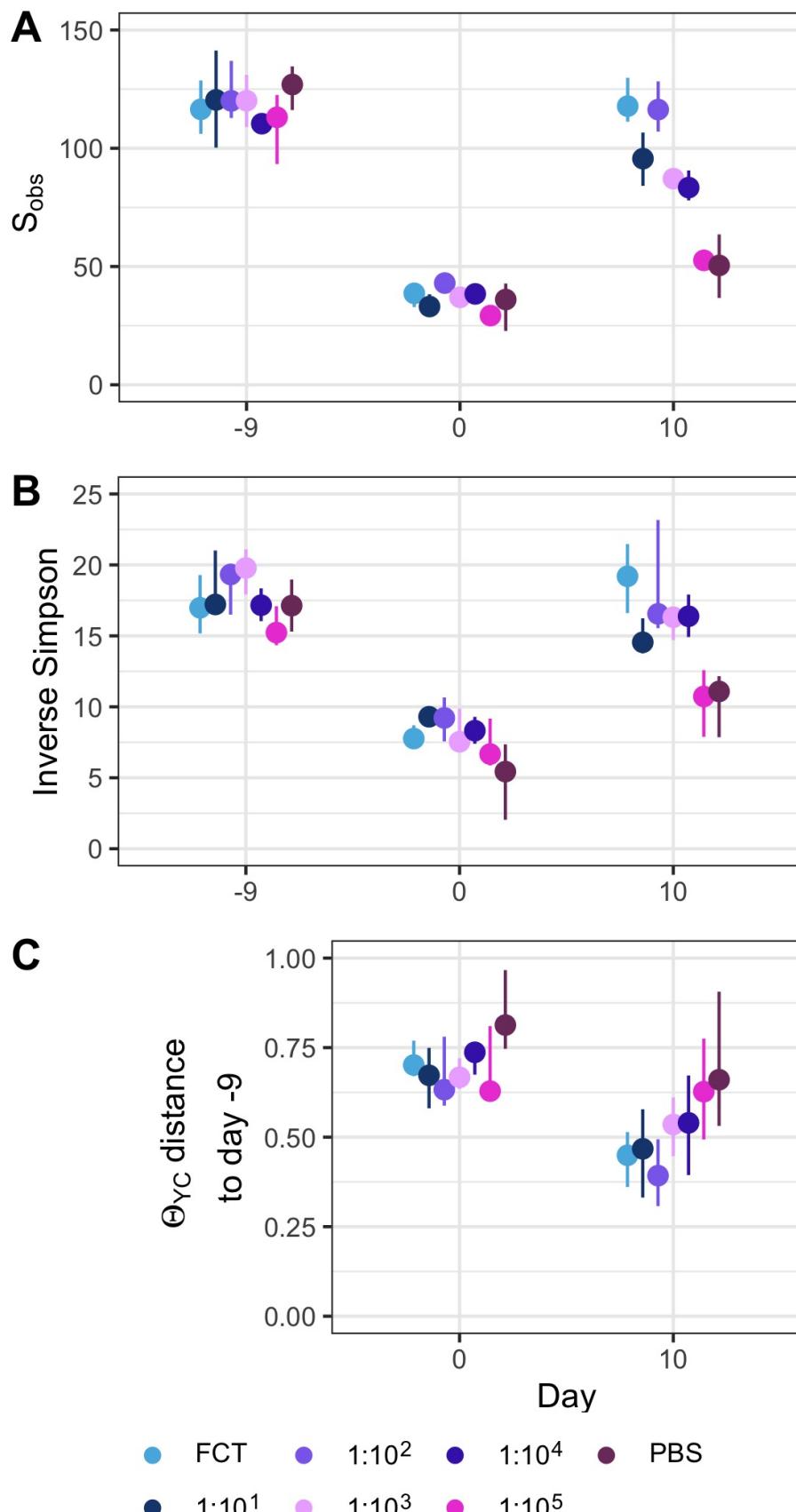


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561 **Figure 3. Diluted FCT inhibited *C. difficile* colonization for mice treated with**
 562 **streptomycin.** *C. difficile* CFU per gram of feces for mice treated with cefoperazone
 563 (blue points) or streptomycin (orange points). Mice were orally gavaged with a dilution of
 564 FCT (1:10 to 1:10⁵) prior to the *C. difficile* infection at (A) one day post *C. difficile* infection
 565 (dpi) and (B) 10 dpi. Each point represents an individual mouse. LOD = limit of detection.

