

# **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, CFU fell below limit of detection, whereas mice treated with cefoperazone and  
62 streptomycin did not. Here, we continued to explore the different effects these three antibiotics  
63 have on *C. difficile* colonization. The purpose of this study was to elucidate the gut bacterial

64 community changes concurrent with clearance of *C. difficile* colonization, specifically the reduction  
65 in CFU below the limit of detection. Other groups have shown *C. difficile* can persist below the  
66 limit of detection and reappear if the community is perturbed, however we were interested in the  
67 community changes involved in clearing or reducing the *C. difficile* population. We hypothesized  
68 that each colonized community has perturbation-specific susceptibilities and requires specific  
69 changes to clear the pathogen. To induce a less severe perturbation, we reduced the doses of  
70 cefoperazone and streptomycin. This resulted in communities that were initially colonized to a high  
71 level ( $>10^6$  CFU/g feces) and then spontaneously cleared *C. difficile*. We found each antibiotic  
72 resulted in unique changes in the microbiota that were associated with the persistence or clearance  
73 of *C. difficile*. These data further support the hypothesis that *C. difficile* can exploit numerous  
74 niches in perturbed communities.

75 **Results**

76 **Reduced doses of cefoperazone and streptomycin allowed communities to spontaneously**  
77 **clear *C. difficile* colonization.** To understand the dynamics of colonization and clearance of *C.*  
78 *difficile*, we first identified conditions which would allow colonization and clearance. Beginning with  
79 clindamycin, mice were treated with an intraperitoneal injection of clindamycin (10 mg/kg) one day  
80 prior to challenge with *C. difficile*. All mice (N = 11) were colonized to a high level (median CFU =  
81  $3.07 \times 10^7$ ) the next day and cleared the colonization within 10 days; 6 mice cleared *C. difficile* within  
82 6 days (Figure 1A). Previous *C. difficile* infection models using cefoperazone and streptomycin  
83 have not demonstrated clearance. So we next explored whether cefoperazone and streptomycin  
84 could permit colonization and subsequent clearance with lower doses. We began with replicating  
85 the previously established *C. difficile* infection models using these antibiotics (20). We treated  
86 mice with cefoperazone or streptomycin in their drinking water for 5 days (0.5 mg/mL and 5 mg/mL,  
87 respectively) and then challenged them with *C. difficile*. For both antibiotics, *C. difficile* colonization  
88 was maintained for the duration of the experiment as previously demonstrated (Figure 1B-C) (20).  
89 Then we repeated the *C. difficile* challenge with reduced doses of the antibiotics (cefoperazone -  
90 0.3 and 0.1 mg/mL; streptomycin - 0.5 and 0.1 mg/mL). For both antibiotic treatments, the lowest

91 dose resulted in either no colonization ( $N = 8$ ) or a transient, low level colonization ( $N = 8$ , median  
92 length = 1 day, median CFU/g =  $2.8 \times 10^3$ ) (Figure 1B-C). The intermediate dose of both antibiotics  
93 resulted in a high level colonization (median CFU/g =  $3.5 \times 10^6$ ) and half ( $N = 8$  of 16) of the mice  
94 clearing the colonization within 10 days. Based on our previous research, which showed each  
95 of these antibiotics uniquely changed the microbiota, we hypothesized that the microbiota varied  
96 across these antibiotic treatments that resulted in colonization clearance. To focus on the changes  
97 related to colonization clearance and not antibiotic dosage, the remaining analysis aggregated  
98 mice which had *C. difficile* present in their stool post-challenge by whether *C. difficile* was detected,  
99 referred to as colonized, or not at the end of the experiment, referred to as cleared.

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

120 We applied the same analysis to the cefoperazone-treated mice to understand what community  
121 features were relevant to clearing *C. difficile*. Increasing the dose of cefoperazone shifted the  
122 dominant community members from relatives of the *Porphyromonadaceae*, *Bacteroides* and  
123 *Akkermansia* to relatives of the *Lactobacillus* and *Enterobacteriaceae* at the time of challenge  
124 (Figure 1E, S1). We saw a similar increase in relatives of *Enterobacteriaceae* with clindamycin.  
125 However, the cefoperazone-treated mice that had larger increases in *Enterobacteriaceae* were  
126 unable to clear *C. difficile*. We next investigated the differences between the cefoperazone-treated  
127 mice that cleared *C. difficile* to those that did not. For the communities that cleared *C. difficile*,  
128 diversity was maintained throughout the experiment (Figure 2B). A subset of mice treated with  
129 cefoperazone that remained colonized experienced an increase in alpha diversity, possibly driven  
130 by the decrease in highly abundant populations and increase in low abundant populations (Figure  
131 1E, S2). These persistently colonized communities also had a large shift away from the initial  
132 community structure caused by the antibiotic treatment ( $P < 0.05$ ), which remained through the end  
133 of the experiment ( $P < 0.05$ ) (Figure 2B). The communities with increased alpha diversity were not  
134 statistically different by dosage, although most of them were from the group treated with 0.3 mg/mL  
135 cefoperazone (Figure S3). These data suggested that it was necessary for cefoperazone-treated  
136 mice to become more similar to the initial pre-antibiotic community structure to clear *C. difficile*. We  
137 next investigated the changes in OTU abundances between the communities that cleared *C. difficile*  
138 and those that did not to elucidate the community members involved in clearance. Communities  
139 that remained colonized were significantly enriched in facultative anaerobic populations including  
140 *Enterococcus*, *Pseudomonas*, *Staphylococcus*, and *Enterobacteriaceae* at the time of challenge.  
141 Communities that cleared *C. difficile* had significant enrichment in 10 different OTUs related to the  
142 *Porphyromonadaceae* at the end of the experiment (Figure 3A). We were also interested in the  
143 temporal changes within each community so we investigated which OTUs changed due to antibiotic  
144 treatment or during the *C. difficile* colonization. The majority of significant temporal differences in  
145 OTUs for cefoperazone-treated mice occurred in persistently colonized communities. Persistently  
146 colonized communities had a persistent loss of numerous relatives of the *Porphyromonadaceae* and  
147 increases in the relative abundance of facultative anaerobes (Figure 4C, S4). Overall, persistent *C.*  
148 *difficile* colonization in cefoperazone-treated mice was associated with a shift in the microbiota to a  
149 new community structure which seemed unable to recover from the antibiotic perturbation, whereas

150 clearance occurred when the community was capable of returning to its original structure.

151 Finally, we identified the differences in *C. difficile* colonization for streptomycin-treated  
152 mice. Increasing the dose of streptomycin maintained the abundance of relatives of the  
153 *Porphyromonadaceae* and *Bacteroides*, but reduced most of the other genera including populations  
154 of the *Lactobacillus*, *Lachnospiraceae*, *Ruminococcaceae*, *Alistipes*, and *Clostridiales* (Figure 1F).  
155 Both communities that cleared and those that remained colonized had similar changes in diversity.  
156 Streptomycin-treated mice became mildly dissimilar ( $P < 0.05$ ) and less diverse ( $P < 0.05$ ) with  
157 streptomycin treatment but by the end of the experiment returned to resemble the pre-antibiotic  
158 community ( $P < 0.05$ ) (Figure 2C). Those communities that remained colonized had slightly lower  
159 alpha-diversity than those that cleared *C. difficile*. ( $P < 0.05$ ). Persistently colonized mice had  
160 reduced relative abundance of relatives of *Alistipes*, *Anaeroplasma*, and *Porphyromonadaceae*  
161 at time of challenge compared to the mice that cleared *C. difficile* (Figure 3B). At the end of the  
162 experiment the mice that were still colonized had lower abundances of *Turicibacter*, *Alistipes*, and  
163 *Lactobacillus*. Since most of the differences were reduced relative abundances in the colonized  
164 mice, we were interested to explore what temporal changes occurred between pre-antibiotic  
165 treatment, the time of challenge, and the end of the experiment for the communities that cleared  
166 *C. difficile*. The temporal changes in streptomycin-treated mice were more subtle than those  
167 observed with the other antibiotic treatments. At the time of challenge, the communities that  
168 remained colonized had reductions in 4 OTUs related to the *Porphyromonadaceae*. Those that  
169 cleared *C. difficile* also had changes in OTUs related to the *Porphyromonadaceae*, however, 2  
170 populations decreased and 2 increased in abundance (Figure 4B, D). At the end of the experiment,  
171 all communities experienced recovery of the abundance of many of the populations changed by the  
172 streptomycin treatment, but the communities that remained colonized did not recover 5 of the OTUs  
173 of *Alistipes*, *Lactobacillus*, and *Porphyromonadaceae* that were reduced by streptomycin. The  
174 differences between the streptomycin-treated mice that remained colonized and those that had  
175 been cleared of *C. difficile* were not as distinct as those observed with the cefoperazone treatment.  
176 The differences between colonized and cleared streptomycin-treated mice were minimal, which  
177 suggested the few differences may be responsible for the clearance. Overall, these data revealed  
178 that while there were commonly affected families across the antibiotic treatments, such as the

179 *Porphyromonadaceae*, *C. difficile* clearance was associated with community and OTU differences  
180 specific to each antibiotic.

