

# **Clearance of *Clostridioides difficile* colonization is associated with antibiotic-specific bacterial changes**

Running title: Clearance of *Clostridioides difficile* colonization

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## **1 Abstract**

2 The gut bacterial community prevents many pathogens from colonizing the intestine. Previous  
3 studies have associated specific bacteria with clearing *Clostridioides difficile* colonization across  
4 different community perturbations. However, those bacteria alone have been unable to clear *C.*  
5 *difficile* colonization. To elucidate the changes necessary to clear colonization, we compared  
6 differences in bacterial abundance between communities able and unable to clear *C. difficile*  
7 colonization. We treated mice with titrated doses of antibiotics prior to *C. difficile* challenge which  
8 resulted in no colonization, colonization and clearance, or persistent colonization. Previously, we  
9 observed that clindamycin-treated mice were susceptible to colonization but spontaneously cleared  
10 *C. difficile*. Therefore, we investigated whether other antibiotics would show the same result. We  
11 found reduced doses of cefoperazone and streptomycin permitted colonization and clearance  
12 of *C. difficile*. Mice that cleared colonization had antibiotic-specific community changes and  
13 predicted interactions with *C. difficile*. Clindamycin treatment led to a bloom in populations related  
14 to *Enterobacteriaceae*. Clearance of *C. difficile* was concurrent with the reduction of those blooming  
15 populations and the restoration of community members related to the *Porphyromonadaceae*  
16 and *Bacteroides*. Cefoperazone created a susceptible community characterized by a drastic  
17 reduction in the community diversity, interactions, and a sustained increase in abundance of  
18 many facultative anaerobes. Lastly, clearance in streptomycin-treated mice was associated with  
19 the recovery of multiple members of the *Porphyromonadaceae*, with little overlap in the specific  
20 *Porphyromonadaceae* observed in the clindamycin treatment. Further elucidation of how *C. difficile*  
21 colonization is cleared from different gut bacterial communities will improve *C. difficile* infection  
22 treatments.

23 **Importance**

24 The community of microorganisms, known as the microbiota, in our intestines prevents pathogens,  
25 such as *C. difficile*, from establishing themselves and causing infection. This is known as  
26 colonization resistance. However, when a person takes antibiotics, their gut microbiota is disturbed.  
27 This disruption allows *C. difficile* to colonize. *C. difficile* infections (CDI) are primarily treated with  
28 antibiotics, which frequently leads to recurrent infections because the microbiota have not yet  
29 returned to a resistant state. The infection cycle often ends when the fecal microbiota from a  
30 presumed resistant person are transplanted into the susceptible person. Although this treatment  
31 is highly effective, we do not understand the mechanism of resistance. We hope to improve the  
32 treatment of CDI through elucidating how the bacterial community eliminates *C. difficile* colonization.  
33 We found *C. difficile* was able to colonize susceptible mice but was spontaneously eliminated  
34 in an antibiotic-treatment specific manner. These data indicate each community had different  
35 requirements for clearing colonization. Understanding how different communities clear colonization  
36 will reveal targets to improve CDI treatments.

37 **Introduction**

38 A complex consortium of bacteria and microbes that inhabits our gut, known as the microbiota,  
39 prevent pathogens from colonizing and causing disease. This protection, known as colonization  
40 resistance, is mediated through many mechanisms such as activating host immune responses,  
41 competing for nutrients, producing antimicrobials, and contributing to the maintenance of the  
42 mucosal barrier (1). However, perturbations to the intestinal community or these functions opens  
43 the possibility that a pathogen can colonize (2). For example, the use of antibiotics perturb the gut  
44 microbiota and can lead to *Clostridioides difficile* infection (CDI).

45 CDI is especially problematic due to its burden on the healthcare system (3, 4). *C. difficile* can  
46 cause severe disease, such as toxic megacolon, diarrhea, and death (5). CDI is primarily treated  
47 with antibiotics (6). CDIs recalcitrant to antibiotics are eliminated by restoring the community with  
48 a fecal microbiota transplant (FMT), returning the perturbed community to a healthier protective  
49 state (7, 8). However, FMTs are not always effective against CDI and have the risk of transferring a  
50 secondary infection (9, 10). Therefore, we need to better understand how the microbiota clears the  
51 infection to develop more effective treatments.

52 Previous research has shown that the microbiota affects *C. difficile* colonization. Mouse models  
53 have identified potential mechanisms of colonization resistance such as bile salt metabolism and  
54 nutrient competition (11–14). However, studies that have restored those functions were unable to  
55 restore complete resistance (15, 16). This could be attributed to the complexity of the community  
56 and the mechanisms of colonization resistance (17, 18). We previously showed that when *C.*  
57 *difficile* colonizes different antibiotic-treated murine communities it modifies its metabolism to fit  
58 each specific environment (14, 19, 20). Therefore, we have investigated the bacterial community  
59 dynamics concurrent with *C. difficile* elimination across uniquely perturbed communities.

60 Jenior et al. (20) observed that clindamycin-treated mice spontaneously cleared *C. difficile*  
61 colonization whereas mice treated with cefoperazone and streptomycin did not. Here, we continued  
62 to explore the different effects these three antibiotics have on *C. difficile* colonization. The  
63 purpose of this study was to elucidate the gut bacterial community changes concurrent with

64 elimination of *C. difficile* colonization. We hypothesized that each colonized community has  
65 perturbation-specific susceptibilities and requires specific changes to clear the pathogen. To  
66 induce a less severe perturbation, we reduced the doses of cefoperazone and streptomycin. This  
67 resulted in communities that were initially colonized to a high level ( $>10^6$  CFU/g feces) and then  
68 spontaneously cleared *C. difficile*. We found each antibiotic resulted in unique changes in the  
69 microbiota that were associated with the persistence or clearance of *C. difficile*. These data further  
70 support the hypothesis that *C. difficile* can exploit numerous niches in perturbed communities.

71 **Results**

72 **Reduced doses of cefoperazone and streptomycin allowed communities to spontaneously**  
73 **clear *C. difficile* colonization.** To understand the dynamics of colonization and clearance of *C.*  
74 *difficile*, we first identified conditions which would allow colonization and clearance. Beginning with  
75 clindamycin, mice were treated with an intraperitoneal injection of clindamycin (10 mg/kg) one day  
76 prior to challenge with *C. difficile*. All mice (N = 11) were colonized to a high level (median CFU =  
77  $3.07 \times 10^7$ ) the next day and cleared the colonization within 10 days; 6 mice cleared *C. difficile* within  
78 6 days (Figure 1A). Previous *C. difficile* infection models using cefoperazone and streptomycin  
79 have not demonstrated clearance. So we next explored whether cefoperazone and streptomycin  
80 could permit colonization and subsequent clearance with lower doses. We began with replicating  
81 the previously established *C. difficile* infection models using these antibiotics (20). We treated  
82 mice with cefoperazone or streptomycin in their drinking water for 5 days (0.5 mg/mL and 5 mg/mL,  
83 respectively) and then challenged them with *C. difficile*. For both antibiotics, *C. difficile* colonization  
84 was maintained for the duration of the experiment as previously demonstrated (Figure 1B-C) (20).  
85 Then we repeated the *C. difficile* challenge with reduced doses of the antibiotics (cefoperazone -  
86 0.3 and 0.1 mg/mL; streptomycin - 0.5 and 0.1 mg/mL). For both antibiotic treatments, the lowest  
87 dose resulted in either no colonization (N = 8) or a transient, low level colonization (N = 8, median  
88 length = 1 day, median CFU/g =  $2.8 \times 10^3$ ) (Figure 1B-C). The intermediate dose of both antibiotics  
89 resulted in a high level colonization (median CFU/g =  $3.5 \times 10^6$ ) and half (N = 8 of 16) of the mice  
90 clearing the colonization within 10 days. Based on our previous research, which showed each

91 of these antibiotics uniquely changed the microbiota, we hypothesized that the microbiota varied  
92 across these antibiotic treatments that resulted in colonization clearance.