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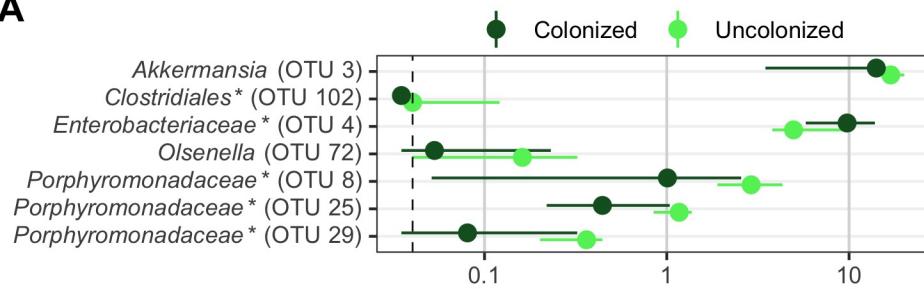
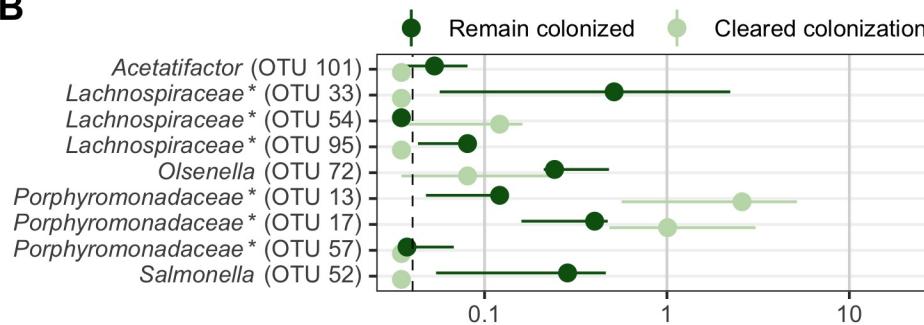
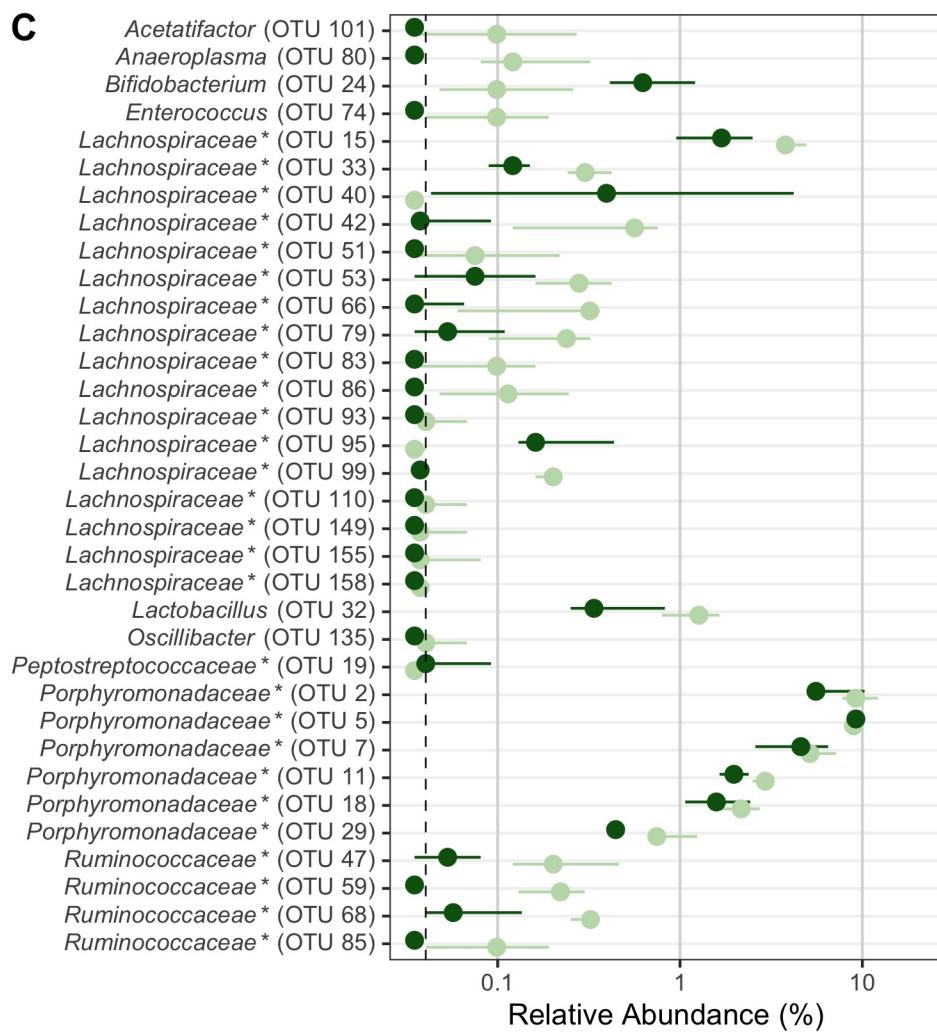
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569 **Figure 4. Diversity of murine gut bacterial community had not recovered at the**
570 **time of *C. difficile* infection in streptomycin-treated mice.** α -diversity, measured by
571 S_{obs} (A) and Inverse Simpson (B), prior to beginning antibiotic treatment (day -9), after
572 fecal community transplant on the day of *C. difficile* challenge (day 0) and at the end of
573 the experiment (day 10). (C) β -diversity, measured by θ_{YC} , distance between community
574 structures on day 0 or 10 compared to the community prior to antibiotic treatment (day
575 -9) community of that individual. Data are grouped by the transplant received, undiluted
576 fecal community (FCT), diluted fecal community (1:10¹-1:10⁵), or PBS. Points are median
577 values and lines represent the interquartile range.

