

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

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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 the changes 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 mice treated with other antibiotics would show the
11 same result. We found reduced doses of cefoperazone and streptomycin permitted colonization
12 and then clearance of *C. difficile*. In mice able to clear colonization, we found each antibiotic
13 caused specific community changes and resulted in different predicted interactions with *C. difficile*.
14 Clindamycin treatment led to a bloom in populations related to *Enterobacteriaceae*. Clearance of
15 *C. difficile* was concurrent with the reduction of those blooming populations and the restoration
16 of community members related to the *Porphyromonadaceae* and *Bacteroides*. Cefoperazone
17 created a susceptible community characterized by a drastic reduction in the community diversity,
18 interactions, and a sustained increase in abundance of many facultative anaerobes, including
19 *Enterobacteriaceae*. Lastly, clearance in streptomycin-treated mice was associated with the
20 recovery of multiple members of the *Porphyromonadaceae*, with little overlap in the specific
21 *Porphyromonadaceae* observed in the clindamycin treatment. Further elucidation of how *C. difficile*
22 colonization is cleared from different gut bacterial communities will improve *C. difficile* infection
23 treatments.

²⁴ **Importance**

²⁵ The community of microorganisms, known as the microbiota, in our intestines prevents pathogens,
²⁶ such as *C. difficile*, from establishing themselves and causing infection. This is known as
²⁷ colonization resistance. However, when a person takes antibiotics, their gut microbiota is disturbed.
²⁸ This disruption allows *C. difficile* to colonize. *C. difficile* infections (CDI) are primarily treated with
²⁹ antibiotics, which frequently leads to recurrent infections because the microbiota have not yet
³⁰ returned to a resistant state. The infection cycle often ends when the fecal microbiota from a
³¹ presumed resistant person are transplanted into the susceptible person. Although this treatment
³² is highly effective, we do not understand the mechanism of resistance. We hope to improve the
³³ treatment of CDI through elucidating how the bacterial community eliminates *C. difficile* colonization.
³⁴ We found *C. difficile* was able to colonize susceptible mice but was spontaneously eliminated
³⁵ in an antibiotic-treatment specific manner. These data indicate each community had different
³⁶ requirements for clearing colonization. Understanding how different communities clear colonization
³⁷ will reveal targets to improve CDI treatments.

38 **Introduction**

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

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

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

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

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

72 Results

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

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

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

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

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

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

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

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

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

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

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

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

220 Discussion

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

235 specific changes within each community to clear *C. difficile*.

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

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

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

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

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

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

305 **Materials and Methods**

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

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

314 ***C. difficile* challenge.** Mice were returned to untreated drinking water for 24 hours before
315 challenging with *C. difficile* strain 630Δerm spores. *C. difficile* spores were aliquoted from a
316 single spore stock stored at 4°C. Spore concentration was determined one week prior to the day of
317 challenge (37). 10³ *C. difficile* spores were orally gavaged into each mouse. Once the gavages
318 were completed, the remaining spore solution was serially diluted and plated to confirm the spore

319 concentration that was delivered.

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

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

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

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

347 (v6.0-85) and previously developed machine learning pipeline (44). Conditional independence
348 networks were calculated 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).

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.