93 **Clearance of *C. difficile* was associated with antibiotic-specific changes to the microbiota.**

94 Beginning with the clindamycin-treated mice, we analyzed their fecal 16S rRNA gene sequences to  
95 identify the community features related to *C. difficile* colonization and clearance. First, we compared  
96 the most abundant bacterial genera of the communities at the time of *C. difficile* challenge. The  
97 clindamycin-treated mice became dominated by relatives of *Enterobacteriaceae* with a concurrent  
98 reduction in the other abundant genera, except for populations of *Lactobacillus* (Figure 1D, S1).  
99 These community changes permitted *C. difficile* to colonize all of these mice, but all of the mice  
100 were also able to clear the colonization. We next investigated how the microbiota diversity  
101 related to *C. difficile* clearance. Clindamycin treatment decreased the alpha diversity ( $P < 0.05$ ) and  
102 similarity to the pre-clindamycin community at the time of *C. difficile* challenge ( $P < 0.05$ ) (Figure  
103 2A). But it was not necessary to restore the community similarity to its initial state to clear *C. difficile*.  
104 Therefore we investigated the temporal differences in the abundance of the operational taxonomic  
105 units (OTUs) between the initial untreated community and post-clindamycin treatment at the time  
106 of challenge and between the time of challenge and the end of the experiment. Clindamycin  
107 treatment resulted in large decreases in 21 OTUs and a bloom of relatives of *Enterobacteriaceae*  
108 (Figure 4A). With the elimination of *C. difficile*, we observed a drastic reduction of the relatives of  
109 *Enterobacteriaceae* and recovery of 10 populations related to *Porphyromonadaceae*, *Bacteroides*,  
110 *Akkermansia*, *Lactobacillus*, *Bifidobacterium*, *Lachnospiraceae*, and *Clostridiales* (Figure 4A). Thus,  
111 clindamycin reduced most of the natural community allowing *C. difficile* to colonize. The recovery of  
112 only a small portion of the community was associated with eliminating the *C. difficile* population.

113 We applied the same analysis to the cefoperazone-treated mice to understand what community  
114 features were relevant to clearing *C. difficile*. Increasing the dose of cefoperazone shifted the  
115 dominant community members from relatives of the *Porphyromonadaceae*, *Bacteroides* and  
116 *Akkermansia* to relatives of the *Lactobacillus* and *Enterobacteriaceae* at the time of challenge  
117 (Figure 1E, S1). We saw a similar increase in relatives of *Enterobacteriaceae* with clindamycin.  
118 However, the cefoperazone-treated mice that had larger increases in *Enterobacteriaceae* were  
119 unable to clear *C. difficile*. We next investigated the differences between the cefoperazone-treated

120 mice that cleared *C. difficile* to those that did not. For the communities that cleared *C. difficile*,  
121 diversity was maintained throughout the experiment (Figure 2B). The mice treated with cefoperazone  
122 that remained colonized experienced an increase in alpha diversity, likely driven by the decrease  
123 in highly abundant populations and increase in low abundant populations (Figure 1E). These  
124 persistently colonized communities also had a large shift away from the initial community structure  
125 caused by the antibiotic treatment ( $P < 0.05$ ), which remained through the end of the experiment ( $P$   
126  $< 0.05$ ) (Figure 2B). These data suggested that it was necessary for cefoperazone-treated mice to  
127 become more similar to the initial pre-antibiotic community structure to clear *C. difficile*. We next  
128 investigated the changes in OTU abundances between the communities that cleared *C. difficile*  
129 and those that did not to elucidate the community members involved in clearance. Communities  
130 that remained colonized were significantly enriched in facultative anaerobic populations including  
131 *Enterococcus*, *Pseudomonas*, *Staphylococcus*, and *Enterobacteriaceae* at the time of challenge.  
132 Communities that cleared *C. difficile* had significant enrichment in 10 different OTUs related to the  
133 *Porphyromonadaceae* at the end of the experiment (Figure 3A). We were also interested in the  
134 temporal changes within each community so we investigated which OTUs changed due to antibiotic  
135 treatment or during the *C. difficile* colonization. The majority of significant temporal differences in  
136 OTUs for cefoperazone-treated mice occurred in persistently colonized communities. Persistently  
137 colonized communities had a persistent loss of numerous relatives of the *Porphyromonadaceae* and  
138 increases in the relative abundance of facultative anaerobes (Figure 4C, S2). Overall, persistent *C.*  
139 *difficile* colonization in cefoperazone-treated mice was associated with a shift in the microbiota to a  
140 new community structure which seemed unable to recover from the antibiotic perturbation, whereas  
141 clearance occurred when the community was capable of returning to its original structure.

142 Finally, we identified the differences in *C. difficile* colonization for streptomycin-treated  
143 mice. Increasing the dose of streptomycin maintained the abundance of relatives of the  
144 *Porphyromonadaceae* and *Bacteroides*, but reduced most of the other genera including populations  
145 of the *Lactobacillus*, *Lachnospiraceae*, *Ruminococcaceae*, *Alistipes*, and *Clostridiales* (Figure 1F).  
146 Both communities that cleared and those that remained colonized had similar changes in diversity.  
147 Streptomycin-treated mice became mildly dissimilar ( $P < 0.05$ ) and less diverse ( $P < 0.05$ ) with  
148 streptomycin treatment but by the end of the experiment returned to resemble the pre-antibiotic

community ( $P < 0.05$ ) (Figure 2C). Those communities that remained colonized had slightly lower alpha-diversity than those that cleared *C. difficile*. ( $P < 0.05$ ). Persistently colonized mice had reduced relative abundance of relatives of *Alistipes*, *Anaeroplasma*, and *Porphyromonadaceae* at time of challenge compared to the mice that cleared *C. difficile* (Figure 3B). At the end of the experiment the mice that were still colonized had lower abundances of *Turicibacter*, *Alistipes*, and *Lactobacillus*. Since most of the differences were reduced relative abundances in the colonized mice, we were interested to explore what temporal changes occurred between pre-antibiotic treatment, the time of challenge, and the end of the experiment for the communities that cleared *C. difficile*. The temporal changes in streptomycin-treated mice were more subtle than those observed with the other antibiotic treatments. At the time of challenge, the communities that remained colonization had reductions in 4 OTUs related to the *Porphyromonadaceae*. Those that cleared *C. difficile* also had changes in OTUs related to the *Porphyromonadaceae*, however, 2 populations decreased and 2 increased in abundance (Figure 4B, D). At the end of the experiment, all communities experienced recovery of the abundance of many of the populations changed by the streptomycin treatment, but the communities that remained colonized did not recover 5 of the OTUs of *Alistipes*, *Lactobacillus*, and *Porphyromonadaceae* that were reduced by streptomycin. The differences between the streptomycin-treated mice that remained colonized and those had been cleared of *C. difficile* were not as distinct as those observed with the cefoperazone treatment. The differences between colonized and cleared streptomycin-treated mice were minimal, which suggested the few differences may be responsible for the clearance. Overall, these data revealed that while there were commonly affected families across the antibiotic treatments, such as the *Porphyromonadaceae*, *C. difficile* clearance was associated with community and OTU differences specific to each antibiotic.

**Distinct features of the bacterial community at the time of infection predicted end point colonization.** To determine whether the community composition at the time of *C. difficile* challenge could predict *C. difficile* clearance, we built a machine learning model using L2 logistic regression. We evaluated the predictive performance of the model using the area under the receiver operating characteristic curve (AUROC), where a value of 0.5 indicated the model is random and 1.0 indicated the model always correctly predicts the outcome. Our model resulted in a AUROC of 0.986 [IQR

178 0.970-1.000], which suggested that the model was able to use the relative abundance of OTUs  
179 at the time of challenge to accurately predict colonization clearance (Figure S3). To assess the  
180 important features, we randomly permuted each OTU feature by removing it from the training set  
181 to determine its effect on the prediction (Figure 5A). The most important feature was an OTU  
182 related to the *Enterobacteriaceae*, whose abundance predicted clearance. This result appears to  
183 have been strongly driven by the clindamycin data (Figure 5B, C). The remaining OTU features  
184 did not have a large effect on the model performance, which suggested that the model decision  
185 was spread across many features. These results revealed the model used the relative abundance  
186 data of the community members and the relationship between those abundances to correctly  
187 classify clearance. There were many OTUs with treatment and outcome specific abundance  
188 patterns that did not agree with the odds ratio of the OTU used by the model. For example,  
189 *Enterobacteriaceae* abundance influenced the model to predict clearance (Figure 5B), however  
190 in experiments that used cefoperazone, the communities that remained colonized had higher  
191 abundances of *Enterobacteriaceae* than the communities that cleared colonization (Figure 5C). The  
192 model arrived at the correct prediction through the influence of other OTUs. Therefore, the model  
193 used different combinations of multiple OTUs and their relative abundances across treatments to  
194 predict *C. difficile* clearance. These data can offer a basis for hypotheses regarding the distinct  
195 combinations of bacteria that promote *C. difficile* clearance.