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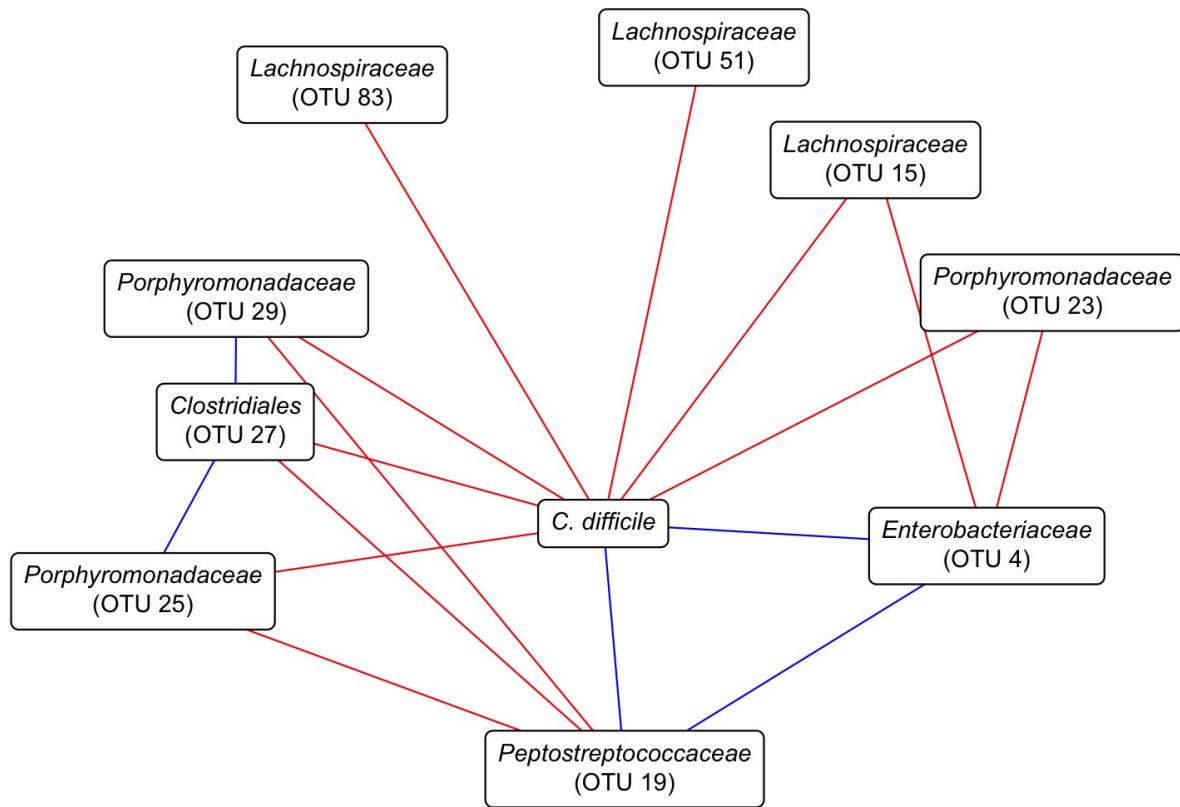
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A**B****C**