181 **Distinct features of the bacterial community at the time of infection predicted end point**  
182 **colonization.** To determine whether the community composition at the time of *C. difficile* challenge  
183 could predict *C. difficile* clearance, we built a machine learning model using L2 logistic regression.  
184 We modeled all treatments together to prevent overfitting of the data and allow the model to reveal  
185 which OTUs were able to correctly predict clearance in the context of the other OTU relative  
186 abundances. We evaluated the predictive performance of the model using the area under the  
187 receiver operating characteristic curve (AUROC), where a value of 0.5 indicated the model is  
188 random and 1.0 indicated the model always correctly predicts the outcome. Our model resulted in a  
189 AUROC of 0.986 [IQR 0.970-1.000], which suggested that the model was able to use the relative  
190 abundance of OTUs at the time of challenge to accurately predict colonization clearance (Figure  
191 S5). To assess the important features, we randomly permuted each OTU feature by removing  
192 it from the training set to determine its effect on the prediction (Figure 5A). The most important  
193 feature was an OTU related to the *Enterobacteriaceae*, whose abundance predicted clearance.  
194 This result appears to have been strongly driven by the clindamycin data (Figure 5B, C). The  
195 remaining OTU features did not have a large effect on the model performance, which suggested  
196 that the model decision was spread across many features. These results revealed the model  
197 used the relative abundance data of the community members and the relationship between those  
198 abundances to correctly classify clearance. There were many OTUs with treatment and outcome  
199 specific abundance patterns that did not agree with the odds ratio of the OTU used by the model.  
200 For example, *Enterobacteriaceae* abundance influenced the model to predict clearance (Figure  
201 5B), however in experiments that used cefoperazone, the communities that remained colonized had  
202 higher abundances of *Enterobacteriaceae* than the communities that cleared colonization (Figure  
203 5C). The model arrived at the correct prediction through the collective influence of other OTUs.  
204 Therefore, the model used different combinations of multiple OTUs and their relative abundances  
205 across treatments to predict *C. difficile* clearance. These data can offer a basis for hypotheses  
206 regarding the distinct combinations of bacteria that promote *C. difficile* clearance.

207 **Conditional independence networks revealed treatment-specific relationships between the**

208 **community members and *C. difficile* during colonization clearance.** We next investigated  
209 the relationship between temporal changes in the community and *C. difficile* by building a  
210 conditional independence network for each treatment using SPIEC-EASI (sparse inverse  
211 covariance estimation for ecological association inference) (21). First, we focused on the first-order  
212 associations of *C. difficile* (Figure 6A). In clindamycin-treated mice, *C. difficile* had positive  
213 associations with relatives of *Enterobacteriaceae*, *Pseudomonas*, and *Olsenella* and negative  
214 associations with relatives of the *Lachnospiraceae* and *Clostridium XIVa*. *C. difficile* had limited  
215 associations in cefoperazone-treated mice; the primary association was positive with relatives  
216 of *Enterobacteriaceae*. In streptomycin-treated mice, *C. difficile* had negative associations  
217 with relatives of the *Porphyromonadaceae* and positive associations with populations of the  
218 *Ruminococcaceae*, *Bacteroidetes*, *Clostridium IV* and *Olsenella*. Next, we quantified the degree  
219 centrality, the number of associations between each OTU for the whole network of each antibiotic  
220 and outcome, and betweenness centrality, the number of associations connecting two OTUs that  
221 pass through an OTU (Figure 6B). This analysis revealed cefoperazone treatment resulted in  
222 networks primarily composed of singular associations with much lower degree centrality ( $P < 0.05$ )  
223 and betweenness centrality ( $P < 0.05$ ) than the other antibiotic treatments. Communities that were  
224 treated with cefoperazone that resulted in cleared or persistent colonization had 10 to 100-fold  
225 lower betweenness centrality values than communities treated with clindamycin or streptomycin.  
226 Collectively, these networks suggest *C. difficile* colonization was affected by unique sets of OTUs in  
227 mice treated with clindamycin and streptomycin, but cefoperazone treatment eliminated bacteria  
228 critical to maintaining community interactions and had few populations that associated with *C.*  
229 *difficile*.

## 230 **Discussion**

231 We have shown that different antibiotic treatments resulted in specific changes to the microbiota  
232 that were associated with *C. difficile* clearance. Clindamycin-treated mice became susceptible  
233 with a dominant bloom in populations related to *Enterobacteriaceae*. Clearance was associated  
234 with the resolution of the bloom and recovery of bacteria that were reduced by the antibiotic

235 treatment. Cefoperazone-treated mice became susceptible with the expansion of numerous  
236 facultative anaerobes. Communities with a sustained presence of these facultative anaerobes  
237 were unable to recover from the initial antibiotic perturbation or clear the colonization, whereas  
238 the communities that returned to their initial community were able to clear *C. difficile* colonization.  
239 Streptomycin-treated mice became susceptible with fewer and smaller changes than the other  
240 treatments. The communities that cleared colonization had slightly higher  $\alpha$ -diversity than those  
241 that remained colonized. Additionally, all communities in mice treated with streptomycin had  
242 similar numbers of OTUs changing through the experiment but the specific OTUs were different for  
243 each outcome. These observations support our hypothesis that each colonized community has  
244 antibiotic-specific changes that create unique conditions for *C. difficile* colonization and requires  
245 specific changes within each community to clear *C. difficile*.

246 Previous studies have identified microbiota associated with reduced *C. difficile* colonization in  
247 either a set of closely related murine communities or collectively across many different susceptible  
248 communities (11, 15, 22). Bacteria from these studies have since been tested in *C. difficile* infection  
249 models. These experiments were able to show decreased colonization but were unable to eliminate  
250 *C. difficile* (11, 23) or only demonstrated elimination in the model it was developed (15). Rather  
251 than looking for similarities across all susceptible communities, we explored the changes that were  
252 associated with *C. difficile* clearance for each antibiotic. Even though these mice all came from the  
253 same breeding colony with similar initial microbiomes, *C. difficile* clearance was associated with  
254 antibiotic-specific changes in community diversity, OTU abundances, and associations between  
255 OTUs. Our data suggest that the set of bacteria necessary to restore colonization resistance  
256 following one antibiotic perturbation may not be effective for all antibiotic perturbations. We have  
257 developed this modeling framework starting from a single mouse community. It should also be  
258 relevant when considering interpersonal variation among humans (24).

259 Recent studies have begun to uncover how communities affect *C. difficile* colonization (17–20, 24).  
260 We attempted to understand the general trends in each antibiotic treatment that lead to clearance  
261 of *C. difficile*. We categorized the general changes and microbial relationships of these experiments  
262 into three models. First, a model of temporary opportunity characterized by the transient dominance  
263 of a facultative anaerobe which permits *C. difficile* colonization but *C. difficile* is not able to persist,

264 as with clindamycin treatment. We hypothesize this susceptibility is due to a transient repression  
265 of community members and interventions which further perturb the community may worsen the  
266 infection. Time alone may be sufficient for the community to clear colonization (15, 22, 25) but  
267 treating the community with an antibiotic or the bowel preparation for an FMT (26, 27) may prolong  
268 susceptibility by eliminating protective functions or opening new niches. Second, a model of an  
269 extensive opportunity characterized by a significant perturbation leading to a persistent increase  
270 in facultative anaerobes and exposing multiple niches, as with cefoperazone treatment. These  
271 communities appear to have been severely depleted of multiple critical community members and  
272 are likely lacking numerous protective functions (20). We hypothesize multiple niches are made  
273 available for *C. difficile* to colonize through reducing physically or chemically inhibitory bacteria or  
274 increasing available resources. In this scenario, a full FMT may be insufficient to provide adequate  
275 diversity and abundance to outcompete and occupy all the exposed niches. Multiple FMTs (28,  
276 29) or transplant of an enriched fecal community (30) may be necessary to recover the microbiota  
277 enough to outcompete *C. difficile* for the nutrient niches and replace the missing protective functions.  
278 Third, a model of a specific opportunity characterized by a perturbation that only affects a select  
279 portion of the microbiota, leading to small changes in relative abundance and a slight decrease  
280 in diversity, opening a limited niche for *C. difficile* to colonize, as with streptomycin treatment. We  
281 hypothesize that a few specific bacteria species with key inhibitory functions would be necessary  
282 to recolonize the exposed niche space and eliminate *C. difficile* colonization (13, 17). A fecal  
283 microbiota transplant may contain the bacterial diversity needed to fill the open niche space and  
284 help supplant *C. difficile* from the exposed niche of the colonized community. Analyzing each of  
285 these colonization models individually allowed us to understand how each may clear *C. difficile*  
286 colonization.