196 **Conditional independence networks revealed treatment-specific relationships between the**  
197 **community members and *C. difficile* during colonization clearance.** We next investigated  
198 the relationship between temporal changes in the community and *C. difficile* by building a  
199 conditional independence network for each treatment using SPIEC-EASI (sparse inverse  
200 covariance estimation for ecological association inference) (21). First, we focused on the first-order  
201 associations of *C. difficile* (Figure 6A). In clindamycin-treated mice, *C. difficile* had positive  
202 associations with relatives of *Enterobacteriaceae*, *Pseudomonas*, and *Olsenella* and negative  
203 associations with relatives of the *Lachnospiraceae* and *Clostridium XIVa*. *C. difficile* had limited  
204 associations in cefoperazone-treated mice; the primary association was positive with relatives  
205 of *Enterobacteriaceae*. In streptomycin-treated mice, *C. difficile* had negative associations  
206 with relatives of the *Porphyromonadaceae* and positive associations with populations of the

207 *Ruminococcaceae*, *Bacteroidetes*, *Clostridium IV* and *Olsenella*. Next, we quantified the degree  
208 centrality, the number of associations between each OTU for the whole network of each antibiotic  
209 and outcome, and betweenness centrality, the number of associations connecting two OTUs that  
210 pass through an OTU (Figure 6B). This analysis revealed cefoperazone treatment resulted in  
211 networks primarily composed of singular associations with much lower degree centrality ( $P < 0.05$ )  
212 and betweenness centrality ( $P < 0.05$ ) than the other antibiotic treatments. Communities that were  
213 treated with cefoperazone that resulted in cleared or persistent colonization had 10 to 100-fold  
214 lower betweenness centrality values than communities treated with clindamycin or streptomycin.  
215 Collectively, these networks suggest *C. difficile* colonization was affected by unique sets of OTUs in  
216 mice treated with clindamycin and streptomycin, but cefoperazone treatment eliminated bacteria  
217 critical to maintaining community interactions and had few populations that associated with *C.*  
218 *difficile*.

## 219 Discussion

220 We have shown that different antibiotic treatments resulted in specific changes to the microbiota  
221 that were associated with *C. difficile* clearance. Clindamycin-treated mice became susceptible  
222 with a dominant bloom in populations related to *Enterobacteriaceae*. Clearance was associated  
223 with the resolution of the bloom and recovery of bacteria that were reduced by the antibiotic  
224 treatment. Cefoperazone-treated mice became susceptible with the expansion of numerous  
225 facultative anaerobes. Communities with a sustained presence of these facultative anaerobes  
226 were unable to recover from the initial antibiotic perturbation or clear the colonization, whereas  
227 the communities that returned to their initial community were able to clear *C. difficile* colonization.  
228 Streptomycin-treated mice became susceptible with fewer and smaller changes than the other  
229 treatments. The communities that cleared colonization had slightly higher  $\alpha$ -diversity than those  
230 that remained colonized. Additionally, all communities in mice treated with streptomycin had  
231 similar numbers of OTUs changing through the experiment but the specific OTUs were different for  
232 each outcome. These observations support our hypothesis that each colonized community has  
233 antibiotic-specific changes that create unique conditions for *C. difficile* colonization and requires

234 specific changes within each community to clear *C. difficile*.  
235 Previous studies have identified microbiota associated with *C. difficile* colonization resistance in  
236 either a set of closely related murine communities or collectively across many different susceptible  
237 communities (11, 15, 22). These bacteria were then tested in *C. difficile* infection models. These  
238 experiments were able to show decreased colonization but were unable to fully clear *C. difficile*  
239 (11, 23). Rather than looking for similarities across all susceptible communities, we explored  
240 the changes that were associated with *C. difficile* clearance for each antibiotic. Even though  
241 these mice all came from the same breeding colony with similar initial microbiomes, *C. difficile*  
242 clearance was associated with antibiotic-specific changes in community diversity, OTU abundances,  
243 and associations between OTUs. Our data suggest that the set of bacteria necessary to restore  
244 colonization resistance following one antibiotic perturbation may not be effective for all antibiotic  
245 perturbations. We have developed this modeling framework starting from a single mouse community.  
246 It should also be relevant when considering interpersonal variation among humans (24).

247 Recent studies have begun to uncover how communities affect *C. difficile* colonization (17–20, 24).  
248 We attempted to understand the general trends in each antibiotic treatment that lead to clearance  
249 of *C. difficile*. We categorized the general changes and microbial relationships of these experiments  
250 into three models. First, a model of temporary opportunity characterized by the transient dominance  
251 of a facultative anaerobe which permits *C. difficile* colonization but *C. difficile* is not able to persist,  
252 as with clindamycin treatment. We hypothesize this susceptibility is due to a transient repression  
253 of community members and interventions which further perturb the community may worsen the  
254 infection. Time alone may be sufficient for the community to clear colonization (15, 22, 25) but  
255 treating the community with an antibiotic or the bowel preparation for an FMT (26, 27) may prolong  
256 susceptibility by eliminating protective functions or opening new niches. Second, a model of an  
257 extensive opportunity characterized by a significant perturbation leading to a persistent increase  
258 in facultative anaerobes and exposing multiple niches, as with cefoperazone treatment. These  
259 communities appear to have been severely depleted of multiple critical community members and  
260 are likely lacking numerous protective functions (20). We hypothesize multiple niches are available  
261 for *C. difficile* to colonize. In this scenario, a full FMT may be insufficient to provide adequate  
262 diversity and abundance to outcompete and occupy all the exposed niches. Multiple FMTs (28,

263 29) or transplant of an enriched fecal community (30) may be necessary to recover the microbiota  
264 enough to outcompete *C. difficile* for the nutrient niches and replace the missing protective functions.  
265 Third, a model of a specific opportunity characterized by a perturbation that only affects a select  
266 portion of the microbiota, leading to small changes in relative abundance and a slight decrease  
267 in diversity, opening a limited niche for *C. difficile* to colonize, as with streptomycin treatment.  
268 We hypothesize that a few specific bacteria would be necessary to recolonize the exposed niche  
269 space and eliminate *C. difficile* colonization (13, 17). A fecal microbiota transplant may contain the  
270 bacterial diversity needed to fill the open niche space and help supplant *C. difficile* from the exposed  
271 niche of the colonized community. Analyzing each of these colonization models individually allowed  
272 us to understand how each may clear *C. difficile* colonization.

273 Future investigations can further identify the exposed niches of susceptible communities and  
274 the requirements to clear *C. difficile* colonization. One common theme for susceptibility across  
275 treatments was the increased abundance of facultative anaerobes. These blooms of facultative  
276 anaerobes could be attributed to the loss of the indigenous obligate anaerobes with antibiotic  
277 treatment (31, 32). However, it is unclear what prevents the succession from the facultative  
278 anaerobes back to the obligate anaerobes in cefoperazone-treated mice. Future studies should  
279 investigate the relationship between facultative anaerobe blooms and susceptibility to colonization  
280 as well as interventions to recover the obligate anaerobes. Another aspect to consider in future  
281 experiments is *C. difficile* strain specificity. Other strains may fill different niche space and fill  
282 other community interactions (33–35). For example, more virulent strains, like *C. difficile* VPI  
283 10463, may have a greater effect on the gut environment since it produces more toxin (15, 36).  
284 Those differences could have different impacts on the susceptible community and change the  
285 requirements to clear *C. difficile*. Finally, we have shown that the functions found in communities  
286 at peak colonization are antibiotic-specific (20). Here, we have shown the community changes  
287 associated with *C. difficile* clearance are antibiotic-specific. It is unknown how the community  
288 functions contributing to *C. difficile* clearance compare across antibiotics. Examining the changes  
289 in transcription and metabolites during clearance will help define the activities necessary to clear *C.*  
290 *difficile* and if they are specific to the perturbation. This information will build upon the community  
291 differences presented in this study and move us closer to elucidating how the microbiota clears *C.*

292 *C. difficile* colonization and developing targeted therapeutics.

293 We have shown that mice became susceptible to *C. difficile* colonization after three different  
294 antibiotic treatments and then differed in their ability to clear the colonization. These experiments  
295 have shown that each antibiotic treatment resulted in different community changes leading to  
296 *C. difficile* clearance. These differences suggest that a single mechanism of infection and one  
297 treatment for all *C. difficile* infections may not be appropriate. While our current use of FMT to  
298 eliminate CDI is highly effective, it does not work in all patients and has even resulted in adverse  
299 consequences (7–10). The findings in this study may help explain why FMTs may be ineffective.  
300 Although an FMT transplants a whole community, it may not be sufficient to replace the missing  
301 community members or functions to clear *C. difficile*. Alternatively, the FMT procedure itself may  
302 disrupt the natural recovery of the community. The knowledge of how a community clears *C. difficile*  
303 colonization will advance our ability to develop targeted therapies to manage CDI.