581 **Figure 5. Bacterial community OTUs differentially abundant in streptomycin-treated**
582 **mice which resisted or cleared colonization.** Murine gut bacterial community OTUs
583 that were significantly different by LEfSe analysis. OTUs from streptomycin-treated mice
584 at the time of *C. difficile* challenge (day 0) which were differentially abundant between
585 (A) mice that were colonized (dark green) and those that were not (no detectable CFU
586 throughout the experiment, bright green) or (B) mice that remained colonized (dark green)
587 and those that cleared colonization (CFU reduced to below the limit of detection by the
588 end of the experiment, faint green). (C) OTUs from streptomycin-treated mice at the end of
589 the experiment (day 10) which were differentially abundant between mice that remained
590 colonized (dark green) and those that cleared colonization (CFU reduced to below the
591 limit of detection by the end of the experiment, faint green). Points are median values and
592 lines represent the interquartile range. Dashed vertical line is the limit of detection. OTUs
593 ordered alphabetically. * indicates that the OTU was unclassified at lower classification
594 rank.

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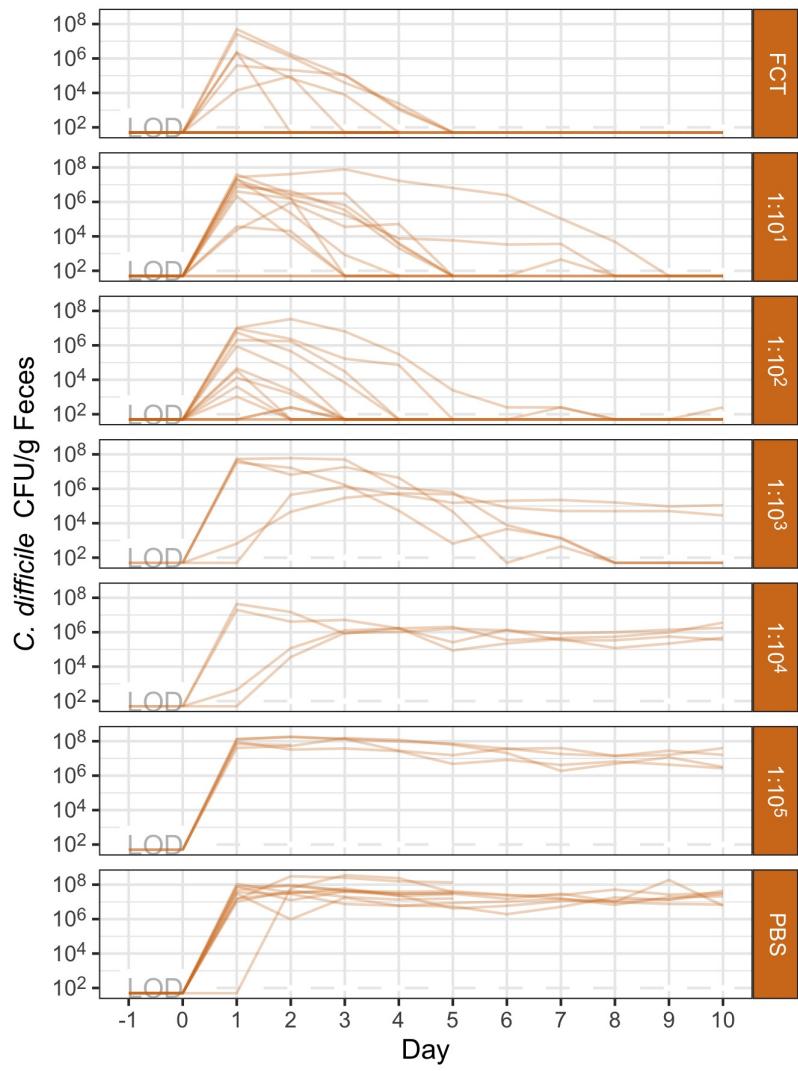
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598 **Figure 6. Streptomycin-treated murine fecal community associations with *C. difficile*.**