287 Future investigations can further identify the exposed niches of susceptible communities and  
288 the requirements to clear *C. difficile* colonization. One common theme for susceptibility across  
289 treatments was the increased abundance of facultative anaerobes. These blooms of facultative  
290 anaerobes could be attributed to the loss of the indigenous obligate anaerobes with antibiotic  
291 treatment (31, 32). However, it is unclear what prevents the succession from the facultative  
292 anaerobes back to the obligate anaerobes in cefoperazone-treated mice. Future studies should

293 investigate the relationship between facultative anaerobe blooms and susceptibility to colonization  
294 as well as interventions to recover the obligate anaerobes. Another aspect to consider in future  
295 experiments is *C. difficile* strain specificity. Other strains may fill different niche space and fill  
296 other community interactions (33–35). For example, more virulent strains, like *C. difficile* VPI  
297 10463, may have a greater effect on the gut environment since it produces more toxin and drives  
298 a stronger immune response (15, 35, 36). Those differences could lead to greater increases in  
299 inflammatory conditions and further increase populations that thrive in these conditions, such as  
300 *Enterobacteriaceae*, and change the requirements to clear *C. difficile* (31, 37, 38). Finally, we have  
301 shown that the functions found in communities at peak colonization are antibiotic-specific (20).  
302 Here, we have shown the bacterial population changes associated with *C. difficile* clearance are  
303 antibiotic-specific. It is unknown how the community functions contributing to *C. difficile* clearance  
304 compare across antibiotics. It is possible while we observed different changes in the bacteria  
305 populations but the functions eliminating *C. difficile* were conserved. Additionally, it is unclear  
306 how specific these functions are to the OTUs we observed. It is possible that phylogenetically  
307 diverse OTUs have similar functional potential as well as phylogenetically similar OTUs having  
308 specific functions. Examining the changes in transcription and metabolites during clearance will  
309 help define the activities necessary to clear *C. difficile* and if they are specific to the perturbation.  
310 This information will build upon the community differences presented in this study and move us  
311 closer to elucidating how the microbiota clears *C. difficile* colonization and developing targeted  
312 therapeutics.

313 We have shown that mice became susceptible to *C. difficile* colonization after three different  
314 antibiotic treatments and then differed in their ability to clear the colonization. These experiments  
315 have shown that each antibiotic treatment resulted in different community changes leading to  
316 *C. difficile* clearance. These differences suggest that a single mechanism of infection and one  
317 treatment for all *C. difficile* infections may not be appropriate. While our current use of FMT to  
318 eliminate CDI is highly effective, it does not work in all patients and has even resulted in adverse  
319 consequences (7–10). The findings in this study may help explain why FMTs may be ineffective.  
320 Although an FMT transplants a whole community, it may not be sufficient to replace the missing  
321 community members or functions to clear *C. difficile*. Alternatively, the FMT procedure itself may

322 disrupt the natural recovery of the community. The knowledge of how a community clears *C. difficile*  
323 colonization will advance our ability to develop targeted therapies to manage CDI.

324 **Materials and Methods**

325 **Animal care.** 5- to 8-week-old C57BL/6 mice were obtained from a single breeding colony. Mice  
326 were housed in cages of 2-5 mice maintained in specific-pathogen-free (SPF) conditions at the  
327 University of Michigan animal facility. Each experiment treatment used 6-11 mice and was repeated  
328 2-4 times. All mouse protocols and experiments were approved by the University Committee on  
329 Use and Care of Animals at the University of Michigan and completed in agreement with approved  
330 guidelines.

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

335 ***C. difficile* challenge.** Mice were returned to untreated drinking water for 24 hours before  
336 challenging with *C. difficile* strain 630Δerm spores. *C. difficile* spores were aliquoted from a  
337 single spore stock stored at 4°C. Spore concentration was determined one week prior to the day of  
338 challenge (39). 10<sup>3</sup> *C. difficile* spores were orally gavaged into each mouse. Once the gavages  
339 were completed, the remaining spore solution was serially diluted and plated to confirm the spore  
340 concentration that was delivered.

341 **Sample collection.** Fecal samples were collected on the day antibiotic treatment was started, on  
342 the day of *C. difficile* challenge and the following 10 days. For the day of challenge and beyond,  
343 a fecal sample was also collected and weighed. Under anaerobic conditions a fecal sample was  
344 serially diluted in anaerobic phosphate-buffered saline and plated on TCCFA plates. After 24 hours  
345 of anaerobic incubation at 37°C, the number of colony forming units (CFU) were determined (40).

346 **DNA sequencing.** Total bacterial DNA was extracted from each fecal sample using MOBIO

347 PowerSoil-htp 96-well soil DNA isolation kit. We created amplicons of the 16S rRNA gene V4 region  
348 and sequenced them using an Illumina MiSeq as described previously (41).

349 **Sequence curation.** Sequences were processed using mothur(v.1.43.0) as previously described  
350 (41). Briefly, we used a 3% dissimilarity cutoff to group sequences into operational taxonomic units  
351 (OTUs). We used a naive Bayesian classifier with the Ribosomal Database Project training set  
352 (version 16) to assign taxonomic classifications to each OTU (43). With the fecal samples, we  
353 also sequenced a mock community with a known community composition and their true 16s rRNA  
354 gene sequences. We processed this mock community along with our samples to determine our  
355 sequence curation resulted in an error rate of 0.019%.

356 **Statistical analysis and modeling.** Diversity comparisons were calculated in mothur. To compare  
357  $\alpha$ -diversity metrics, we calculated the number of OTUs ( $S_{obs}$ ) and the Inverse Simpson diversity  
358 index. To compare across communities, we calculated dissimilarity matrices based on metric of  
359 Yue and Clayton (44). All calculations were made by rarifying samples to 1,200 sequences per  
360 sample to limit biases due to uneven sampling. OTUs were subsampled to 1,200 counts per sample  
361 and remaining statistical analysis and data visualization was performed in R (v3.5.1) with the  
362 tidyverse package (v1.3.0). Significance of pairwise comparisons of  $\alpha$ -diversity ( $S_{obs}$  and Inverse  
363 Simpson),  $\beta$ -diversity ( $\theta_{YC}$ ), OTU abundance, and network centrality (betweenness and degree)  
364 were calculated by pairwise Wilcoxon rank sum test and then  $P$  values were corrected for multiple  
365 comparisons with a Benjamini and Hochberg adjustment for a type I error rate of 0.05 (45). Logistic  
366 regression models were constructed with OTUs from all day 0 samples using half of the samples  
367 to train and the other half to test the model. The model was developed from the caret R package  
368 (v6.0-85) and previously developed machine learning pipeline (46). For each antibiotic treatment,  
369 conditional independence networks were calculated from the day 1 through 10 samples of all  
370 mice initially colonized using SPIEC-EASI (sparse inverse covariance estimation for ecological  
371 association inference) methods from the SpiecEasi R package after optimizing lambda to 0.001  
372 with a network stability between 0.045 and 0.05 (v1.0.7) (21). Network centrality measures degree  
373 and betweenness were calculated on whole networks using functions from the igraph R package  
374 (v1.2.4.1).