304 **Materials and Methods**

305 **Animal care.** All mice were obtained from a single breeding colony and maintained in  
306 specific-pathogen-free (SPF) conditions at the University of Michigan animal facility. All mouse  
307 protocols and experiments were approved by the University Committee on Use and Care of  
308 Animals at the University of Michigan and completed in agreement with approved guidelines.

309 **Antibiotic administration.** Mice were given one of three antibiotics, cefoperazone, clindamycin,  
310 or streptomycin. Cefoperazone (0.5, 0.3, or 0.1 mg/ml) and streptomycin (5, 0.5, or 0.1 mg/ml)  
311 were delivered via drinking water for 5 days. Clindamycin (10 mg/kg) was administered through  
312 intraperitoneal injection.

313 ***C. difficile* challenge.** Mice were returned to untreated drinking water for 24 hours before  
314 challenging with *C. difficile* strain 630Δerm spores. *C. difficile* spores were aliquoted from a  
315 single spore stock stored at 4°C. Spore concentration was determined one week prior to the day of  
316 challenge (37). 10<sup>3</sup> *C. difficile* spores were orally gavaged into each mouse. Once the gavages  
317 were completed, the remaining spore solution was serially diluted and plated to confirm the spore

318 concentration that was delivered.

319 **Sample collection.** Fecal samples were collected on the day antibiotic treatment was started, on  
320 the day of *C. difficile* challenge and the following 10 days. For the day of challenge and beyond,  
321 a fecal sample was also collected and weighed. Under anaerobic conditions a fecal sample was  
322 serially diluted in anaerobic phosphate-buffered saline and plated on TCCFA plates. After 24 hours  
323 of anaerobic incubation at 37°C, the number of colony forming units (CFU) were determined (38).

324 **DNA sequencing.** Total bacterial DNA was extracted from each fecal sample using MOBIO  
325 PowerSoil-htp 96-well soil DNA isolation kit. We created amplicons of the 16S rRNA gene V4 region  
326 and sequenced them using an Illumina MiSeq as described previously (39).

327 **Sequence curation.** Sequences were processed using mothur(v.1.43.0) as previously described  
328 (39). Briefly, we used a 3% dissimilarity cutoff to group sequences into operational taxonomic units  
329 (OTUs). We used a naive Bayesian classifier with the Ribosomal Database Project training set  
330 (version 16) to assign taxonomic classifications to each OTU (41). With the fecal samples, we  
331 also sequenced a mock community with a known community composition and their true 16s rRNA  
332 gene sequences. We processed this mock community along with our samples to determine our  
333 sequence curation resulted in an error rate of 0.019%.

334 **Statistical analysis and modeling.** Diversity comparisons were calculated in mothur. To compare  
335 α-diversity metrics, we calculated the number of OTUs ( $S_{obs}$ ) and the Inverse Simpson diversity  
336 index. To compare across communities, we calculated dissimilarity matrices based on metric of  
337 Yue and Clayton (42). All calculations were made by rarifying samples to 1,200 sequences per  
338 sample to limit biases due to uneven sampling. OTUs were subsampled to 1,200 counts per sample  
339 and remaining statistical analysis and data visualization was performed in R (v3.5.1) with the  
340 tidyverse package (v1.3.0). Significance of pairwise comparisons of α-diversity ( $S_{obs}$  and Inverse  
341 Simpson), β-diversity ( $\theta_{YC}$ ), OTU abundance, and network centrality (betweenness and degree)  
342 were calculated by pairwise Wilcoxon rank sum test and then  $P$  values were corrected for multiple  
343 comparisons with a Benjamini and Hochberg adjustment for a type I error rate of 0.05 (43). Logistic  
344 regression models were constructed with OTUs from all day 0 samples using half of the samples  
345 to train and the other half to test the model. The model was developed from the caret R package

346 (v6.0-85) and previously developed machine learning pipeline (44). For each antibiotic treatment,  
347 conditional independence networks were calculated from the day 1 through 10 samples of all  
348 mice initially colonized using SPIEC-EASI (sparse inverse covariance estimation for ecological  
349 association inference) methods from the SpiecEasi R package after optimizing lambda to 0.001  
350 with a network stability between 0.045 and 0.05 (v1.0.7) (21). Network centrality measures degree  
351 and betweenness were calculated on whole networks using functions from the igraph R package  
352 (v1.2.4.1).

353 **Code availability.** Scripts necessary to reproduce our analysis and this paper are available in an  
354 online repository ([https://github.com/SchlossLab/Lesniak\\_Clearance\\_XXXX\\_2020](https://github.com/SchlossLab/Lesniak_Clearance_XXXX_2020)).

355 **Sequence data accession number.** All 16S rRNA gene sequence data and associated metadata  
356 are available through the Sequence Read Archive via accession PRJNA674858.

## 357 Acknowledgements

358 Thank you to Begüm Topçuoglu and Sarah Tomkovich for critical discussion in the development and  
359 execution of this project. This work was supported by several grants from the National Institutes  
360 for Health R01GM099514, U19AI090871, U01AI12455, and P30DK034933. Additionally, NAL  
361 was supported by the Molecular Mechanisms of Microbial Pathogenesis training grant (NIH T32  
362 AI007528). The funding agencies had no role in study design, data collection and analysis, decision  
363 to publish, or preparation of the manuscript.