599 Network constructed with SpiecEasi from the OTU relative abundances and *C. difficile*
600 CFU data from 1 through 5 days post *C. difficile* infection. Red lines represent negative
601 associations and blue lines indicate positive associations. *C. difficile* is based on CFU
602 counts and *Peptostreptococcaceae* (OTU 19), the OTU most closely related to *C. difficile*,
603 is based on sequence counts. Only *C. difficile* subnetwork shown.

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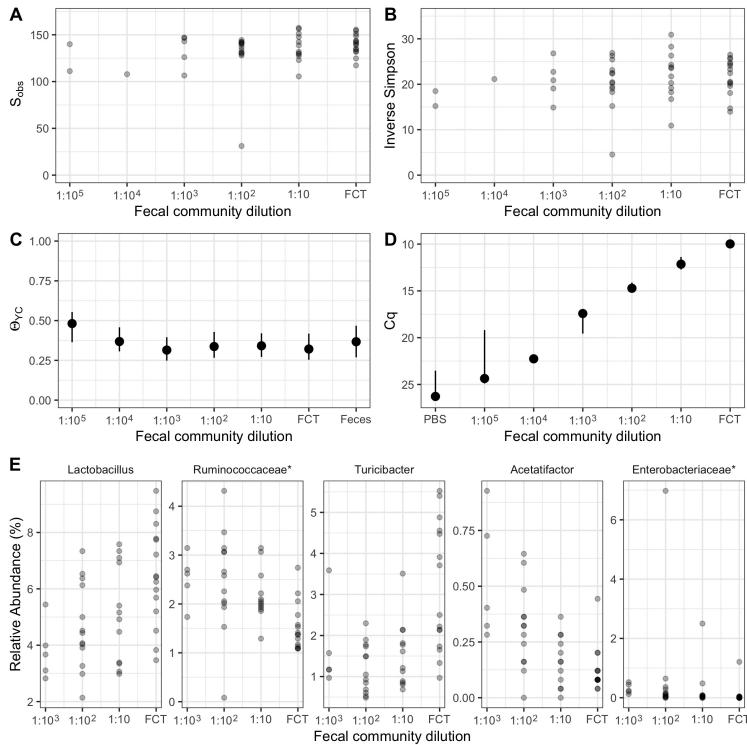


606

607 **Figure S1. *C. difficile* colonization dynamics in streptomycin-treated mice**
 608 **across all prophylactic transplant treatments.** *C. difficile* CFU per gram of feces for
 609 streptomycin-treated mice orally gavaged PBS, fecal community transplant (FCT), or
 610 diluted FCT (1:10-1:10⁵) prior to the *C. difficile* infection. Each semi-transparent line
 611 represents an individual mouse. Mice challenged with 10^3 *C. difficile* 630 spores on day 0.
 612 Lines grouped by the transplant treatment received. LOD = limit of detection.