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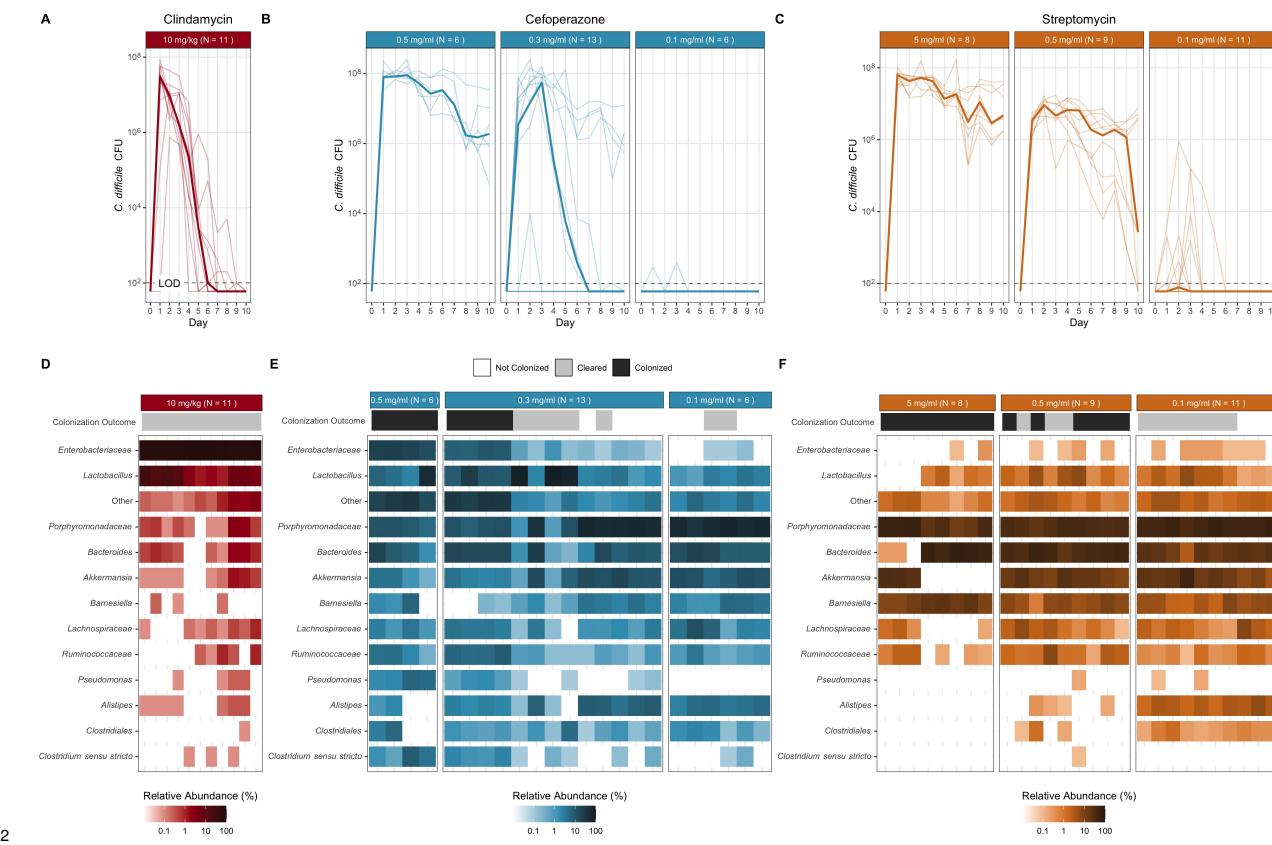
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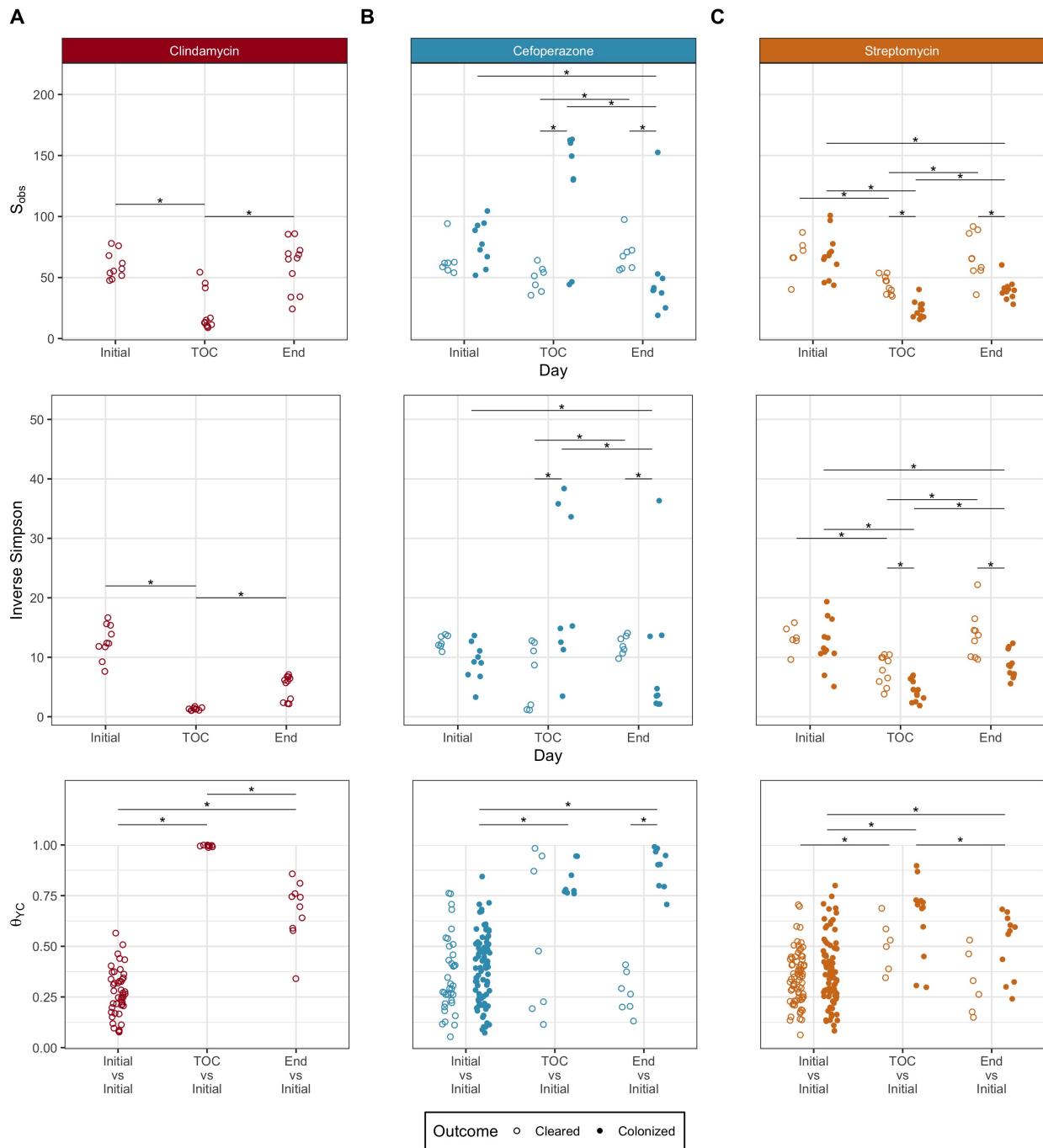
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512 **Figure 1. Reduced antibiotic doses permitted murine communities to be colonized and**
513 spontaneously clear that *C. difficile* colonization (A-C) Daily CFU of *C. difficile* in fecal samples
514 of mice treated with clindamycin, cefoperazone,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