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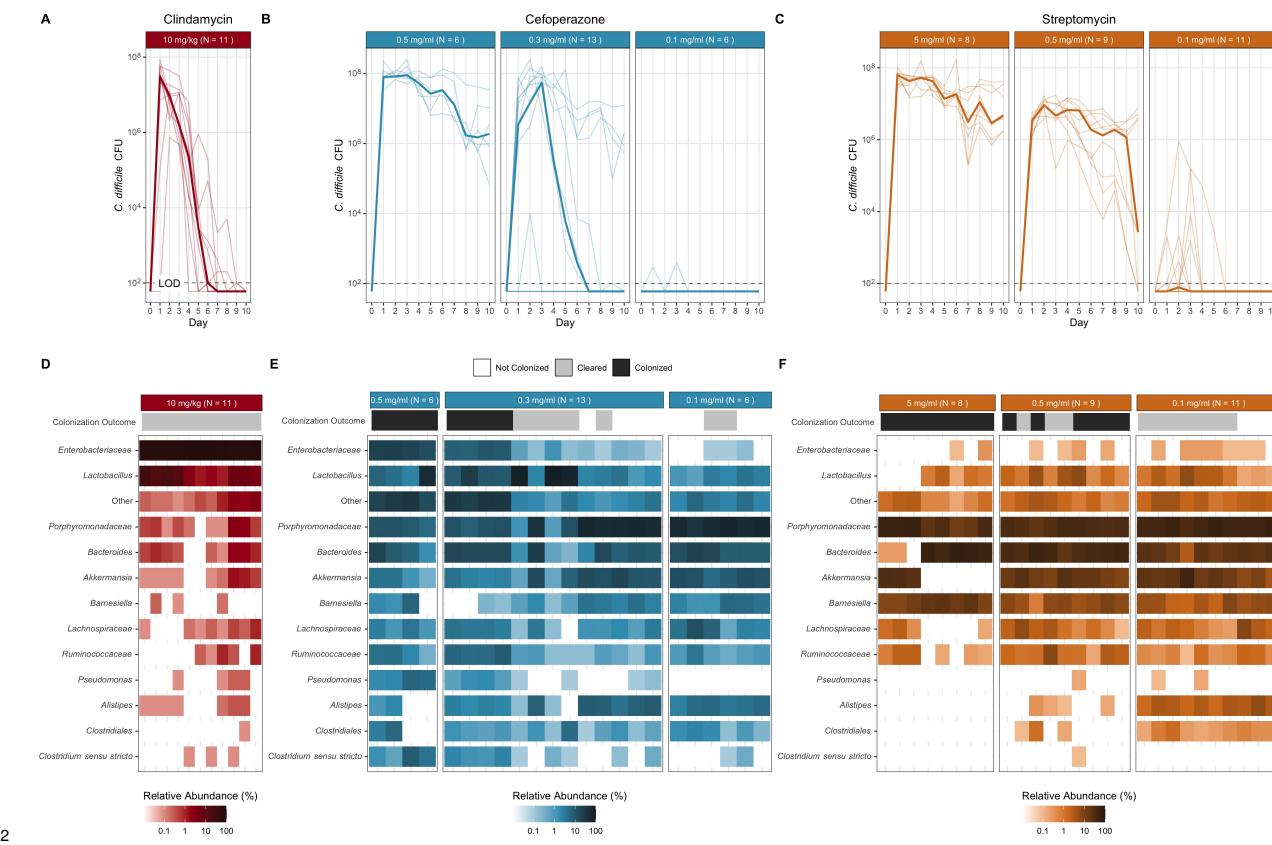
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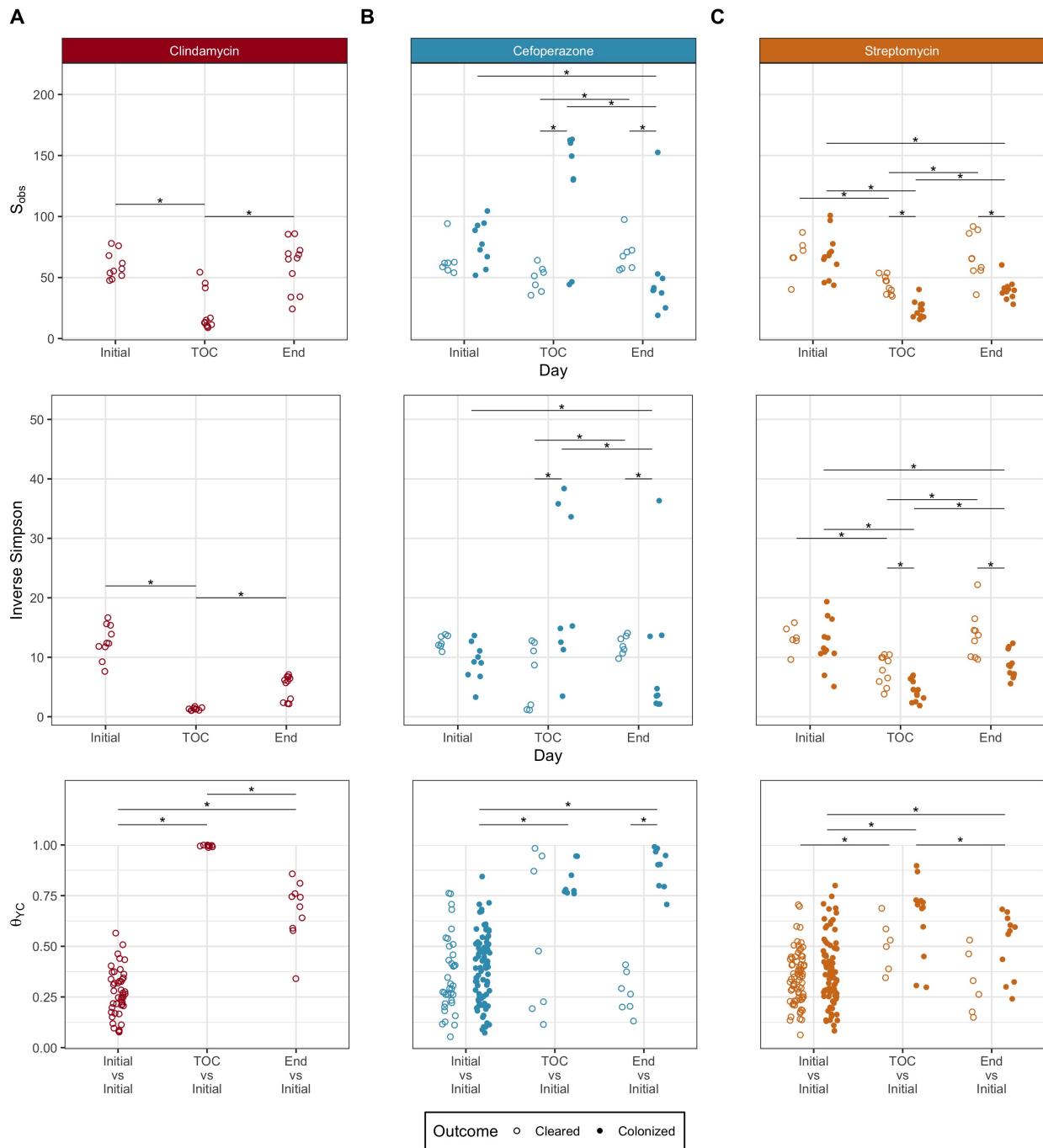


512

513 **Figure 1. Reduced antibiotic doses permitted murine communities to be colonized and**  
 514 **spontaneously clear that *C. difficile* colonization.** (A-C) Daily CFU of *C. difficile* in fecal samples  
 515 of mice treated with clindamycin, cefoperazone, or streptomycin from time of challenge (Day 0)  
 516 through 10 days post infection (dpi). The bold line is the median CFU of the group and the  
 517 transparent lines are the individual mice. (D-F) Relative abundance of twelve most abundant genera  
 518 at the time of *C. difficile* challenge, all other genera grouped into Other. Each column is an individual  
 519 mouse. LOD = Limit of detection. (clindamycin - 10 mg/kg N = 11; cefoperazone - 0.5 mg/mL N = 5,  
 520 0.3 mg/mL N = 9, 0.1 mg/mL N = 2; streptomycin - 5.0 mg/mL N = 8, 0.5 mg/mL N = 7, 0.1 mg/mL  
 521 N = 7).

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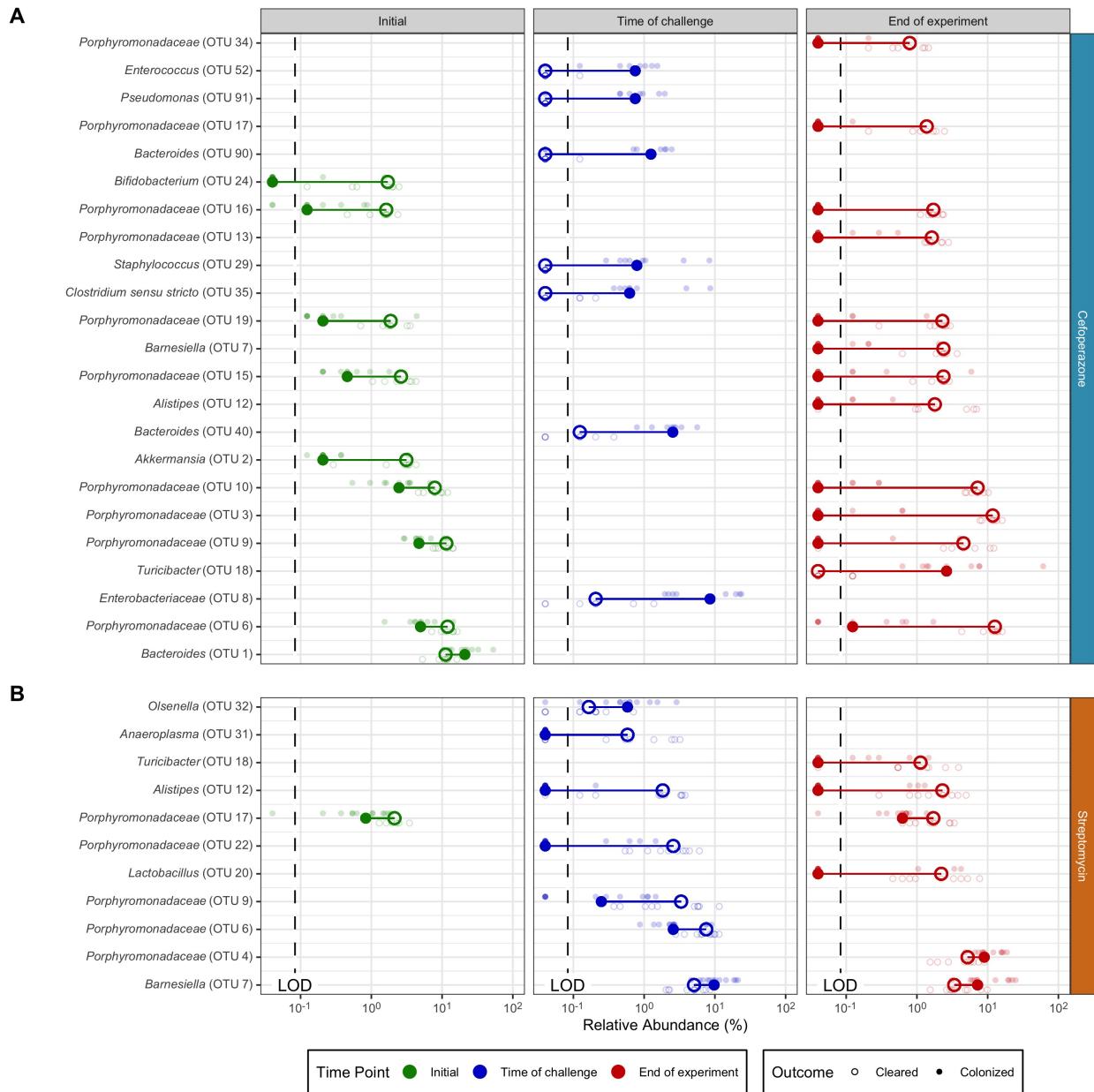
524

525 **Figure 2. Microbiota community diversity showed antibiotic-specific trends associated with**  
526 ***C. difficile* colonization clearance.** For communities colonized with *C. difficile* from mice treated  
527 with clindamycin (A), cefoperazone (B), and streptomycin (C), microbiota  $\alpha$ -diversity ( $S_{\text{obs}}$  and  
528 Inverse Simpson) and  $\beta$ -diversity ( $\theta_{YC}$ ) were compared at the initial pre-antibiotic treatment state,  
529 time of *C. difficile* challenge (TOC), and end of the experiment.  $\beta$ -diversity ( $\theta_{YC}$ ) was compared

530 between the initial pre-antibiotic treatment to all other initial pre-antibiotic treatment communities  
531 treated with the same antibiotic, the initial community to the same community at the time of *C.*  
532 *difficile* challenge, and the initial community to the same community at end of the experiment.  
533 (clindamycin - cleared N = 11; cefoperazone - cleared N = 7, colonized N = 9; streptomycin - cleared  
534 N = 9, colonized N = 11). \* indicates statistical significance of  $P < 0.05$ , calculated by Wilcoxon  
535 rank sum test with Benjamini-Hochberg correction.