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

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

379 **Acknowledgements**

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

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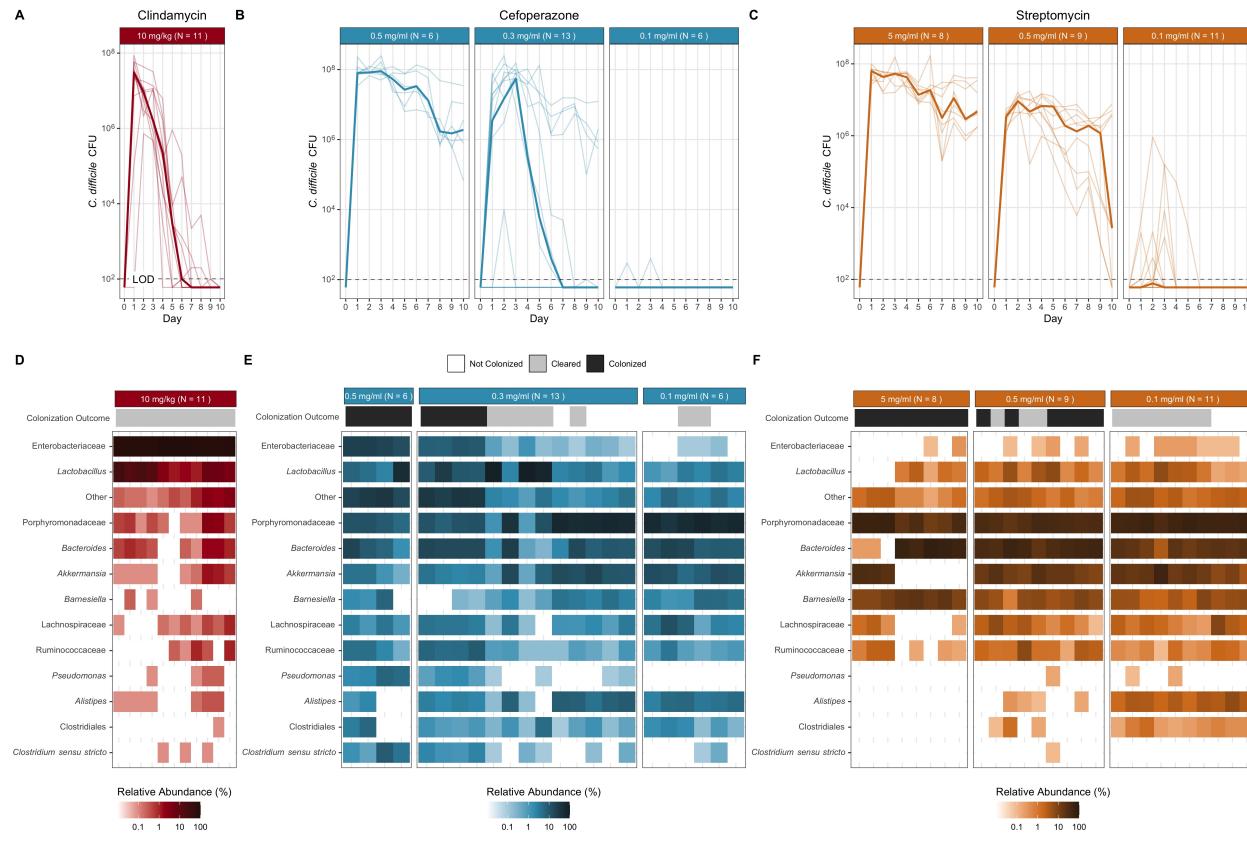
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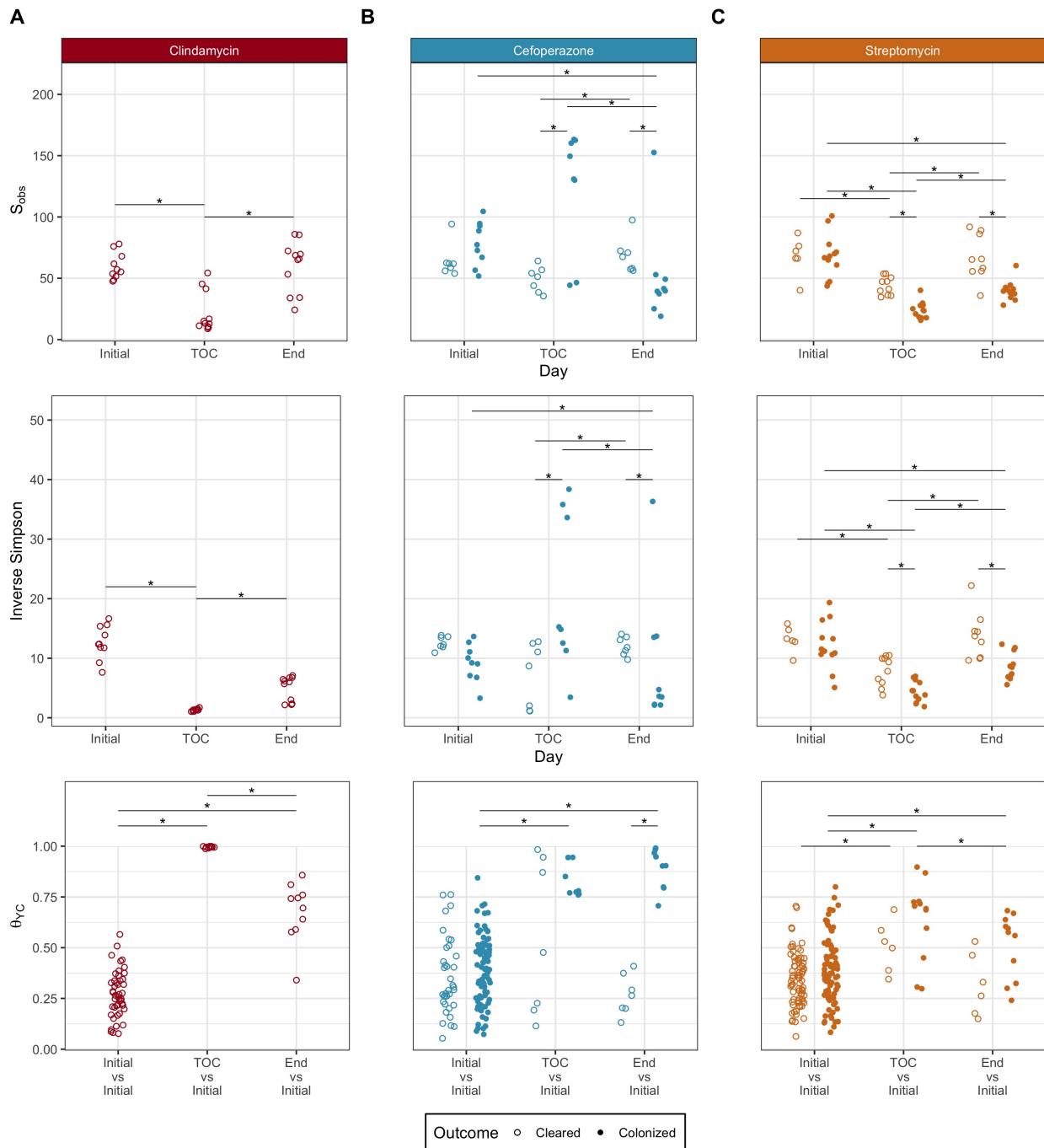


542

543 **Figure 1. Reduced antibiotic doses permitted murine communities to be colonized and**  
 544 **spontaneously clear that *C. difficile* colonization.** (A-C) Daily CFU of *C. difficile* in fecal samples  
 545 of mice treated with clindamycin, cefoperazone, or streptomycin from time of challenge (Day 0)  
 546 with  $10^3$  *C. difficile* strain 630 $\Delta$ erm spores through 10 days post infection (dpi). The bold line is  
 547 the median CFU of the group and the transparent lines are the individual mice. (D-F) Relative  
 548 abundance of twelve most abundant taxonomic groups, labeled with the lowest level of classification,  
 549 at the time of *C. difficile* challenge, all other taxonomic groups are combined into Other. Each  
 550 column is an individual mouse. LOD = Limit of detection.

551

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553

554 **Figure 2. Microbiota community diversity showed antibiotic-specific trends associated with**

555 ***C. difficile* colonization clearance.** For communities colonized with *C. difficile* from mice treated

556 with clindamycin (A), cefoperazone (B), and streptomycin (C), microbiota  $\alpha$ -diversity ( $S_{obs}$  and

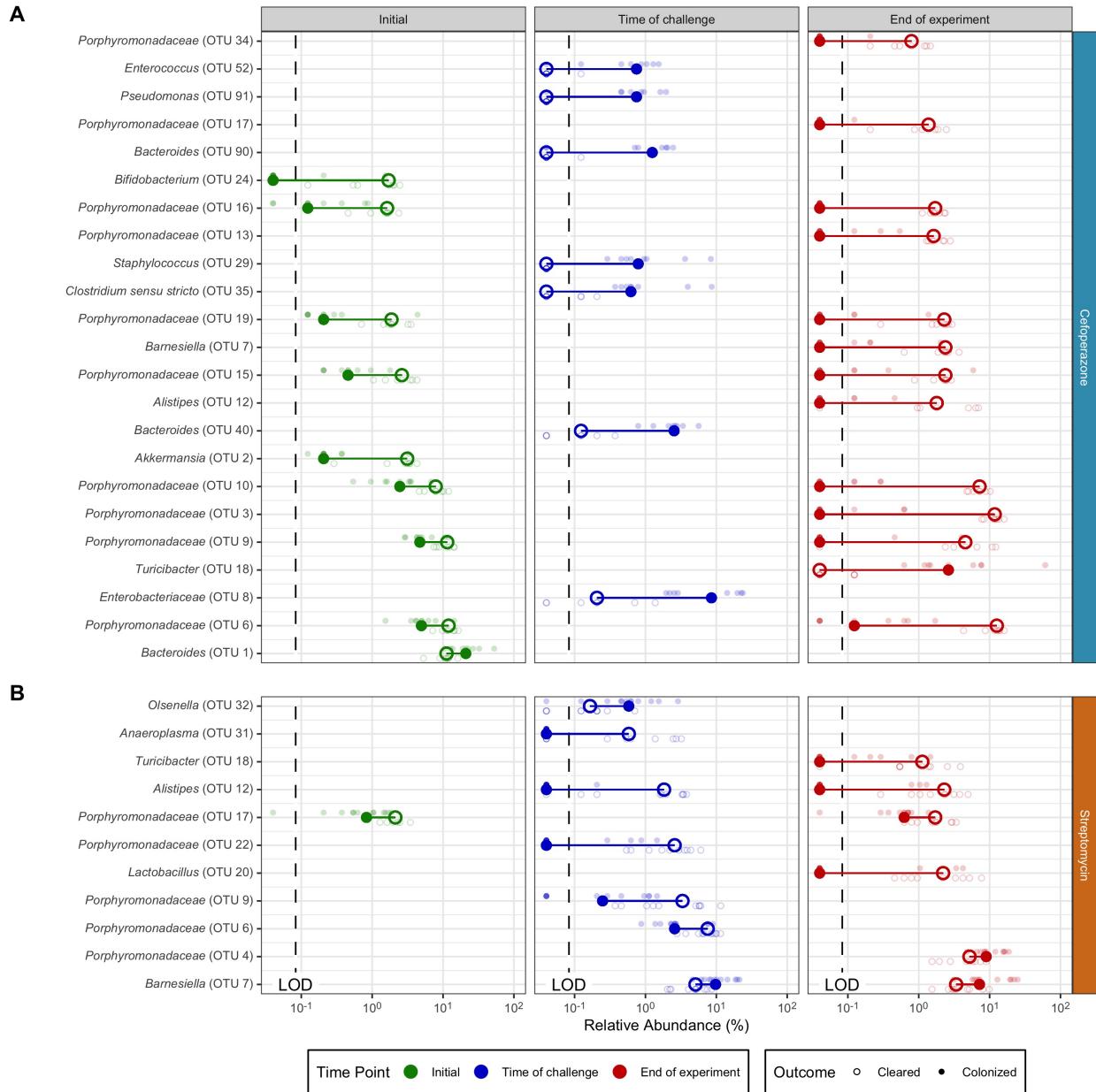
557 Inverse Simpson) and  $\beta$ -diversity ( $\theta_{YC}$ ) were compared at the initial pre-antibiotic treatment state,