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

526 ***C. difficile* colonization clearance** For clindamycin (A), cefoperazone (B), and streptomycin (C),

527 microbiota α -diversity (S_{obs} and Inverse Simpson) and β -diversity (θ_{YC}) were compared at the

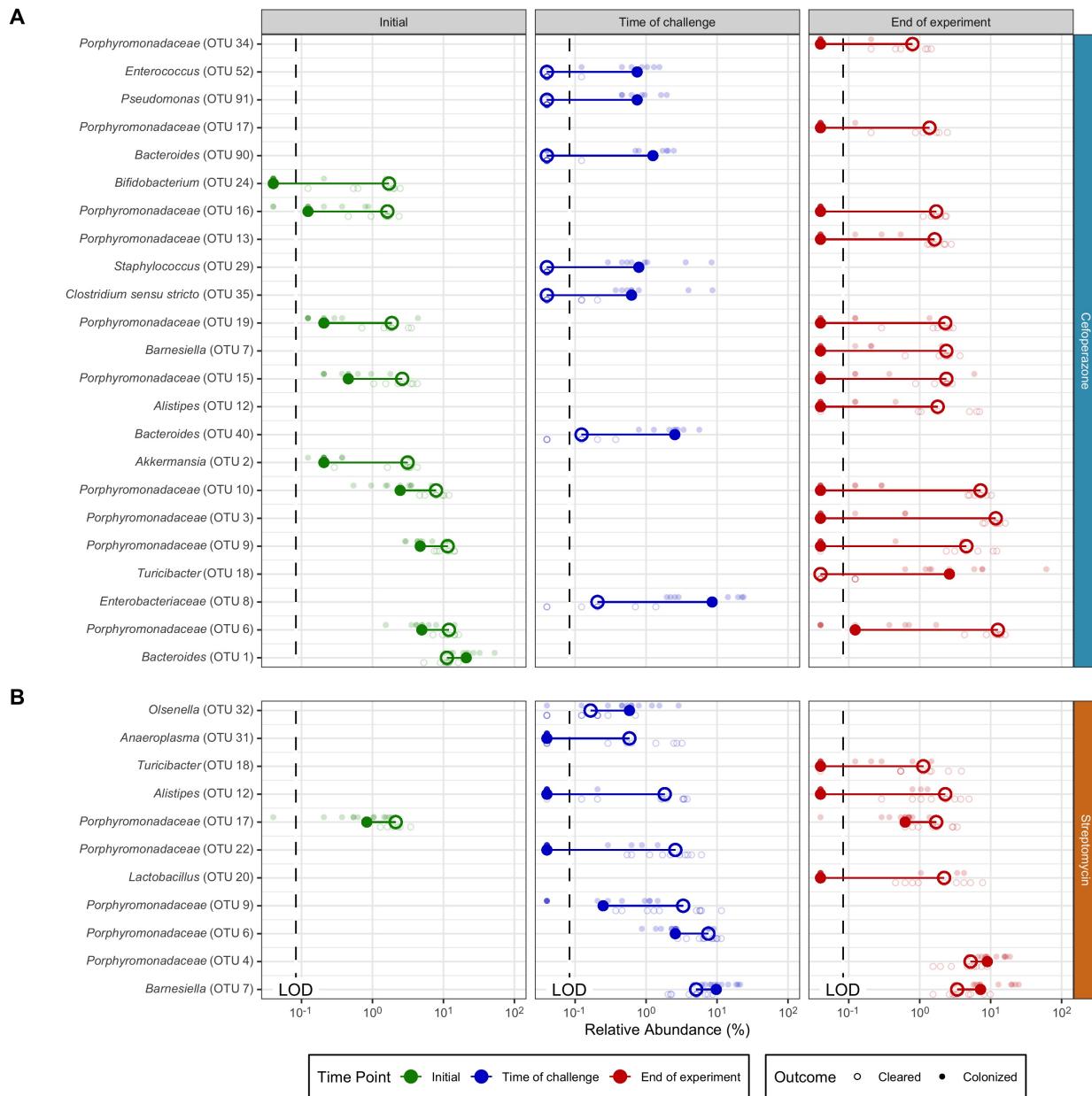
528 initial pre-antibiotic treatment state, time of *C. difficile* challenge (TOC), and end of the experiment.

529 β -diversity (θ_{YC}) was compared between the initial pre-antibiotic treatment state relative and all other

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

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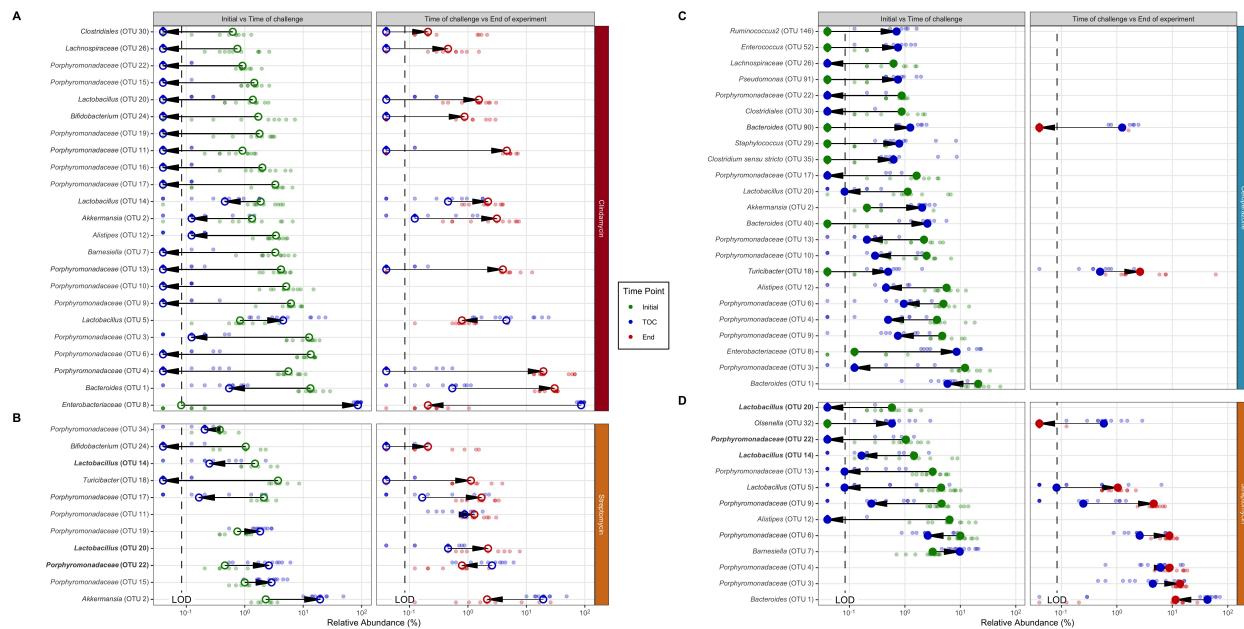