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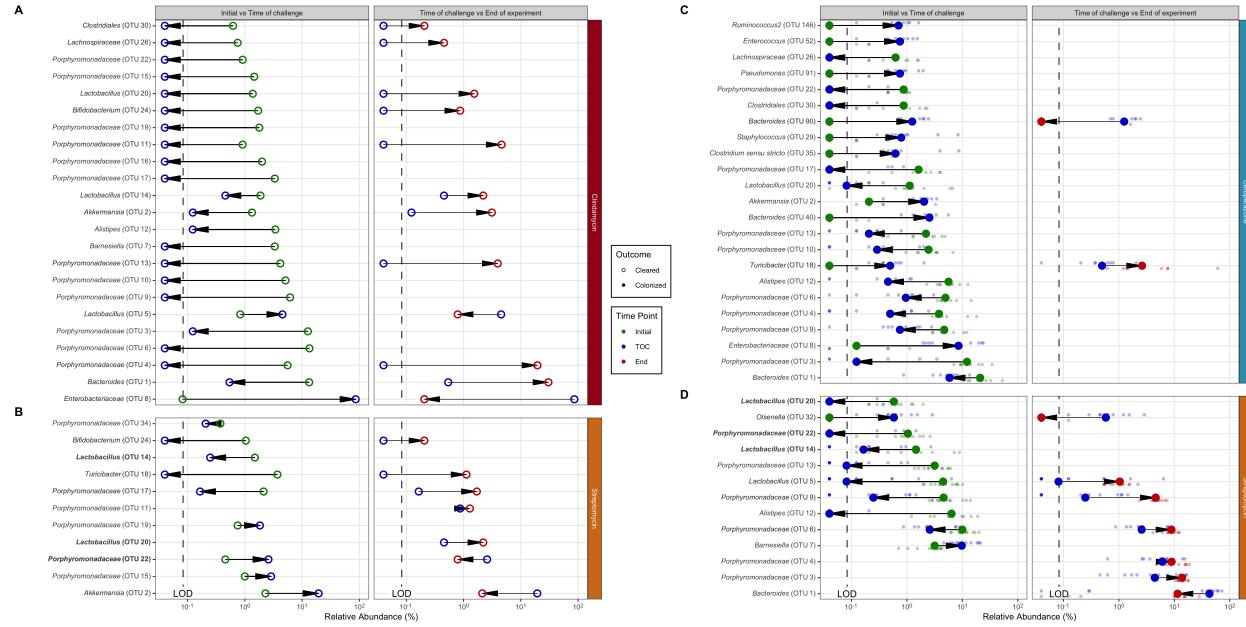
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539 **Figure 3. OTU abundance differences between communities that cleared *C. difficile***  
 540 **colonization and remained colonized are unique to each treatment.** For cefoperazone (A) and  
 541 streptomycin (B), the difference in the relative abundance of OTUs that were significantly different  
 542 between communities that eliminated *C. difficile* colonization and those that remained colonized  
 543 within each antibiotic treatment for each time point. Bold points are median relative abundance and  
 544 transparent points are relative abundance of individual mice. Lines connect points within each  
 545 comparison to show difference in medians. Only OTUs at time points with statistically significant

546 differences,  $P < 0.05$ , were plotted (calculated by Wilcoxon rank sum test with Benjamini-Hochberg  
 547 correction). Limit of detection (LOD).

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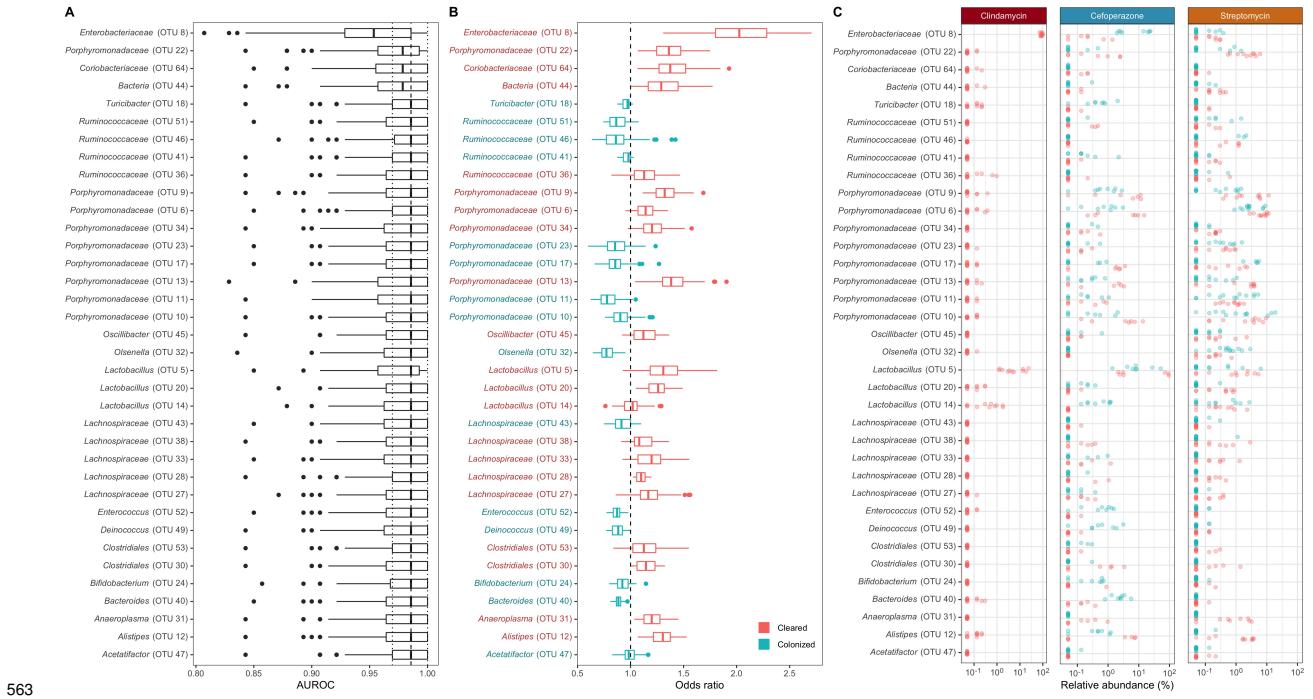


550

551 **Figure 4. Each antibiotic had specific sets of temporal changes in OTU abundance**  
 552 **associated with *C. difficile* colonization and clearance.** For clindamycin (A), cefoperazone (C),  
 553 and streptomycin (B, D), the difference in the relative abundance of OTUs that were significantly  
 554 different between time points within each *C. difficile* colonization outcome for each antibiotic  
 555 treatment. Bold points are median relative abundance and transparent points are relative  
 556 abundance of individual mice. Lines connect points within each comparison to show difference in  
 557 medians. Arrows point in the direction of the temporal change of the relative abundance. Only  
 558 OTUs at time points with statistically significant differences,  $P < 0.05$ , were plotted (calculated  
 559 by Wilcoxon rank sum test with Benjamini-Hochberg correction). Bold OTUs were shared across  
 560 outcomes. Limit of detection (LOD).

