

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
67 induce a less severe perturbation, we reduced the doses of cefoperazone and streptomycin. This
68 resulted in communities that were initially colonized to a high level ($>10^6$ CFU/g feces) and then
69 spontaneously cleared *C. difficile*. We found each antibiotic resulted in unique changes in the
70 microbiota that were associated with the persistence or clearance of *C. difficile*. These data further
71 support the hypothesis that *C. 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 temporary opportunity characterized by the transient dominance
252 of 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 identify 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). For each antibiotic treatment,
348 conditional independence networks were calculated from the day 1 through 10 samples of all
349 mice initially colonized using SPIEC-EASI (sparse inverse covariance estimation for ecological
350 association inference) methods from the SpiecEasi R package after optimizing lambda to 0.001
351 with a network stability between 0.045 and 0.05 (v1.0.7) (21). Network centrality measures degree
352 and betweenness were calculated on whole networks using functions from the igraph R package
353 (v1.2.4.1).

354 **Code availability.** Scripts necessary to reproduce our analysis and this paper are available in an
355 online repository (https://github.com/SchlossLab/Lesniak_Clearance_XXXX_2020).

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

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364 to publish, or preparation of the manuscript.

365 **References**

- 366 1. **Ducarmon QR, Zwittink RD, Hornung BVH, Schaik W van, Young VB, Kuijper EJ.** 2019.
367 Gut microbiota and colonization resistance against bacterial enteric infection. *Microbiology and*
368 *Molecular Biology Reviews* **83**. doi:10.1128/mmbr.00007-19.
- 369 2. **Britton RA, Young VB.** 2012. Interaction between the intestinal microbiota and host
370 in clostridium difficile colonization resistance. *Trends in Microbiology* **20**:313–319.
371 doi:10.1016/j.tim.2012.04.001.
- 372 3. **Lessa FC, Mu Y, Bamberg WM, Beldavs ZG, Dumyati GK, Dunn JR, Farley MM, Holzbauer
373 SM, Meek JI, Phipps EC, Wilson LE, Winston LG, Cohen JA, Limbago BM, Fridkin SK,
374 Gerding DN, McDonald LC.** 2015. Burden of Clostridium difficile Infection in the united states.
375 *New England Journal of Medicine* **372**:825–834. doi:10.1056/nejmoa1408913.
- 376 4. **Zimlichman E, Henderson D, Tamir O, Franz C, Song P, Yamin CK, Keohane C, Denham
377 CR, Bates DW.** 2013. Health careAssociated infections. *JAMA Internal Medicine* **173**:2039.
378 doi:10.1001/jamainternmed.2013.9763.
- 379 5. **Spigaglia P, Barbanti F, Morandi M, Moro ML, Mastrantonio P.** 2016. Diagnostic
380 testing for clostridium difficile in italian microbiological laboratories. *Anaerobe* **37**:29–33.
381 doi:10.1016/j.anaerobe.2015.11.002.
- 382 6. **Dieterle MG, Rao K, Young VB.** 2018. Novel therapies and preventative strategies for primary
383 and recurrent Clostridium difficile infections. *Annals of the New York Academy of Sciences*
384 **1435**:110–138. doi:10.1111/nyas.13958.
- 385 7. **Juul FE, Garborg K, Brethauer M, Skudal H, Øines MN, Wiig H, Rose, Seip B, Lamont JT,
386 Midtvedt T, Valeur J, Kalager M, Holme, Helsing L, Løberg M, Adami H-O.** 2018. Fecal
387 microbiota transplantation for primary clostridium difficile infection. *New England Journal of*
388 *Medicine* **378**:2535–2536. doi:10.1056/nejmc1803103.