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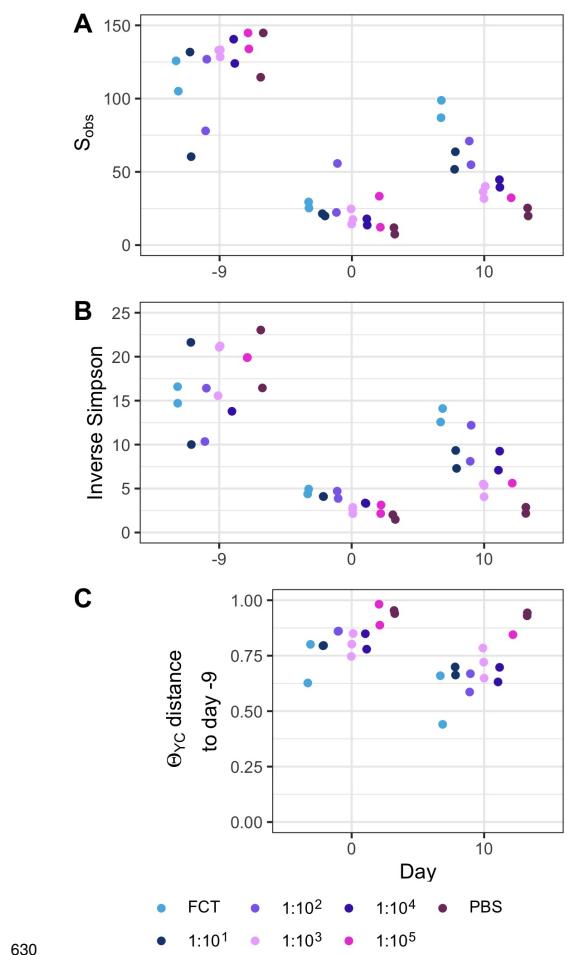


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616 **Figure S2. Diversity and quantification of fecal community dilutions used for**
 617 **prophylactic transplants in antibiotic-treated mice.** (A-C) Diversity of fecal community
 618 dilutions. α -diversity, measured by (A) S_{obs} and (B) Inverse Simpson for undiluted fecal
 619 community (FCT) and diluted fecal communities (1:10-1:10⁵). Points are individual
 620 samples. (C) β -diversity, measured by θ_{YC} , community structure of feces collected
 621 from untreated mice, undiluted fecal community (FCT), and diluted fecal communities
 622 (1:10-1:10⁵) compared to untreated feces. Points are median values and lines represent
 623 the interquartile range. (D) Cq values for qPCR of FCT and its dilutions for eubacterial
 624 16S rRNA gene. Points are median values and lines represent the interquartile range. (E)
 625 Relative abundance of bacterial taxonomic groups that significantly correlate with fecal
 626 community dilutions (FCT-1:10³) by Spearman correlation. Points are individual mice. *
 627 indicates that the bacterial taxonomic group was unclassified at lower classification rank.

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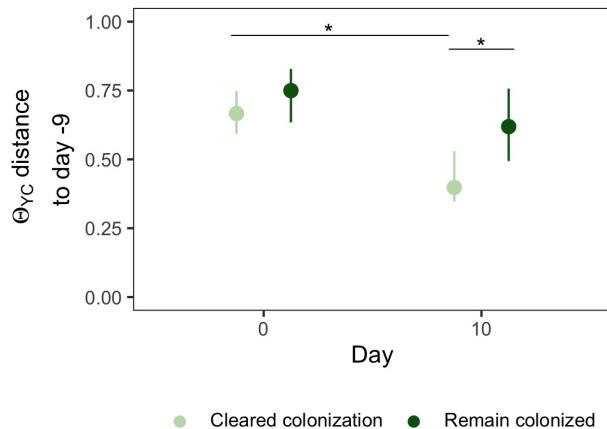
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631 **Figure S3. Diversity of murine gut bacterial community was not recovered at the**
 632 **time of *C. difficile* infection in cefoperazone-treated mice.** Diversity changes through
 633 experiments with cefoperazone-treated mice. α -diversity, measured by (A) S_{obs} and (B)
 634 Inverse Simpson, prior to beginning antibiotic treatment (day -9), after fecal community
 635 transplant on the day of *C. difficile* infection (day 0) and at the end of the experiment (day
 636 10). (C) β -diversity, measured by θ_{YC} , distance between community structures on day 0
 637 or 10 compared to the community prior to antibiotic treatment (day -9) community of that
 638 individual. Data are grouped by the transplant received, undiluted fecal community (FCT),
 639 diluted fecal community ($1:10^1$ - $1:10^5$), or PBS. Points are individual mice.