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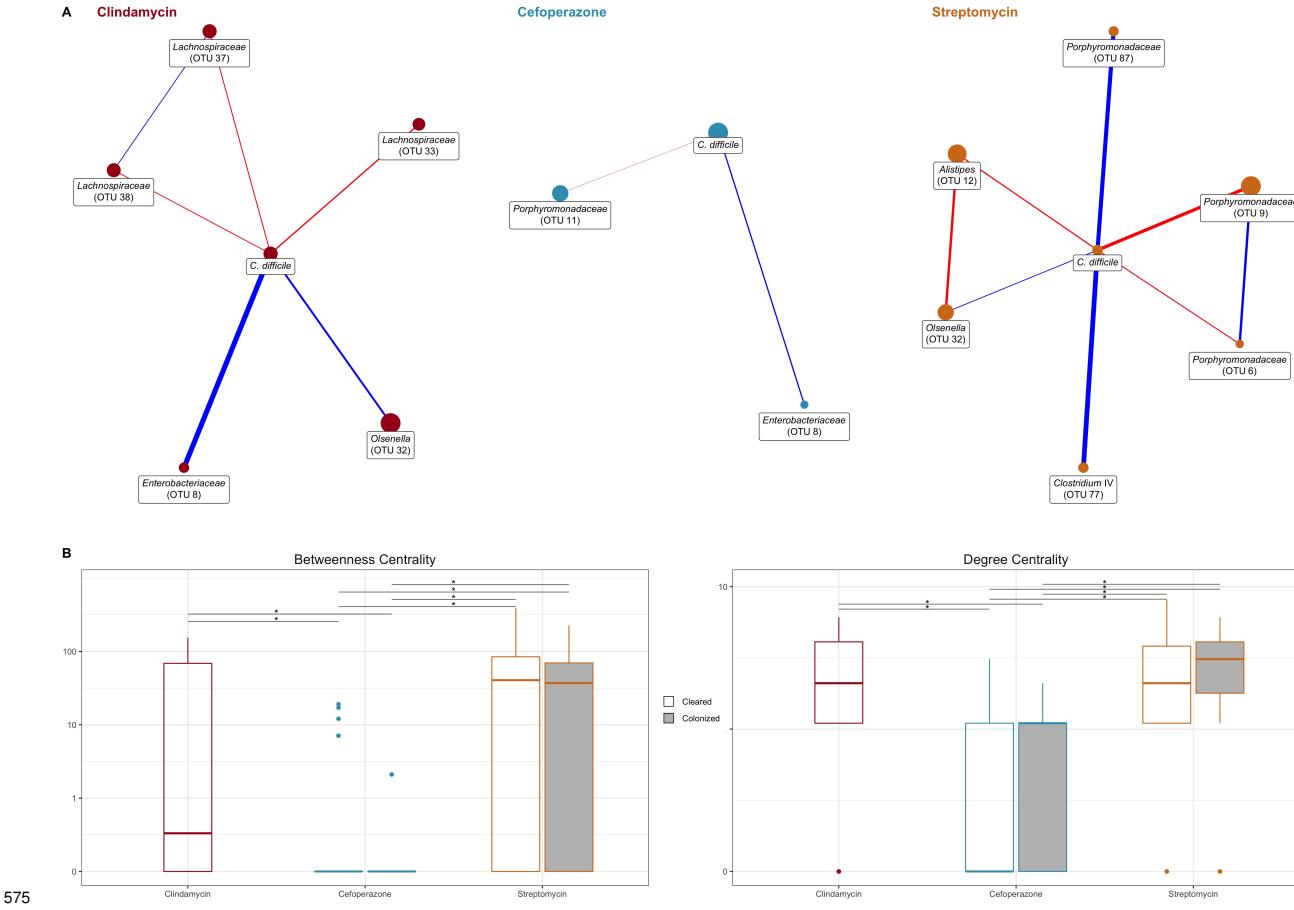
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564 **Figure 5. Distinct features of the bacterial community at the time of infection can classify**  
 565 **end point colonization.** (A) L2 logistic regression model features' importance determined by  
 566 the decrease in model performance when randomizing an individual feature. All OTUs affecting  
 567 performance shown. Dashed lines show performance range of final model with all features  
 568 included. (B) Distribution of odds ratio used in L2 logistic regression model. Values above 1  
 569 indicate abundance predicted the community cleared colonization (red) and values below 1 indicate  
 570 abundance predicted *C. difficile* remained colonized (blue). Feature label and boxplot are colored  
 571 to match the median odds ratio. (C) Relative abundance difference in features used by L2 logistic  
 572 regression model displayed by antibiotic treatment.

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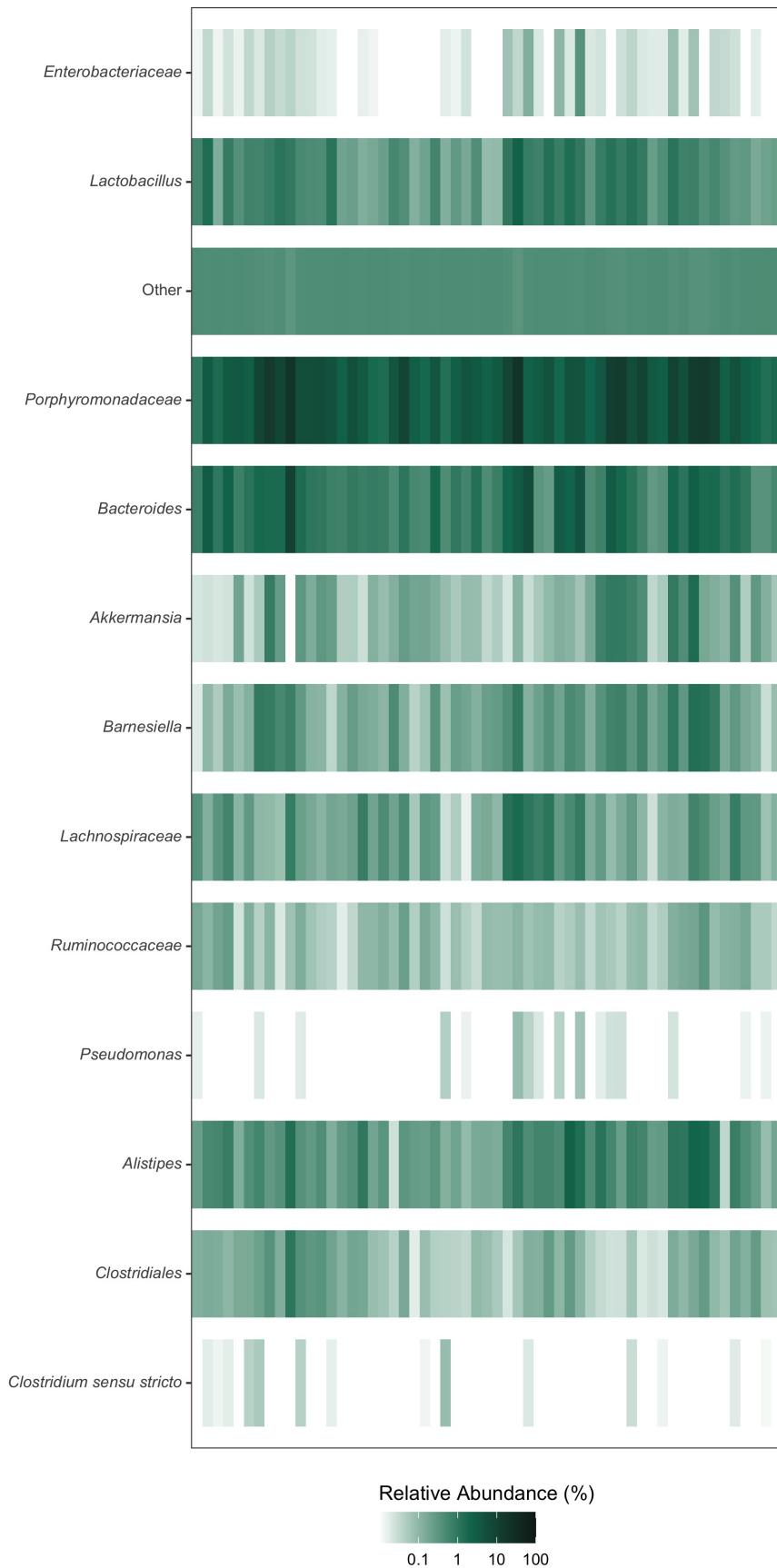
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576 **Figure 6. Conditional independence networks reveal treatment-specific relationships**  
 577 **between the community and *C. difficile* during colonization clearance.** (A) SPIEC-EASI  
 578 (sparse inverse covariance estimation for ecological association inference) networks showing  
 579 conditionally independent first-order relationships between *C. difficile* and the community as *C.*  
 580 *difficile* was cleared from the gut environment. Nodes are sized by median relative abundance  
 581 of the OTU. A red colored edge indicates a negative interaction and blue indicates a positive  
 582 interaction, while edge thickness indicates the interaction strength. (B) Network centrality measured  
 583 with betweenness, i.e. how many paths between two OTUs pass through an individual, and degree,  
 584 i.e. how many connections an OTU had. \* indicates statistical significance of  $P < 0.05$ , calculated  
 585 by Wilcoxon rank sum test with Benjamini-Hochberg correction.