558 time of *C. difficile* challenge (TOC), and end of the experiment.  $\beta$ -diversity ( $\theta_{YC}$ ) was compared

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

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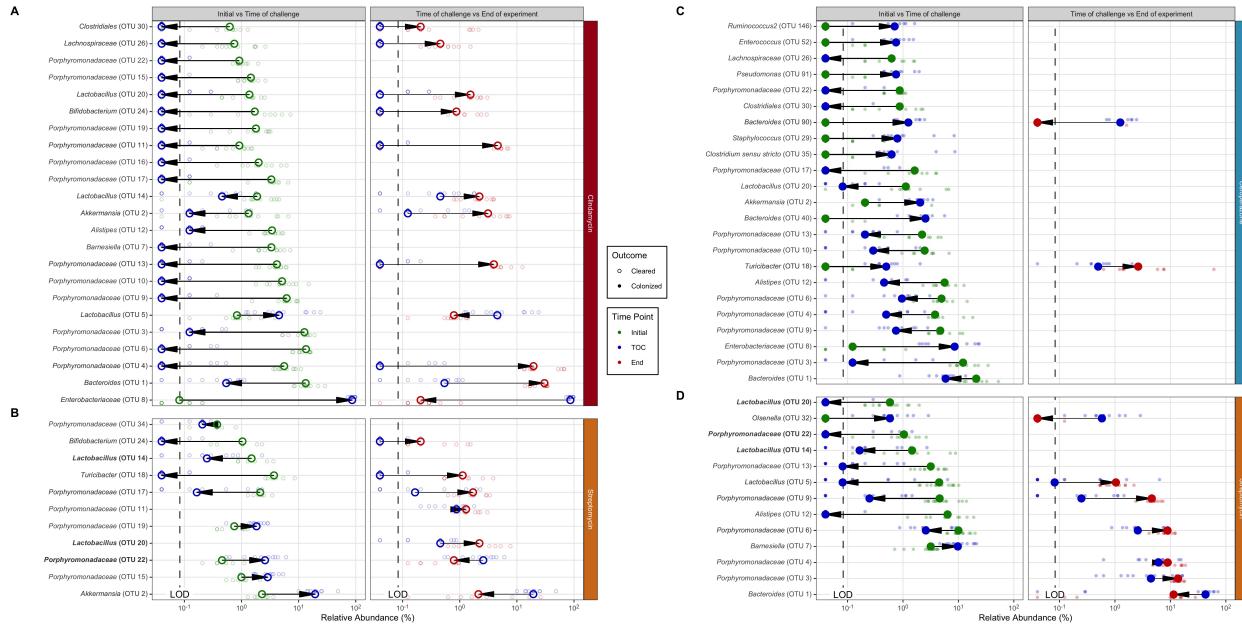
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568 **Figure 3. OTU abundance differences between communities that cleared *C. difficile***  
 569 **colonization and remained colonized are unique to each treatment.** For cefoperazone (A)  
 570 and streptomycin (B), the difference in the relative abundance of OTUs that were significantly  
 571 different between communities that eliminated *C. difficile* colonization and those that remained  
 572 colonized within each antibiotic treatment for each time point were identified. Dark larger points  
 573 in foreground are median relative abundance and transparent smaller points in background are  
 574 relative abundance of individual mice. Lines connect points within each comparison to show

575 difference in medians. Only OTUs at time points with statistically significant differences,  $P < 0.05$ ,  
 576 were plotted (calculated by Wilcoxon rank sum test with Benjamini-Hochberg correction). Limit of  
 577 detection (LOD).

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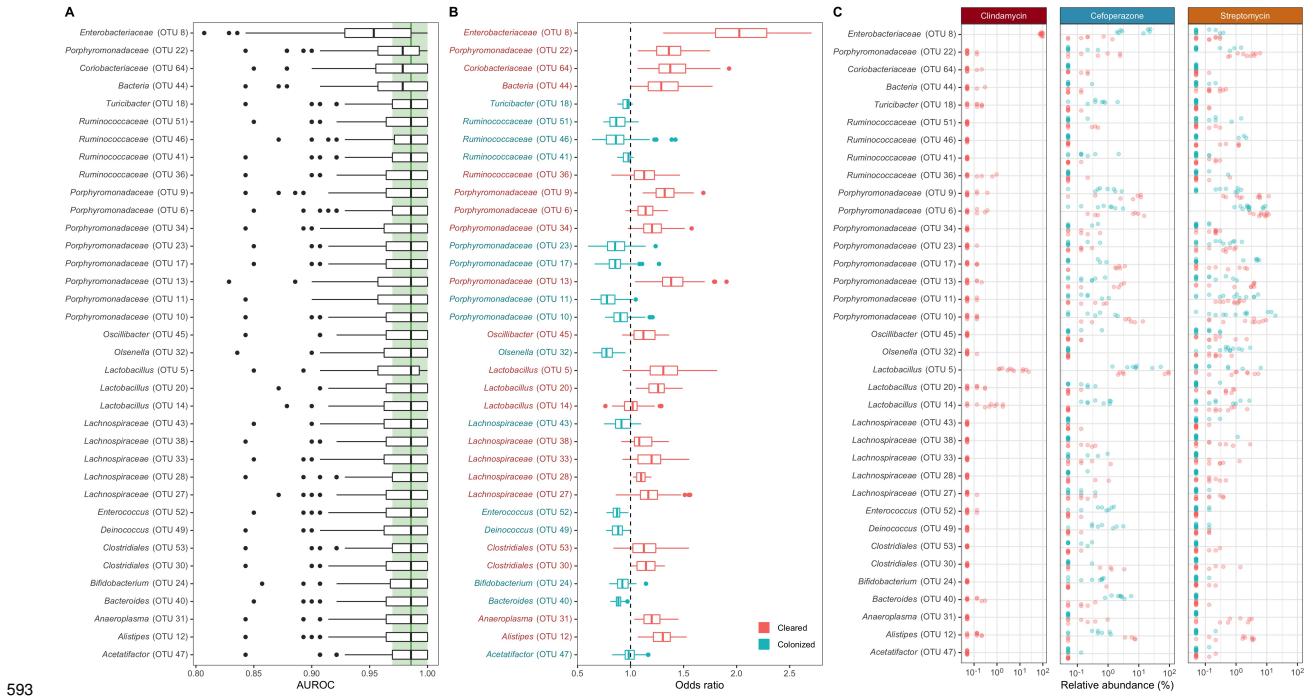


580

581 **Figure 4. Each antibiotic had specific sets of temporal changes in OTU abundance**  
 582 **associated with *C. difficile* colonization and clearance.** For clindamycin (A), cefoperazone (C),  
 583 and streptomycin (B, D), the difference in the relative abundance of OTUs that were significantly  
 584 different between time points within each *C. difficile* colonization outcome for each antibiotic  
 585 treatment were identified. Dark larger points in foreground are median relative abundance and  
 586 transparent smaller points in background are relative abundance of individual mice. Lines connect  
 587 points within each comparison to show difference in medians. Arrows point in the direction of the  
 588 temporal change of the relative abundance. Only OTUs at time points with statistically significant  
 589 differences,  $P < 0.05$ , were plotted (calculated by Wilcoxon rank sum test with Benjamini-Hochberg  
 590 correction). Bold OTUs were shared across outcomes. Limit of detection (LOD).