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538 **Figure 3. OTU abundance differences between communities that cleared *C. difficile***
 539 **colonization and remained colonized are unique to each treatment** For cefoperazone (A)
 540 and streptomycin (B), the difference in the relative abundance of OTUs that were significantly
 541 different between communities that eliminated *C. difficile* colonization and those that remained
 542 colonized within each antibiotic treatment for each time point. Lines connect points by OTU
 543 within each comparison to show difference in medians. Only plotted OTUs at time points which
 544 differences were statistically significant, $P < 0.05$, and calculated by Wilcoxon rank sum test with
 545 Benjamini-Hochberg correction. Bold points are median relative abundance and transparent points
 546 are relative abundance of individual mice. Limit of detection (LOD).

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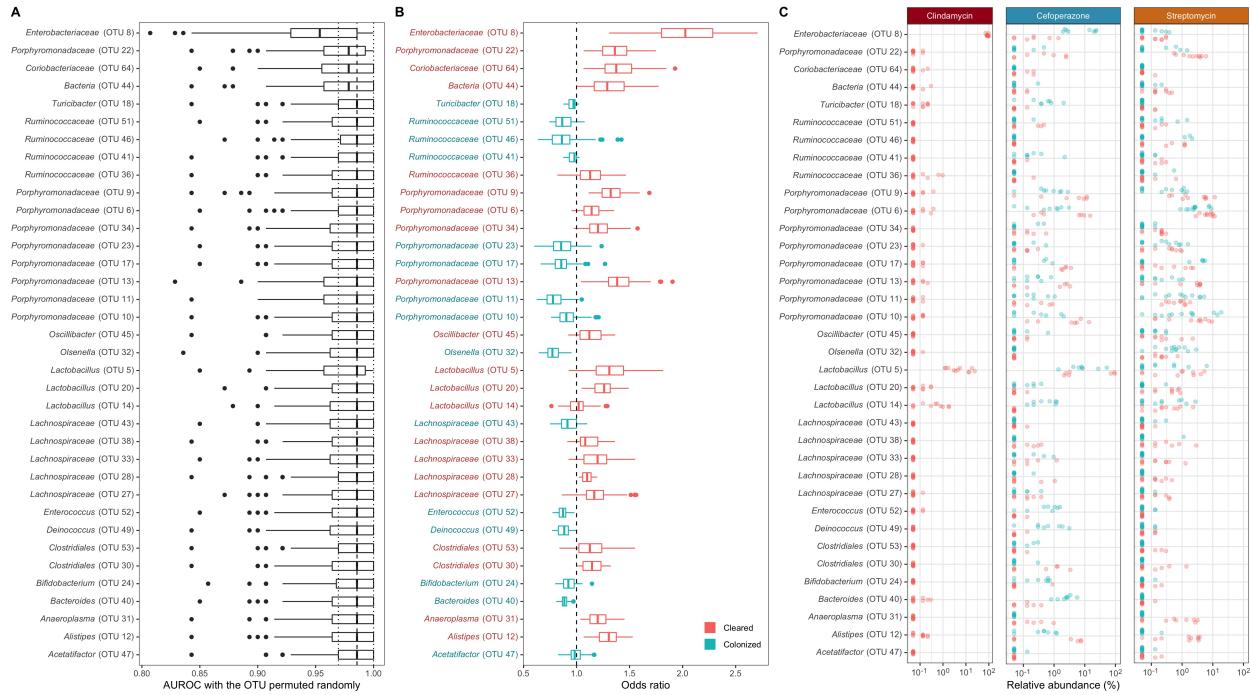
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550 **Figure 4. Each antibiotic had specific sets of temporal changes in OTU abundance**
 551 **associated with *C. difficile* colonization and clearance** For clindamycin (A), cefoperazone (C),
 552 and streptomycin (B, D), the difference in the relative abundance of OTUs that were significantly
 553 different between time points within each *C. difficile* colonization outcome for each antibiotic
 554 treatment. Arrows point in the direction of the temporal change of the relative abundance. Lines
 555 connect points by OTU within each comparison to show difference in medians. Bold OTUs were