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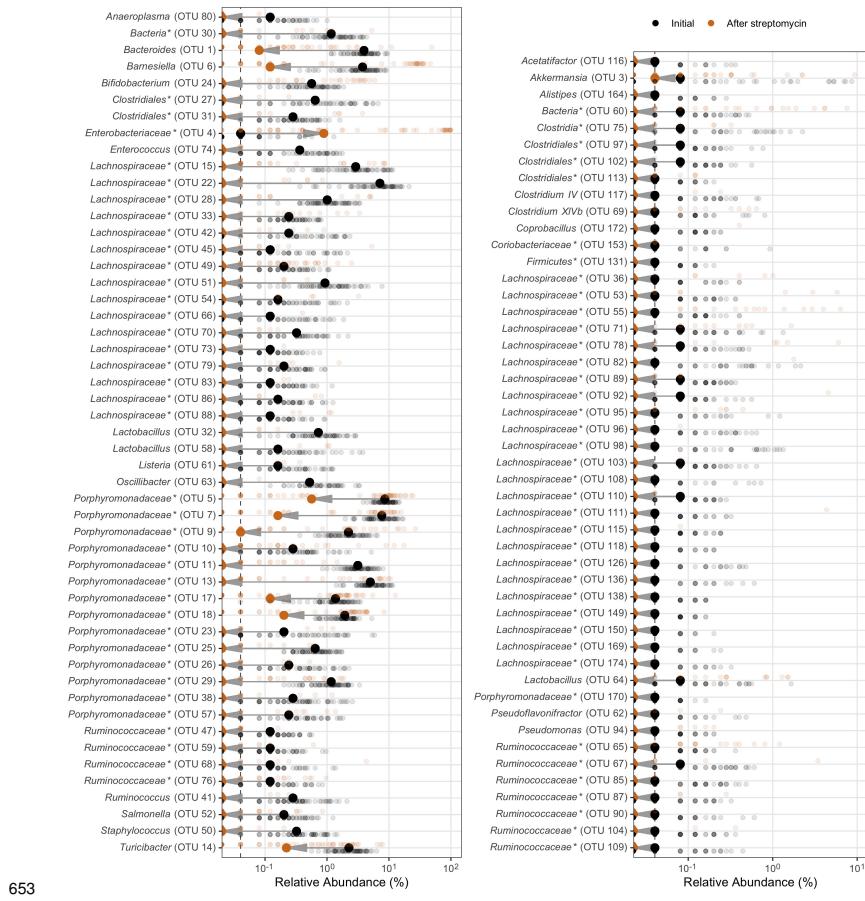


642

643 **Figure S4. Gut bacterial community of streptomycin-treated mice that cleared**
 644 **colonization were more similar to their initial community.** Diversity differences by
 645 outcome in streptomycin-treated mice. β -diversity, measured by θ_{YC} , distance between
 646 community structures on day 0 or 10 compared to the community prior to antibiotic
 647 treatment (day -9) community of that individual. Data are grouped by the outcome, cleared
 648 colonization (faint green) or remain colonized (dark green). Points are median values and
 649 lines represent the interquartile range. * indicates significant difference by Wilcoxon rank
 650 sum test with Bonferroni correction.