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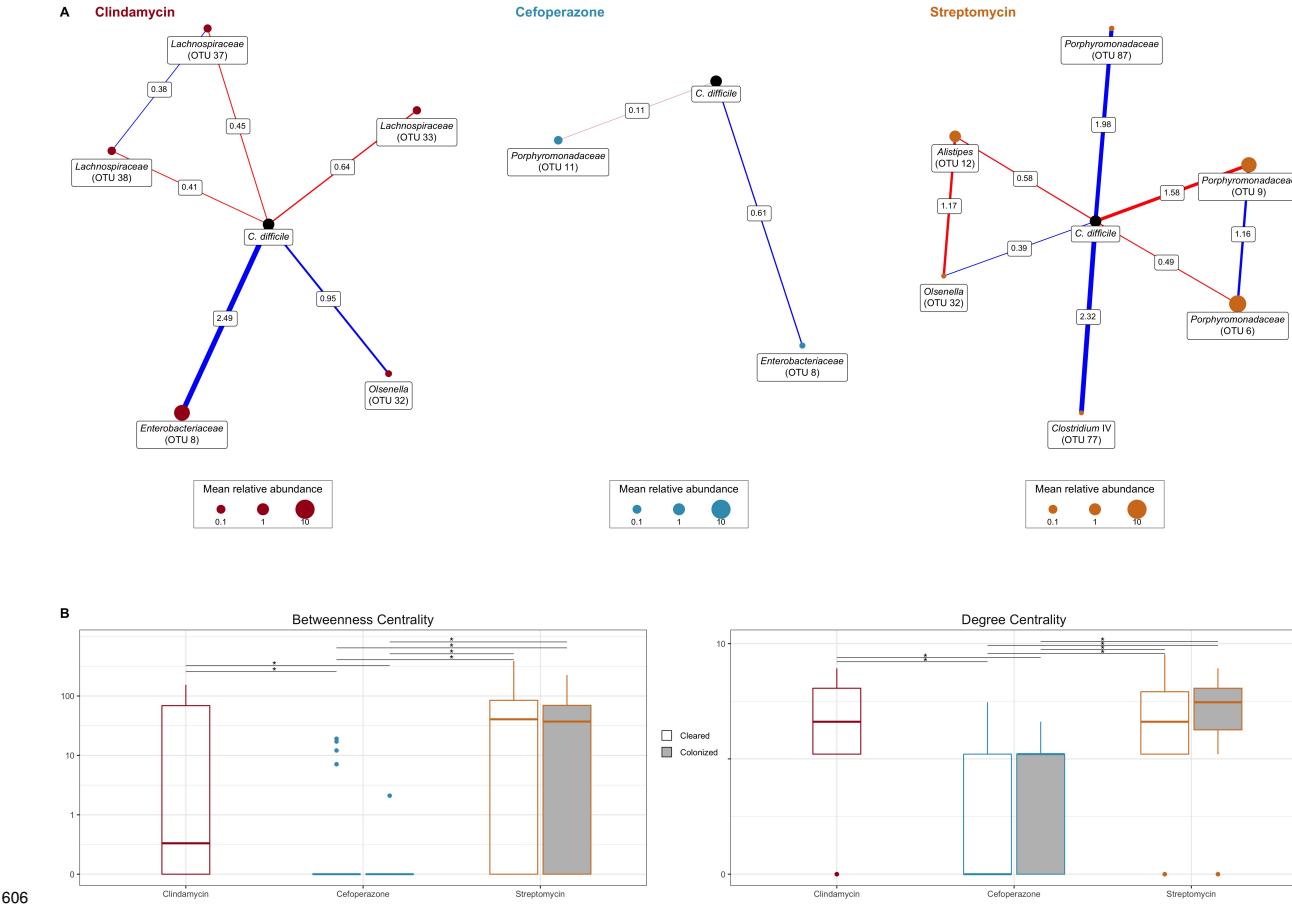
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594 **Figure 5. Distinct features of the bacterial community at the time of infection can classify**  
 595 **end point colonization.** (A) L2 logistic regression model features' importance determined by  
 596 the decrease in model performance when randomizing an individual feature. All OTUs affecting  
 597 performance shown. Light green band in the background shows the interquartile range and the dark  
 598 green line shows the median AUROC of the final model with all features included. (B) Distribution of  
 599 odds ratio used in L2 logistic regression model. Values above 1 indicate abundance predicted the  
 600 community cleared colonization (red) and values below 1 indicate abundance predicted *C. difficile*  
 601 remained colonized (blue). Feature label and boxplot are colored to match the median odds ratio.  
 602 (C) Relative abundance difference in features used by L2 logistic regression model displayed by  
 603 antibiotic treatment.

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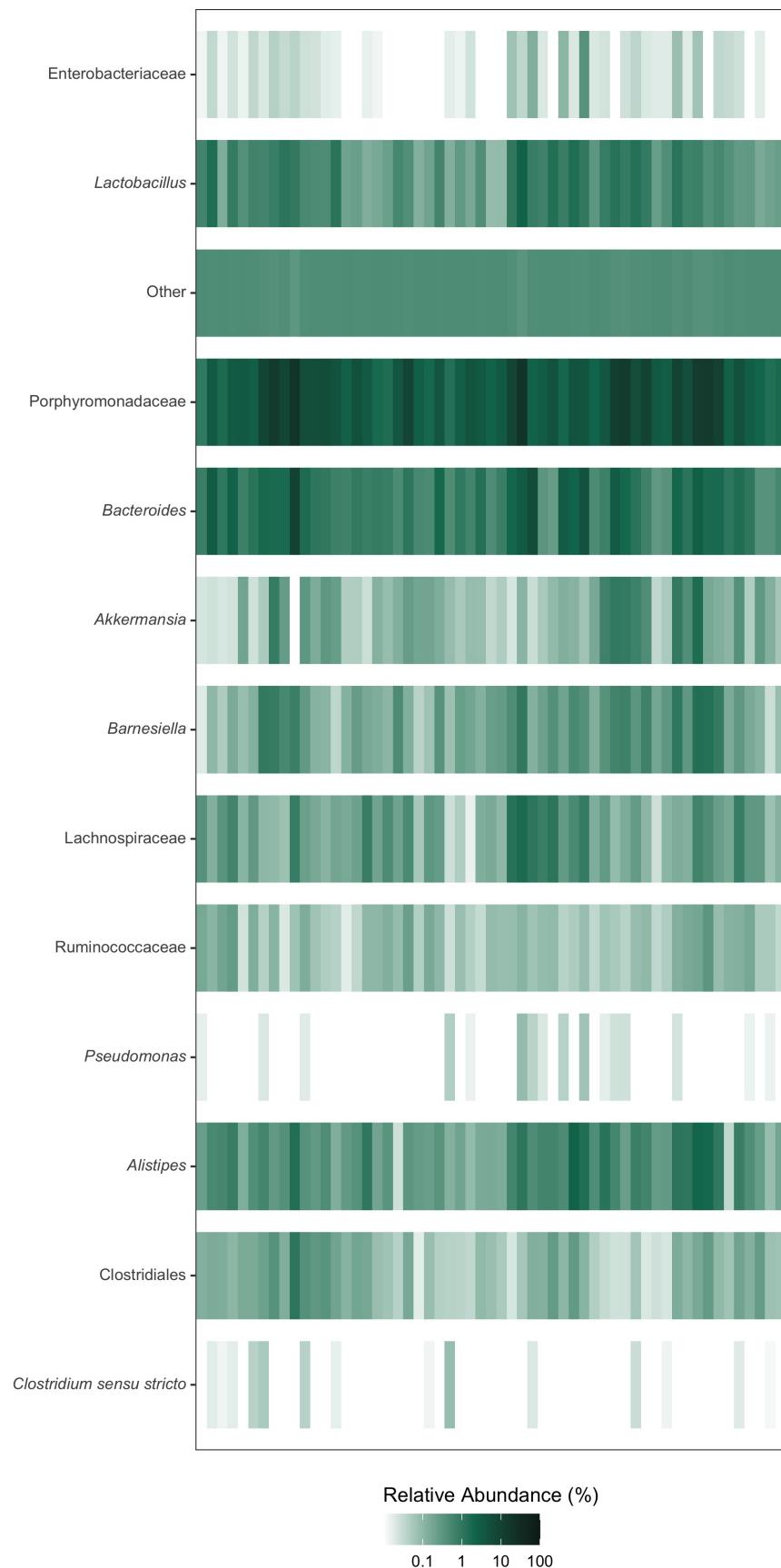
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607 **Figure 6. Conditional independence networks reveal treatment-specific relationships**  
608 **between the community and *C. difficile* during colonization clearance.** (A) SPIEC-EASI  
609 (sparse inverse covariance estimation for ecological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