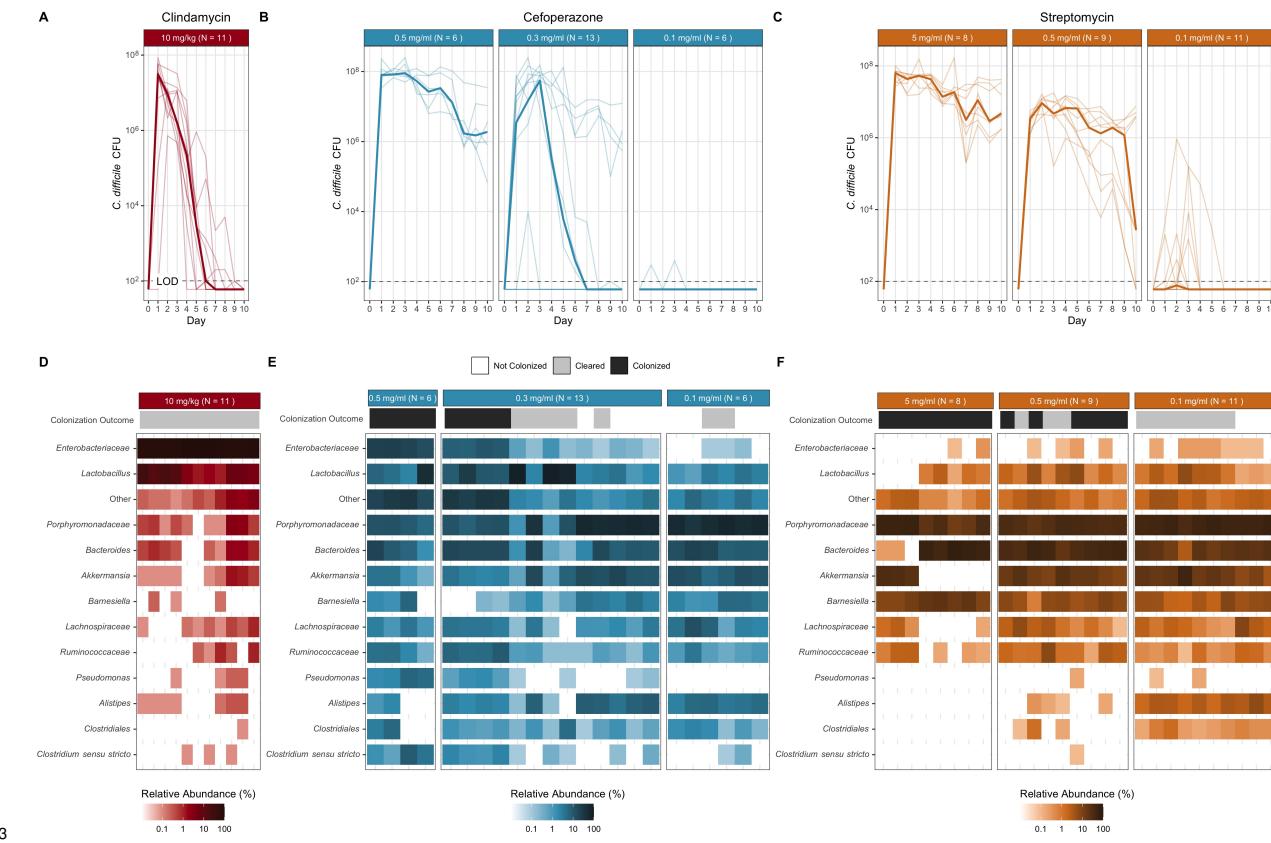
- 389 8. **Seekatz AM, Aas J, Gessert CE, Rubin TA, Saman DM, Bakken JS, Young VB.** 2014.
390 Recovery of the gut microbiome following fecal microbiota transplantation. *mBio* **5**.
391 doi:10.1128/mbio.00893-14.
- 392 9. **Patron RL, Hartmann CA, Allen S, Griesbach CL, Kosiorek HE, DiBaise JK, Orenstein
393 R.** 2017. Vancomycin taper and risk of failure of fecal microbiota transplantation in patients
394 with recurrent clostridium difficile infection. *Clinical Infectious Diseases* **65**:1214–1217.
395 doi:10.1093/cid/cix511.
- 396 10. **DeFilipp Z, Bloom PP, Soto MT, Mansour MK, Sater MRA, Huntley MH, Turbett
397 S, Chung RT, Chen Y-B, Hohmann EL.** 2019. Drug-resistant *e. Coli* bacteremia
398 transmitted by fecal microbiota transplant. *New England Journal of Medicine* **381**:2043–2050.
399 doi:10.1056/nejmoa1910437.
- 400 11. **Buffie CG, Bucci V, Stein RR, McKenney PT, Ling L, Gobourne A, No D, Liu H, Kinnebrew
401 M, Viale A, Littmann E, Brink MRM van den, Jenq RR, Taur Y, Sander C, Cross JR,
402 Toussaint NC, Xavier JB, Pamer EG.** 2014. Precision microbiome reconstitution restores bile
403 acid mediated resistance to clostridium difficile. *Nature* **517**:205–208. doi:10.1038/nature13828.
- 404 12. **Fletcher JR, Erwin S, Lanzas C, Theriot CM.** 2018. Shifts in the gut metabolome and
405 clostridium difficile transcriptome throughout colonization and infection in a mouse model.
406 *mSphere* **3**. doi:10.1128/msphere.00089-18.
- 407 13. **Reed AD, Nethery MA, Stewart A, Barrangou R, Theriot CM.** 2020. Strain-dependent
408 inhibition of *clostridioides difficile* by commensal clostridia carrying the bile acid-inducible (*bai*)
409 operon. *Journal of Bacteriology* **202**. doi:10.1128/jb.00039-20.
- 410 14. **Jenior ML, Leslie JL, Young VB, Schloss PD.** 2017. Clostridium difficile colonizes
411 alternative nutrient niches during infection across distinct murine gut microbiomes. *mSystems* **2**.
412 doi:10.1128/msystems.00063-17.
- 413 15. **Lawley TD, Clare S, Walker AW, Stares MD, Connor TR, Raisen C, Goulding D, Rad R,
414 Schreiber F, Brandt C, Deakin LJ, Pickard DJ, Duncan SH, Flint HJ, Clark TG, Parkhill J,**