556 shared across comparisons. Only plotted OTUs at time points which differences were statistically
 557 significant, $P < 0.05$, and calculated by Wilcoxon rank sum test with Benjamini-Hochberg correction.
 558 Bold points are median relative abundance and transparent points are relative abundance of
 559 individual mice. Limit of detection (LOD).

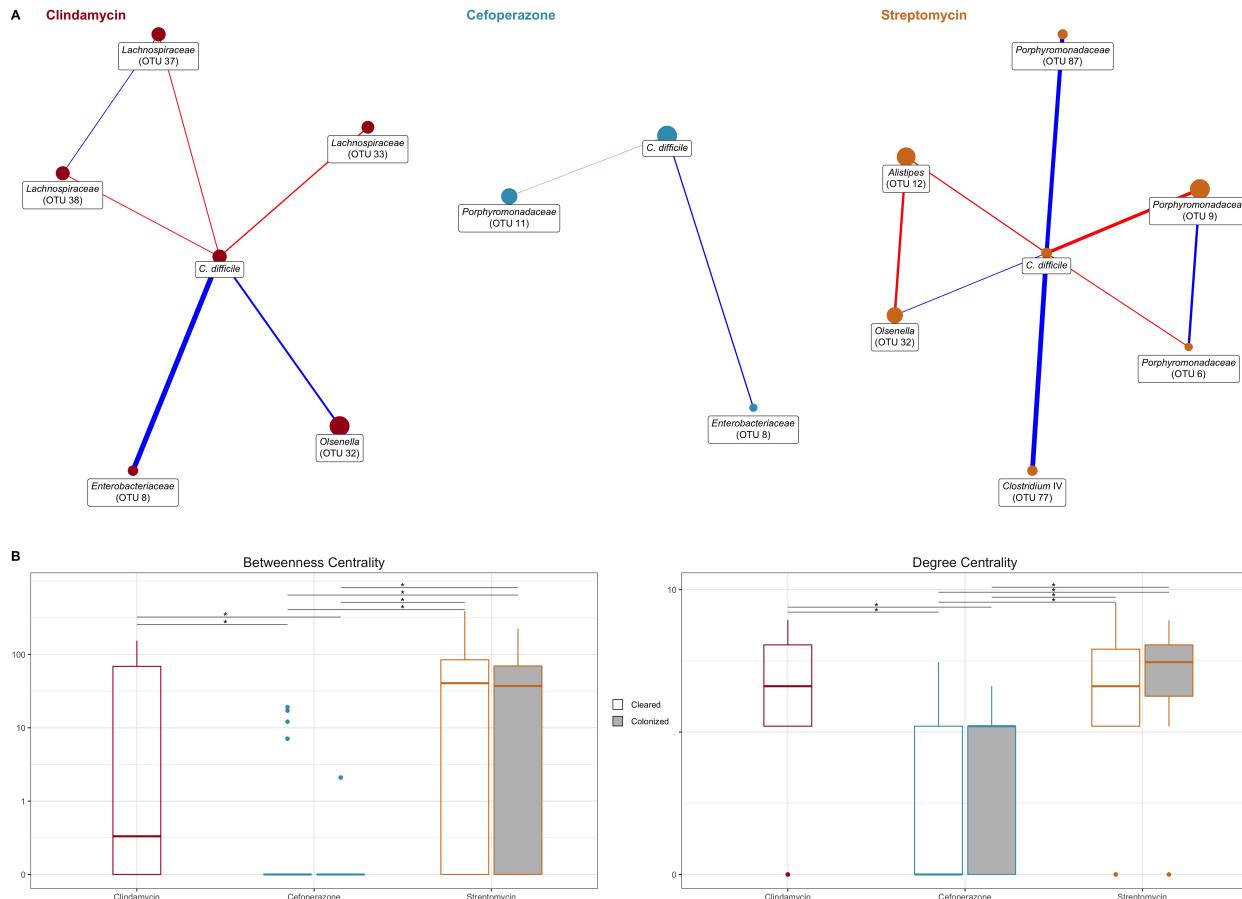
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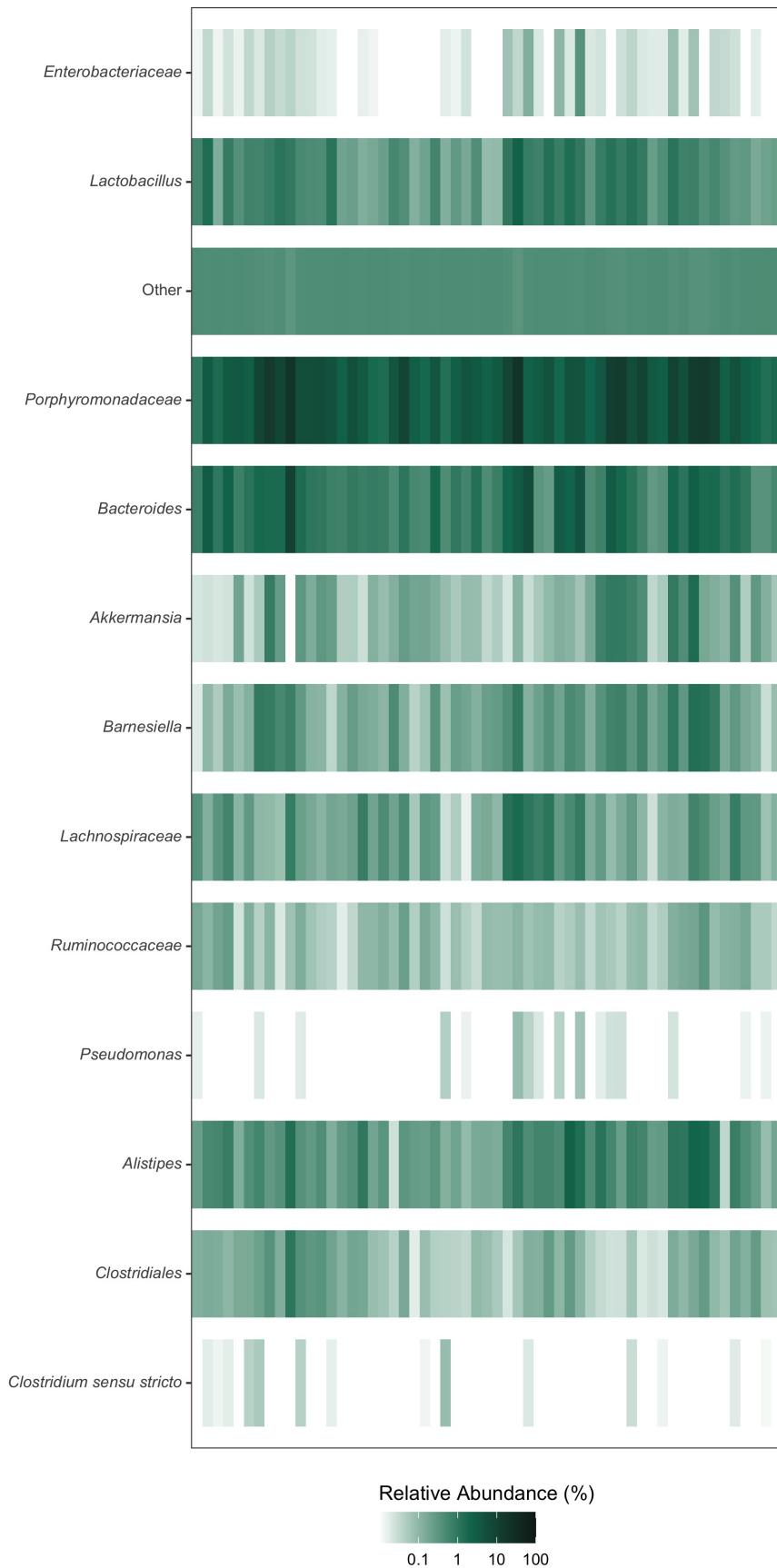
563 **Figure 5. Distinct features of the bacterial community at the time of infection can classify**
 564 **end point colonization** (A) L2 logistic regression model feature importances determined by the
 565 negative effect randomizing an individual feature has on the model performance. All OTUs affecting
 566 performance shown. Gray column shows performance range of final model with all features
 567 included. (B) Distribution of odds ratio used in L2 logistic regression model. Values above 1
 568 indicated abundance predicted the community cleared colonization (red) and values below 1
 569 indicated abundance predicted *C. difficile* remained colonized (blue). Feature label and boxplot
 570 colored to match the median odds ratio. (C) Relative abundance difference in features used by L2
 571 logistic regression model faceted by antibiotic treatment.