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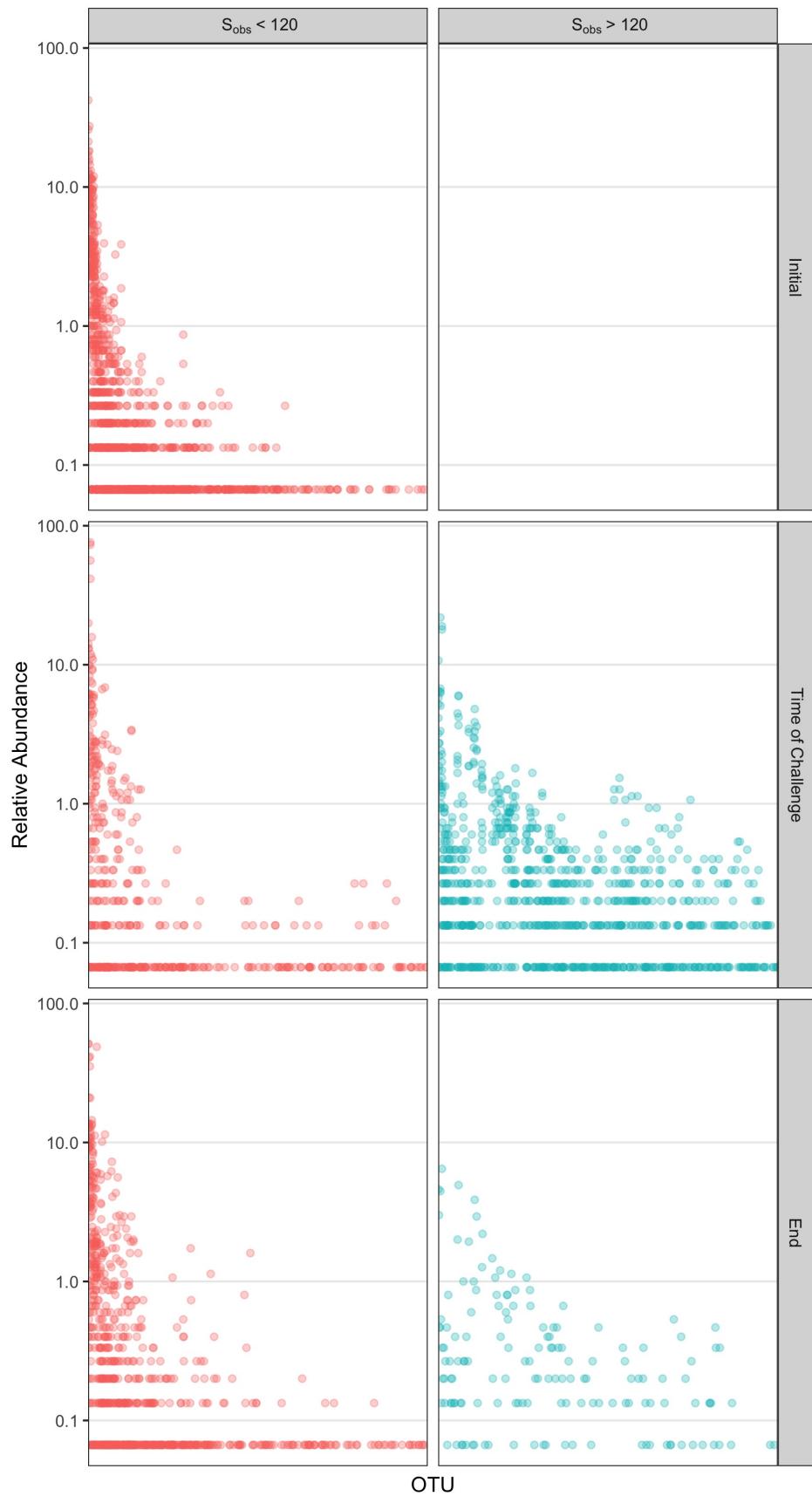
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620 **Figure S1. Initial microbiota relative abundance of mice prior to antibiotic treatment.** Initial  
621 community shows the most abundant taxa. The plot shows the relative abundance at the beginning  
622 of the experiment prior to antibiotic treatment of twelve most abundant taxonomic groups, labeled  
623 with the lowest level of classification. All other taxonomic groups are combined into Other. Each  
624 column is an individual mouse fecal community. Color intensity is  $\log_{10}$ -transformed mean percent  
625 relative abundance.

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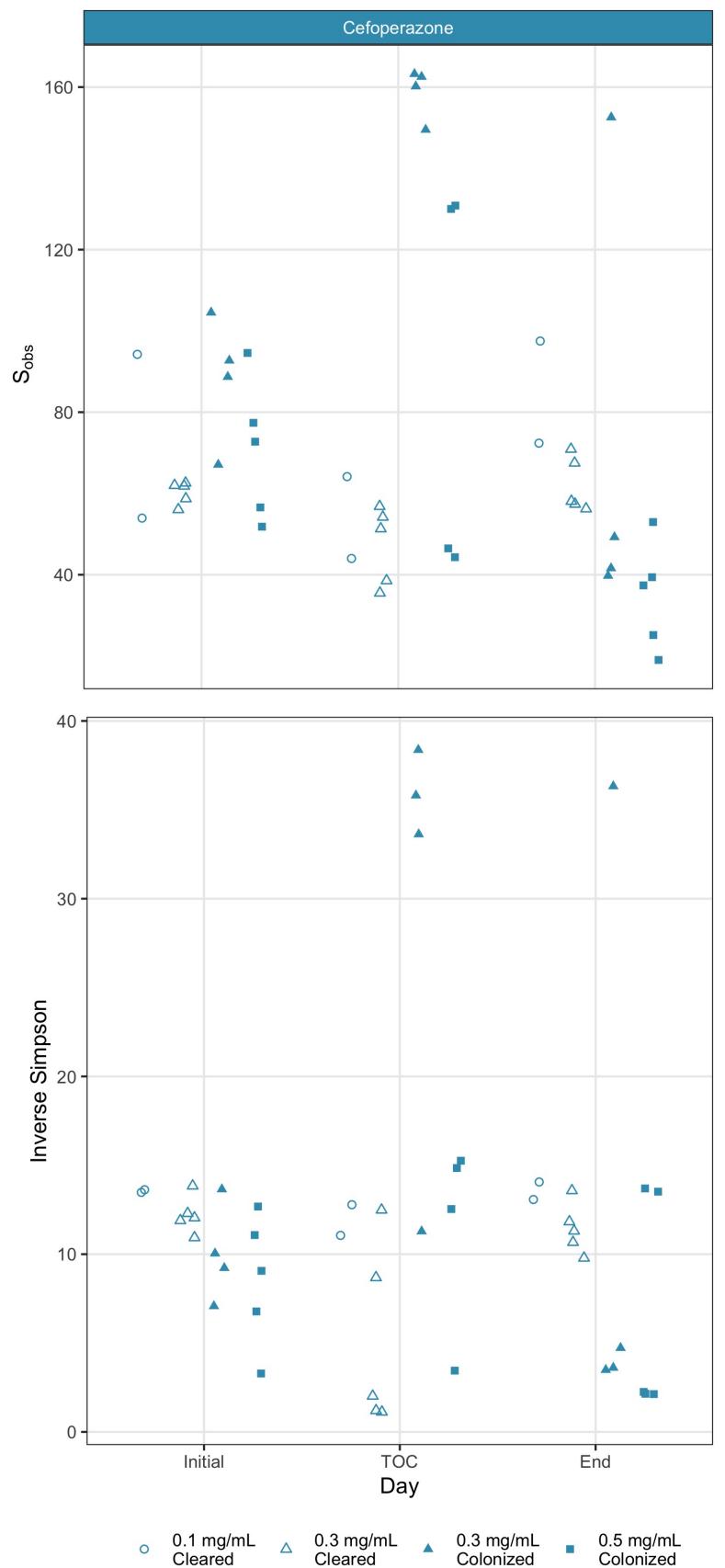
627



629 **Figure S2. Cefoperazone-treated mice with increased  $S_{obs}$  have increased abundance**  
630 **initially low abundant OTUs.** Relative abundance of each OTU plotted for mice treated with  
631 cefoperazone. OTUs arranged numerically along the x-axis. Each point is the relative abundance  
632 of a single OTU of an individual mouse. Split at  $S_{obs} = 120$  to separate the communities that  
633 increased in alpha diversity above the alpha diversity in the untreated initial communities (Figure 2).  
634  $S_{obs} < 120$  - Initial N = 16, Time of Challenge N = 9, End N = 15;  $S_{obs} > 120$  - Initial N = 0, Time of  
635 Challenge N = 6, End N = 1.