- 415 **Dougan G.** 2012. Targeted restoration of the intestinal microbiota with a simple, defined
416 bacteriotherapy resolves relapsing clostridium difficile disease in mice. PLoS Pathogens
417 **8**:e1002995. doi:10.1371/journal.ppat.1002995.
- 418 **16. McDonald JAK, Mullish BH, Pechlivanis A, Liu Z, Brignardello J, Kao D, Holmes E, Li**
419 **JV, Clarke TB, Thorsz MR, Marchesi JR.** 2018. Inhibiting growth of clostridioides difficile by
420 restoring valerate, produced by the intestinal microbiota. Gastroenterology **155**:1495–1507.e15.
421 doi:10.1053/j.gastro.2018.07.014.
- 422 **17. Ghimire S, Roy C, Wongkuna S, Antony L, Maji A, Keena MC, Foley A, Scaria J.** 2020.
423 Identification of clostridioides difficile-inhibiting gut commensals using culturomics, phenotyping,
424 and combinatorial community assembly. mSystems **5**. doi:10.1128/msystems.00620-19.
- 425 **18. Auchtung JM, Preisner EC, Collins J, Lerma AI, Britton RA.** 2020. Identification of simplified
426 microbial communities that inhibit clostridioides difficile infection through dilution/extinction.
427 mSphere **5**. doi:10.1128/msphere.00387-20.
- 428 **19. Schubert AM, Sinani H, Schloss PD.** 2015. Antibiotic-induced alterations of the murine gut
429 microbiota and subsequent effects on colonization resistance against clostridium difficile. mBio
430 **6**. doi:10.1128/mbio.00974-15.
- 431 **20. Jenior ML, Leslie JL, Young VB, Schloss PD.** 2018. Clostridium difficile alters the structure
432 and metabolism of distinct cecal microbiomes during initial infection to promote sustained
433 colonization. mSphere **3**. doi:10.1128/msphere.00261-18.
- 434 **21. Kurtz ZD, Müller CL, Miraldi ER, Littman DR, Blaser MJ, Bonneau RA.** 2015. Sparse and
435 compositionally robust inference of microbial ecological networks. PLOS Computational Biology
436 **11**:e1004226. doi:10.1371/journal.pcbi.1004226.
- 437 **22. Reeves AE, Theriot CM, Bergin IL, Huffnagle GB, Schloss PD, Young VB.** 2011.
438 The interplay between microbiome dynamics and pathogen dynamics in a murine model
439 ofClostridium difficileInfection. Gut Microbes **2**:145–158. doi:10.4161/gmic.2.3.16333.