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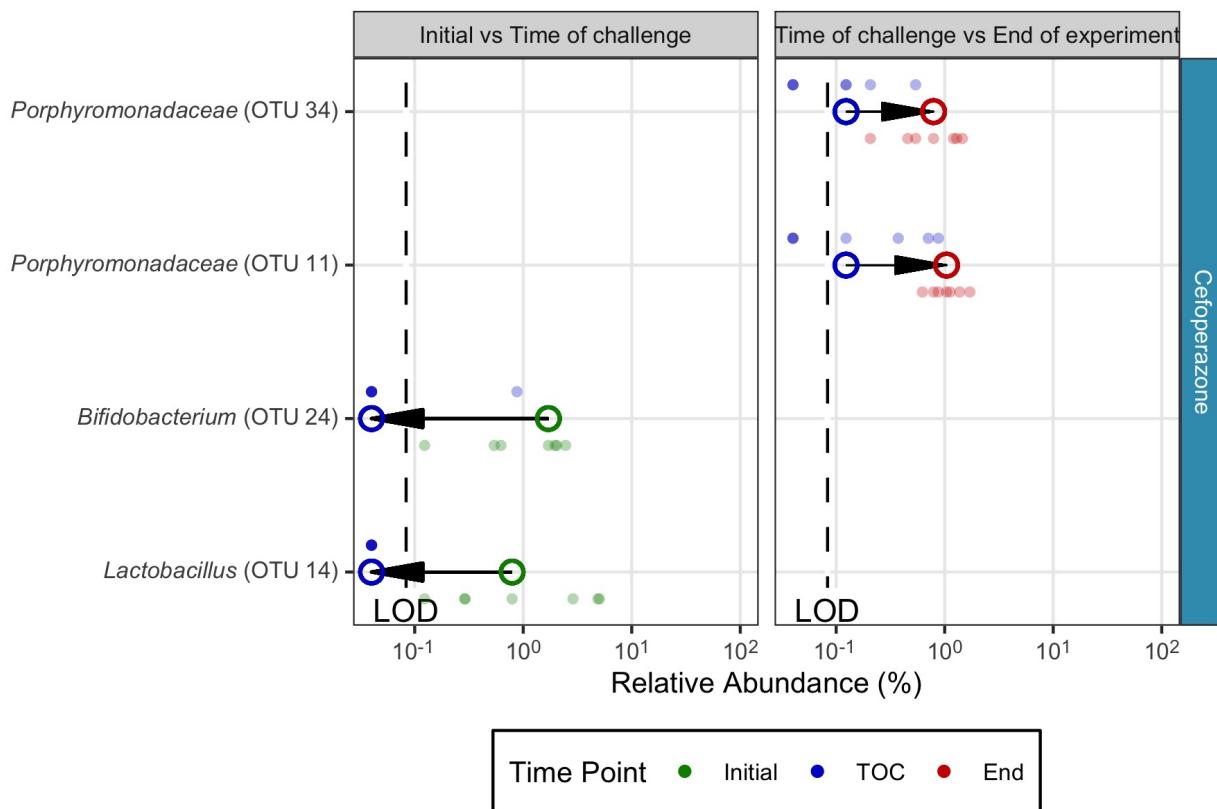
587



589 **Figure S1. Initial microbiota relative abundance of mice prior to antibiotic treatment.**  
 590 Relative abundance at the beginning of the experiment prior to antibiotic treatment of twelve most  
 591 abundant genera post antibiotic treatment, all other genera grouped into Other. Each column is an  
 592 individual mouse. Color intensity is  $\log_{10}$ -transformed mean percent relative abundance of each  
 593 day. (N = 57).

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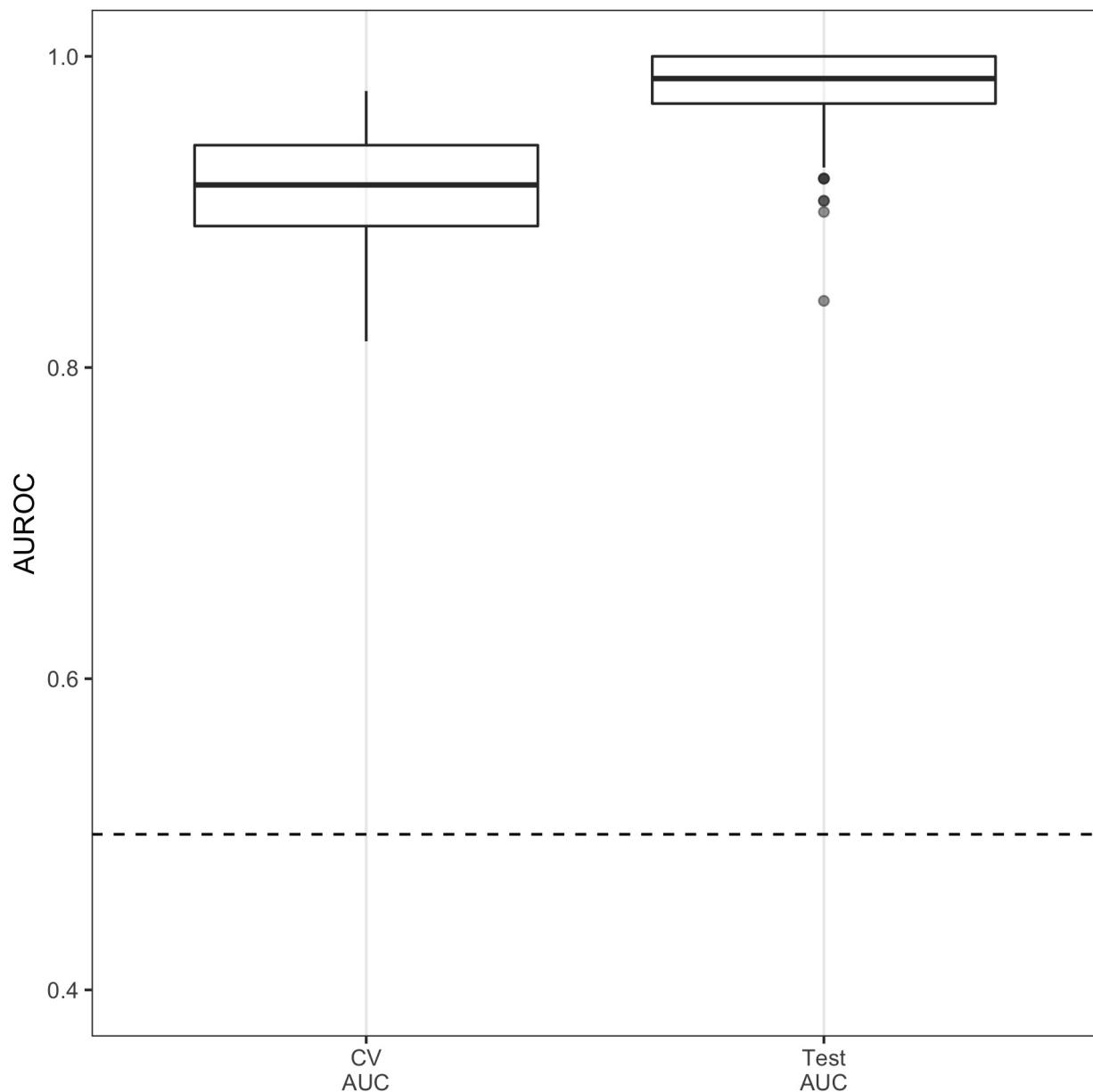


596

597 **Figure S2. Temporally differing OTU for cefoperazone-treated mice that cleared *C. difficile* colonization.** Bold points are median relative abundance and transparent points are relative  
 598 abundance of individual mice. Lines connect points within each comparison to show difference  
 599 in medians. Arrows point in the direction of the temporal change of the relative abundance. Only  
 600 OTUs at time points with statistically significant differences,  $P < 0.05$ , were plotted (calculated by  
 601 Wilcoxon rank sum test with Benjamini-Hochberg correction). Limit of detection (LOD).

603

604



605

**Figure S3. Bacterial community at the time of infection can classify endpoint colonization.**

Classification performance of L2 logistic regression. Area under the receiver-operator curve for classifying if the community will remain colonized based on the OTUs present at the time of *C. difficile* infection (Day 0). Cross-validation of model performed on half of the data to tune model (CV AUC) and then tuned model was tested on the held-out data (Test AUC).