636

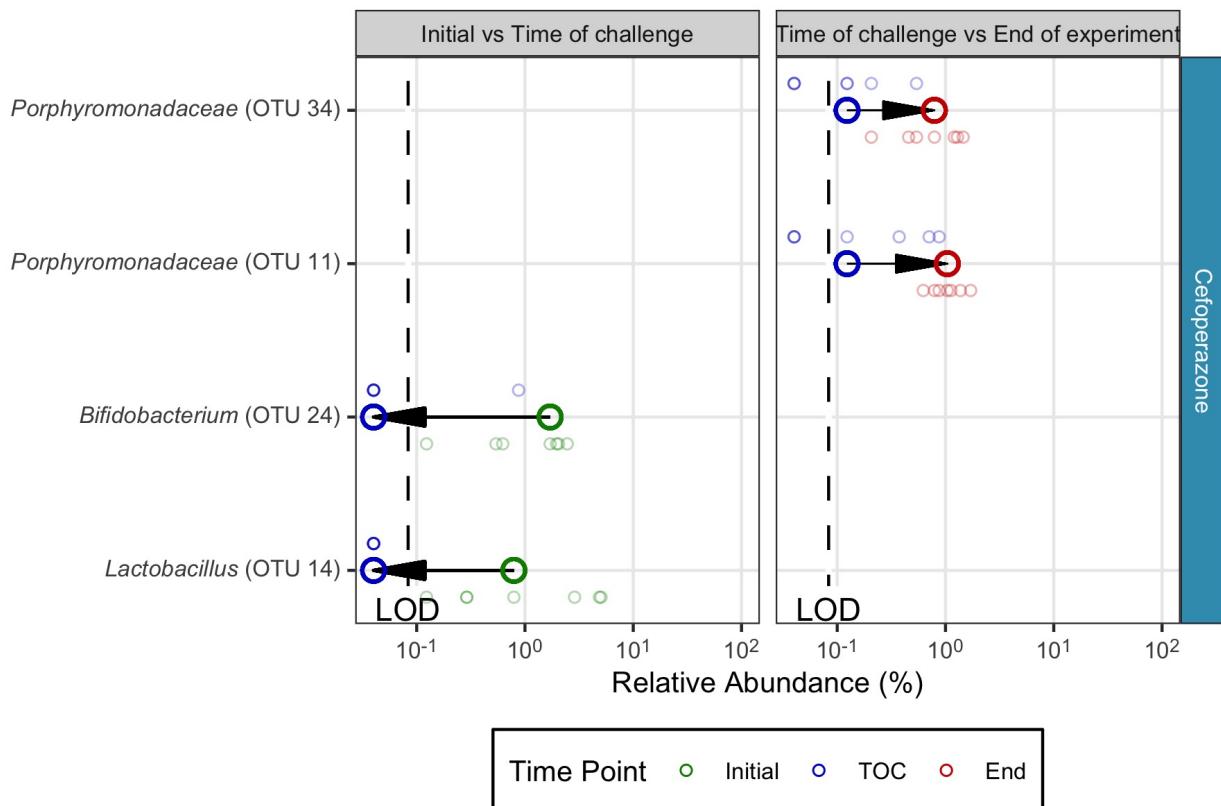
637



639 **Figure S3. Alpha diversity of communities from cefoperazone-treated mice that remained**  
 640 **colonized with *C. difficile* was not different by antibiotic dosage.**  $S_{obs}$  and inverse simpson  
 641 were plotted by the time point, *C. difficile* colonization outcome, and cefoperazone dosage and  
 642 tested by Wilcoxon rank sum test with Benjamini-Hochberg correction for differences. The group  
 643 with the largest difference, at the time of challenge for mice that remained colonized, was not  
 644 significant ( $P = 0.1142857$ ). Mice that remained colonized are represented with filled points and  
 645 those that cleared are unfilled. Points are shaped by cefoperazone dosage - circle 0.1 mg/mL,  
 646 triangle 0.3 mg/mL, 0.5 mg/mL.

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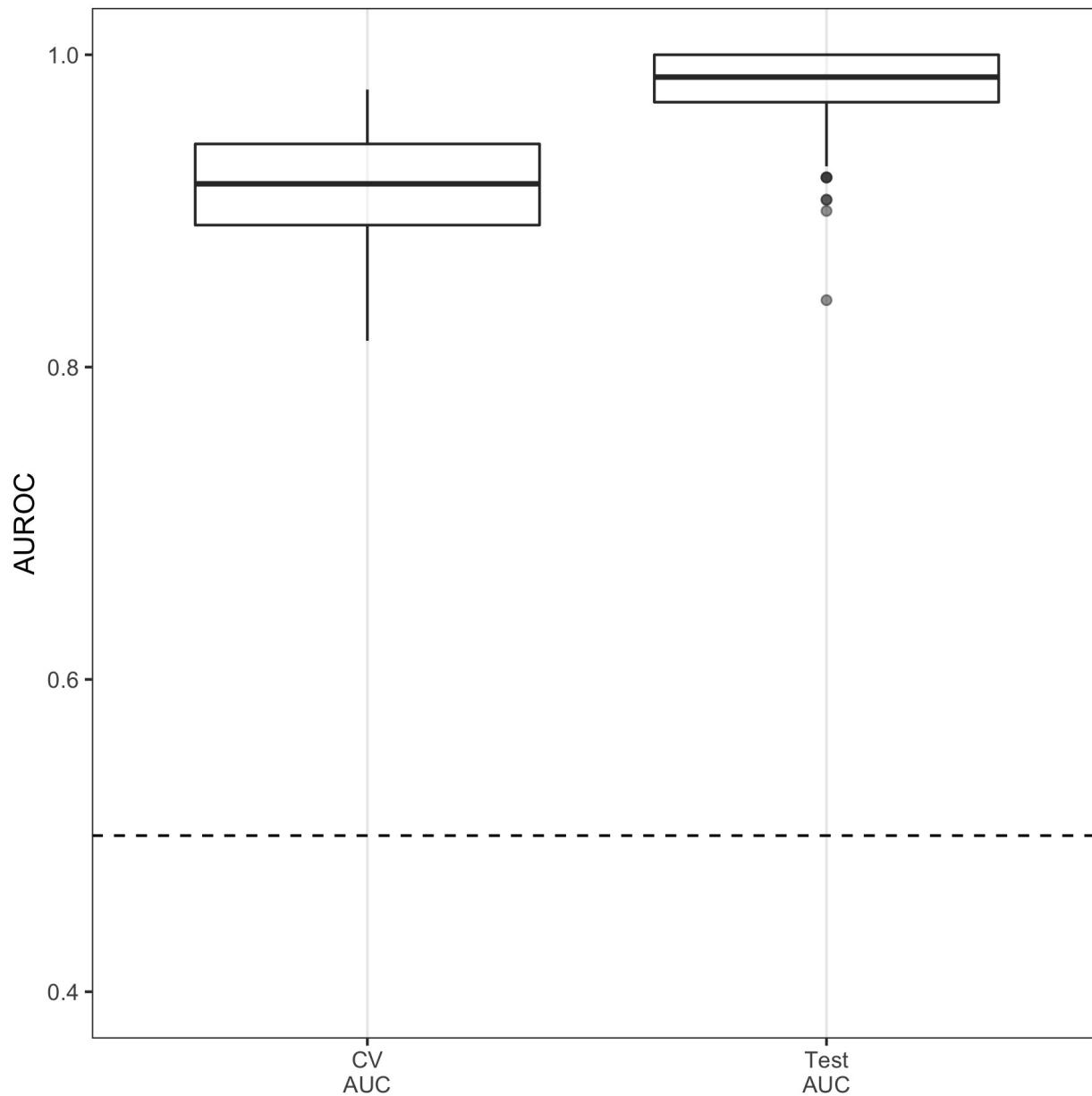
649

650 **Figure S4. Temporally differing OTU for cefoperazone-treated mice that cleared *C. difficile***  
 651 **colonization.** Bold points are median relative abundance and transparent points are relative  
 652 abundance of individual mice. Lines connect points within each comparison to show difference  
 653 in medians. Arrows point in the direction of the temporal change of the relative abundance. Only

654 OTUs at time points with statistically significant differences,  $P < 0.05$ , were plotted (calculated by  
655 Wilcoxon rank sum test with Benjamini-Hochberg correction). Limit of detection (LOD).

656

657



658

659 **Figure S5. Bacterial community at the time of infection can classify endpoint colonization.**  
660 Classification performance of L2 logistic regression. Area under the receiver-operator curve for  
661 classifying if the community will remain colonized based on the OTUs present at the time of C.

662 *difficile* infection (Day 0). Cross-validation of model performed on half of the data to tune model  
663 (CV AUC) and then tuned model was tested on the held-out data (Test AUC).