- 440 23. **Reeves AE, Koenigsknecht MJ, Bergin IL, Young VB.** 2012. Suppression of clostridium
441 difficile in the gastrointestinal tracts of germfree mice inoculated with a murine isolate from the
442 family lachnospiraceae. *Infection and Immunity* **80**:3786–3794. doi:10.1128/iai.00647-12.
- 443 24. **Tomkovich S, Stough JMA, Bishop L, Schloss PD.** 2020. The initial gut microbiota and
444 response to antibiotic perturbation influence clostridioides difficile clearance in mice. *mSphere*
445 **5**. doi:10.1128/msphere.00869-20.
- 446 25. **Peterfreund GL, Vandivier LE, Sinha R, Marozsan AJ, Olson WC, Zhu J, Bushman
447 FD.** 2012. Succession in the gut microbiome following antibiotic and antibody therapies for
448 clostridium difficile. *PLoS ONE* **7**:e46966. doi:10.1371/journal.pone.0046966.
- 449 26. **Fukuyama J, Rumker L, Sankaran K, Jeganathan P, Dethlefsen L, Relman
450 DA, Holmes SP.** 2017. Multidomain analyses of a longitudinal human microbiome
451 intestinal cleanout perturbation experiment. *PLOS Computational Biology* **13**:e1005706.
452 doi:10.1371/journal.pcbi.1005706.
- 453 27. **Suez J, Zmora N, Zilberman-Schapira G, Mor U, Dori-Bachash M, Bashiardes S, Zur
454 M, Regev-Lehavi D, Brik RB-Z, Federici S, Horn M, Cohen Y, Moor AE, Zeevi D, Korem
455 T, Kotler E, Harmelin A, Itzkovitz S, Maherak N, Shibolet O, Pevsner-Fischer M,
456 Shapiro H, Sharon I, Halpern Z, Segal E, Elinav E.** 2018. Post-antibiotic gut mucosal
457 microbiome reconstitution is impaired by probiotics and improved by autologous FMT. *Cell*
458 **174**:1406–1423.e16. doi:10.1016/j.cell.2018.08.047.
- 459 28. **Ianiro G, Maida M, Burisch J, Simonelli C, Hold G, Ventimiglia M, Gasbarrini A,
460 Cammarota G.** 2018. Efficacy of different faecal microbiota transplantation protocols for
461 clostridium difficile infection: A systematic review and meta-analysis. *United European
462 Gastroenterology Journal* **6**:1232–1244. doi:10.1177/2050640618780762.
- 463 29. **Allegretti JR, Mehta SR, Kassam Z, Kelly CR, Kao D, Xu H, Fischer M.** 2020. Risk factors
464 that predict the failure of multiple fecal microbiota transplants for clostridioides difficile
465 infection. *Digestive Diseases and Sciences*. doi:10.1007/s10620-020-06198-2.