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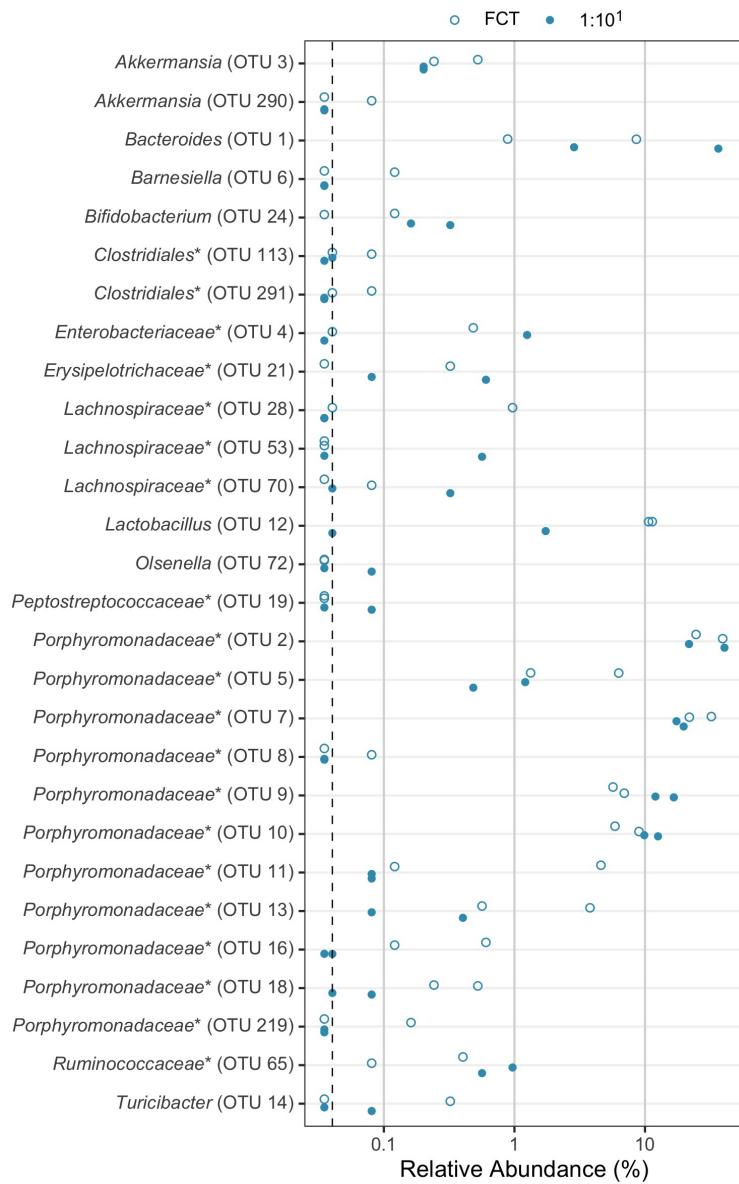


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654 **Figure S5. Murine gut bacterial community OTUs differentially abundant with**
 655 **streptomycin treatment.** Murine gut bacterial community OTUs that were significantly
 656 different by LEfSe analysis between untreated mice (Initial, black) and after 5 days of water
 657 with streptomycin (5 mg/ml) and 2 days of untreated water (After streptomycin, orange).
 658 Large bold points represent the group median. Small, semi-transparent points represent an
 659 individual mouse. Gray arrow indicates the direction the relative abundance shifted with the
 660 streptomycin treatment. Left plot displays OTUs with a median relative abundance greater
 661 than 0.1%, the OTUs lower are displayed in the right plot. Dashed vertical line is the limit
 662 of detection. OTUs ordered alphabetically. * indicates that the OTU was unclassified at
 663 lower classification rank.

664

665



666 **Figure S6. Murine gut bacterial community OTUs of cefoperazone-treated mice at**
667 **the time of challenge.** Murine gut bacterial community OTUs that were present in at
668 least one sample at the time of *C. difficile* challenge (day 0). Mice were pre-treated with
669 either fecal community transplant (FCT, open circles) or FCT diluted 1:10 (filled circles).
670 Points are individual samples. Dashed vertical line is the limit of detection. OTUs ordered
671 alphabetically. * indicates that the OTU was unclassified at lower classification rank.
672