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

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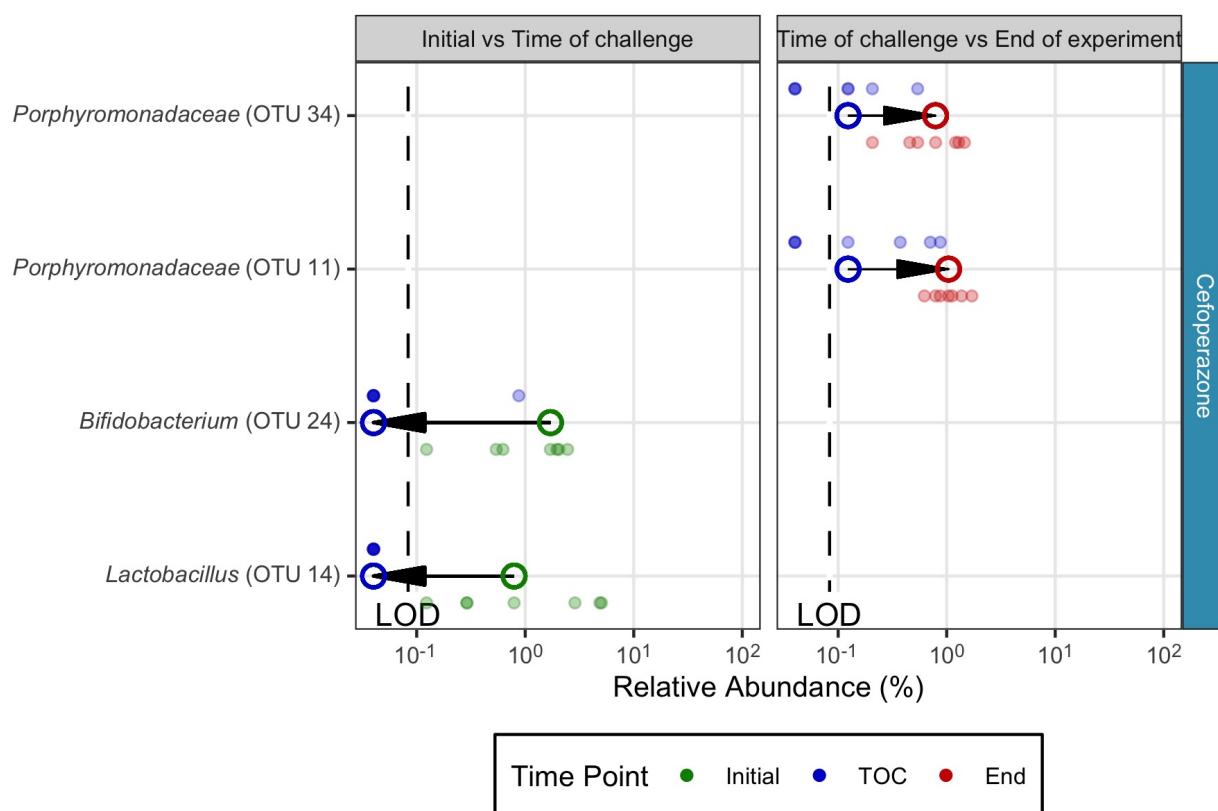
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588 **Figure S1. Initial microbiota relative abundance of mice prior to antibiotic treatment** Relative
 589 abundance at the beginning of the experiment prior to antibiotic treatment of twelve most abundant
 590 genera post antibiotic treatment, all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

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596 **Figure S2. Temporally differing OTU for cefoperazone-treated mice that cleared *C. difficile***
 597 **colonization** Arrow shows the change over time. Only plotted OTUs at time points which
 598 differences were statistically significant, $P < 0.05$, and calculated by Wilcoxon rank sum test
 599 with Benjamini-Hochberg correction.

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