- 466 30. **Garza-González E, Mendoza-Olazarán S, Morfin-Otero R, Ramírez-Fontes A,**
467 **Rodríguez-Zulueta P, Flores-Treviño S, Bocanegra-Ibarias P, Maldonado-Garza H,**
468 **Camacho-Ortiz A.** 2019. Intestinal microbiome changes in fecal microbiota transplant (FMT)
469 vs. FMT enriched with lactobacillus in the treatment of recurrent clostridioides difficile infection.
470 Canadian Journal of Gastroenterology and Hepatology **2019**:1–7. doi:10.1155/2019/4549298.
- 471 31. **Winter SE, Lopez CA, Bäumler AJ.** 2013. The dynamics of gut-associated microbial
472 communities during inflammation. EMBO reports **14**:319–327. doi:10.1038/embor.2013.27.
- 473 32. **Rivera-Chávez F, Lopez CA, Bäumler AJ.** 2017. Oxygen as a driver of gut dysbiosis. Free
474 Radical Biology and Medicine **105**:93–101. doi:10.1016/j.freeradbiomed.2016.09.022.
- 475 33. **Carlson PE, Walk ST, Bourgis AET, Liu MW, Kopliku F, Lo E, Young VB, Aronoff DM,**
476 **Hanna PC.** 2013. The relationship between phenotype, ribotype, and clinical disease in human
477 clostridium difficile isolates. Anaerobe **24**:109–116. doi:10.1016/j.anaerobe.2013.04.003.
- 478 34. **Thanissery R, Winston JA, Theriot CM.** 2017. Inhibition of spore germination, growth, and
479 toxin activity of clinically relevant c. difficile strains by gut microbiota derived secondary bile
480 acids. Anaerobe **45**:86–100. doi:10.1016/j.anaerobe.2017.03.004.
- 481 35. **Theriot CM, Koumpouras CC, Carlson PE, Bergin II, Aronoff DM, Young VB.** 2011.
482 Cefoperazone-treated mice as an experimental platform to assess differential virulence
483 of Clostridium difficile strains. Gut Microbes **2**:326–334. doi:10.4161/gmic.19142.
- 484 36. **Rao K, Micic D, Natarajan M, Winters S, Kiel MJ, Walk ST, Santhosh K, Mogle JA, Galecki
485 AT, LeBar W, Higgins PDR, Young VB, Aronoff DM.** 2015. Clostridium difficile Ribotype 027:
486 Relationship to age, detectability of toxins a or b in stool with rapid testing, severe infection, and
487 mortality. Clinical Infectious Diseases **61**:233–241. doi:10.1093/cid/civ254.
- 488 37. **Sorg JA, Dineen SS.** 2009. Laboratory maintenance of Clostridium difficile. Current Protocols
489 in Microbiology **12**. doi:10.1002/9780471729259.mc09a01s12.
- 490 38. **Winston JA, Thanissery R, Montgomery SA, Theriot CM.** 2016. Cefoperazone-treated
491 mouse model of clinically-relevant Δclostridium difficile strain r20291. Journal of Visualized

- 492 Experiments. doi:10.3791/54850.
- 493 39. **Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD.** 2013. Development
494 of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence
495 data on the MiSeq illumina sequencing platform. *Applied and Environmental Microbiology*
496 **79**:5112–5120. doi:10.1128/aem.01043-13.
- 497 40. **Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA,**
498 **Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Horn DJV, Weber**
499 **CF.** 2009. Introducing mothur: Open-source, platform-independent, community-supported
500 software for describing and comparing microbial communities. *Applied and Environmental*
501 *Microbiology* **75**:7537–7541. doi:10.1128/aem.01541-09.
- 502 41. **Wang Q, Garrity GM, Tiedje JM, Cole JR.** 2007. Naïve bayesian classifier for rapid assignment
503 of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology*
504 **73**:5261–5267. doi:10.1128/aem.00062-07.
- 505 42. **Yue JC, Clayton MK.** 2005. A similarity measure based on species proportions.
506 *Communications in Statistics - Theory and Methods* **34**:2123–2131. doi:10.1080/sta-200066418.
- 507 43. **Benjamini Y, Hochberg Y.** 1995. Controlling the false discovery rate: A practical and powerful
508 approach to multiple testing. *Journal of the Royal Statistical Society: Series B (Methodological)*
509 **57**:289–300. doi:10.1111/j.2517-6161.1995.tb02031.x.
- 510 44. **Topçuoğlu BD, Lesniak NA, Ruffin MT, Wiens J, Schloss PD.** 2020. A framework for
511 effective application of machine learning to microbiome-based classification problems. *mBio* **11**.
512 doi:10.1128/mbio.00434-20.

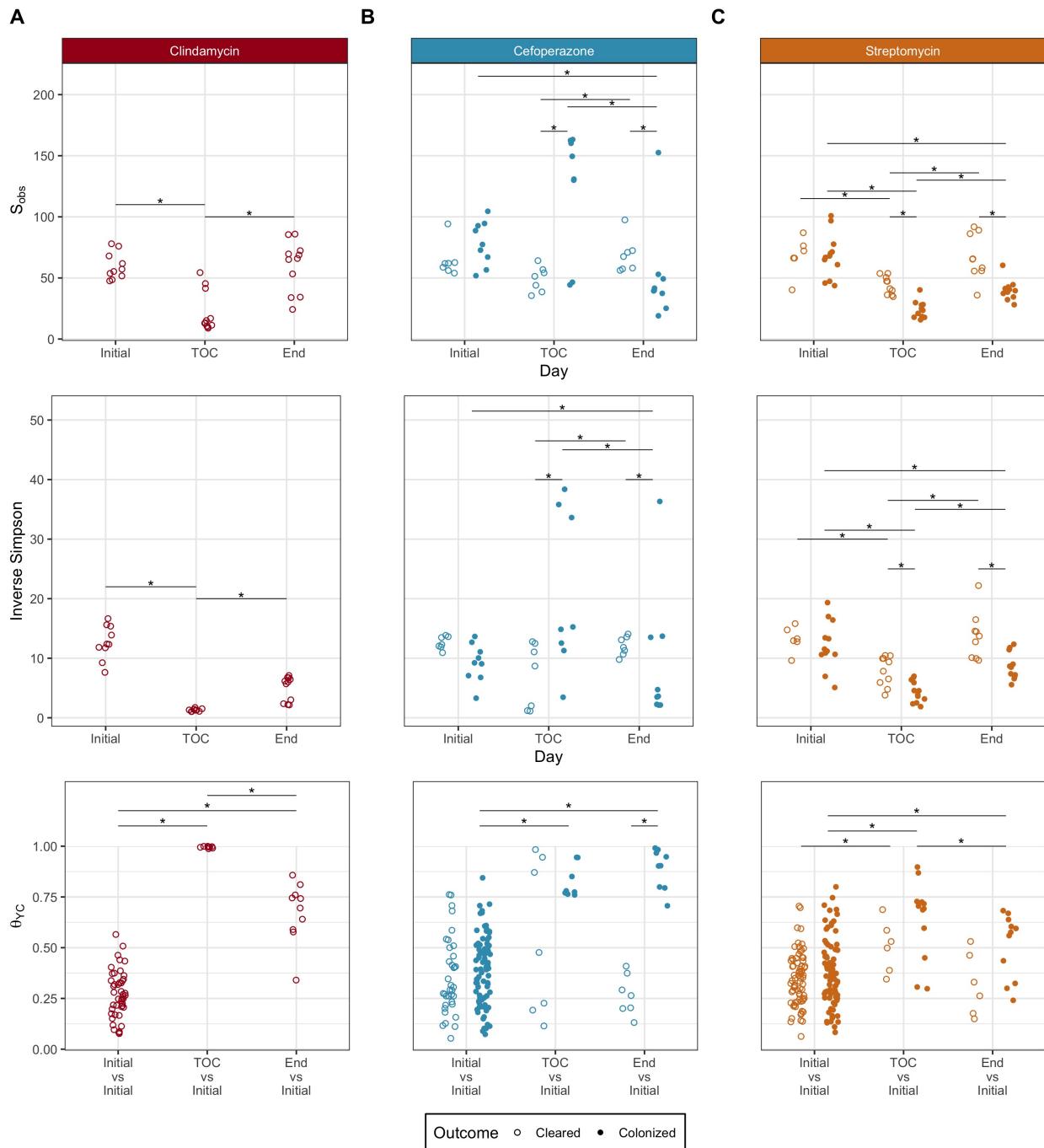


513

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

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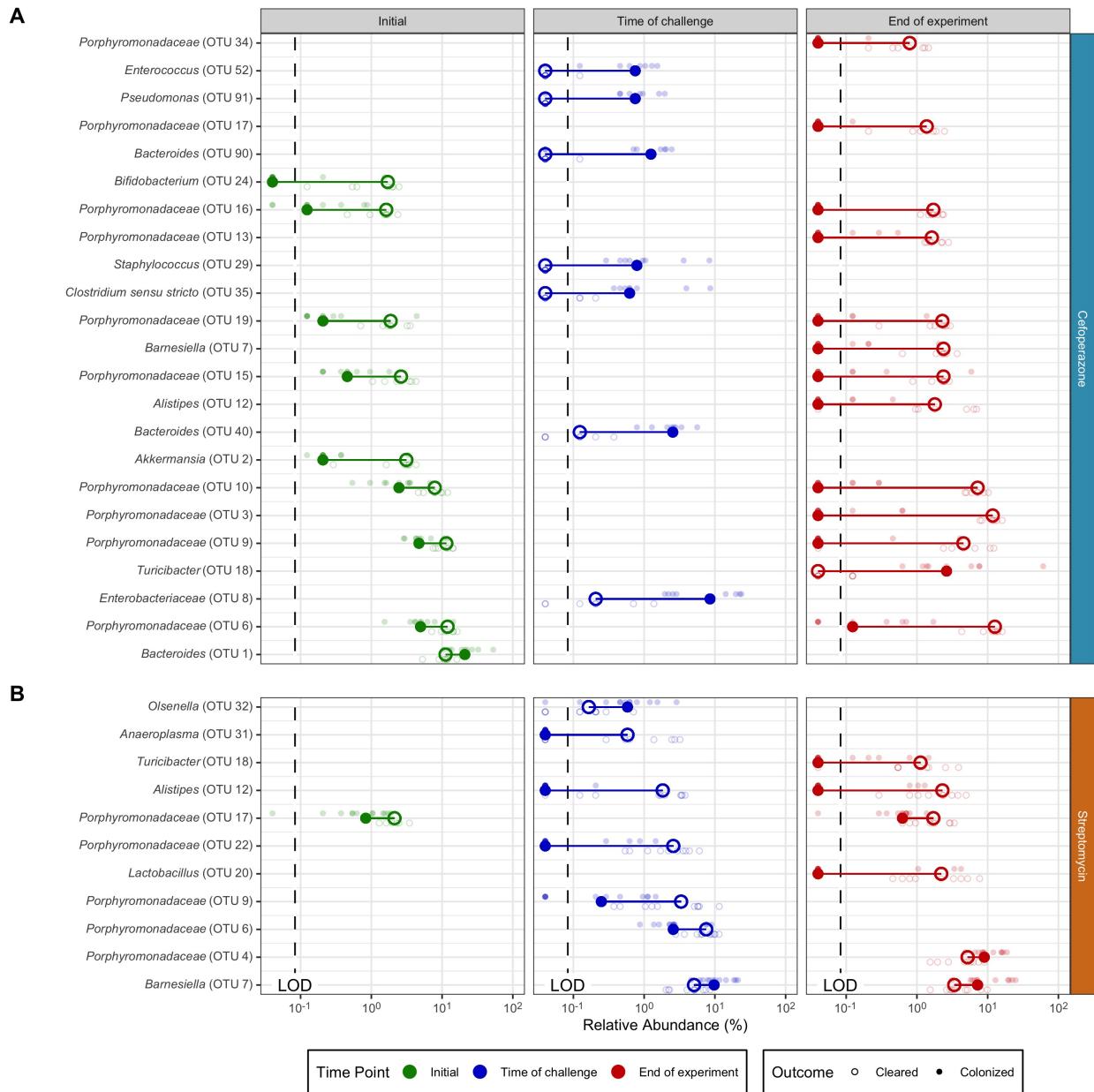
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526 **Figure 2. Microbiota community diversity showed antibiotic-specific trends associated with**
 527 ***C. difficile* colonization clearance.** For communities colonized with *C. difficile* from mice treated
 528 with clindamycin (A), cefoperazone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

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

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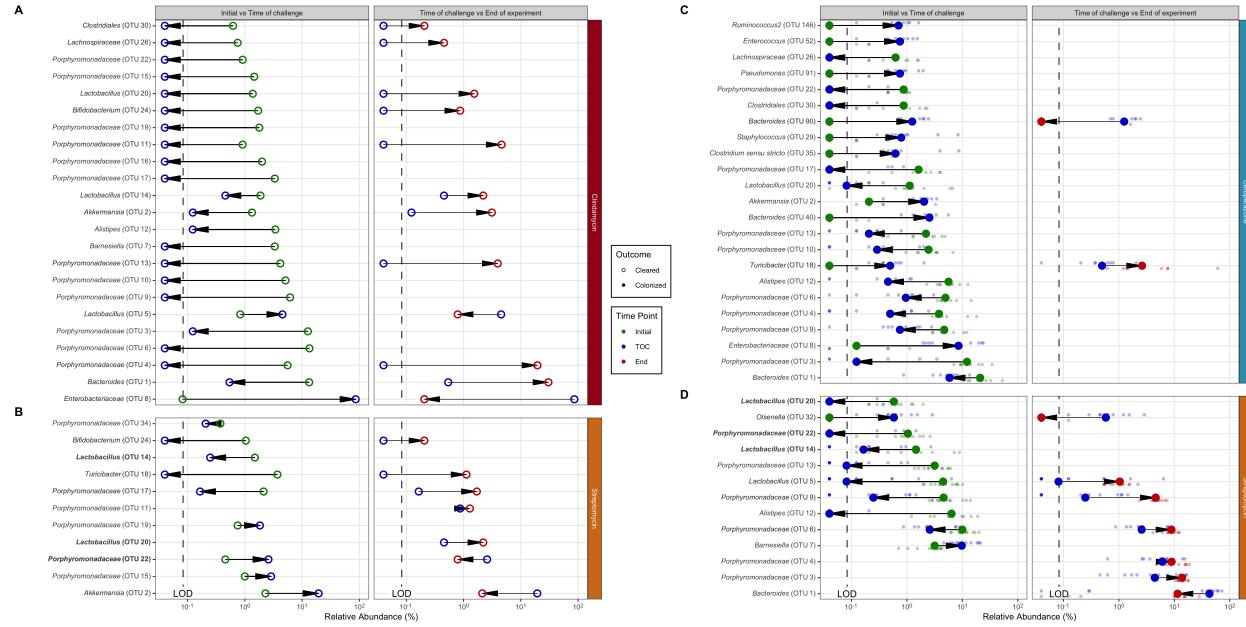
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540 **Figure 3. OTU abundance differences between communities that cleared *C. difficile***
541 **colonization and remained colonized are unique to each treatment.** For cefoperazone (A) and
542 streptomycin (B), the difference in the relative abundance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

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

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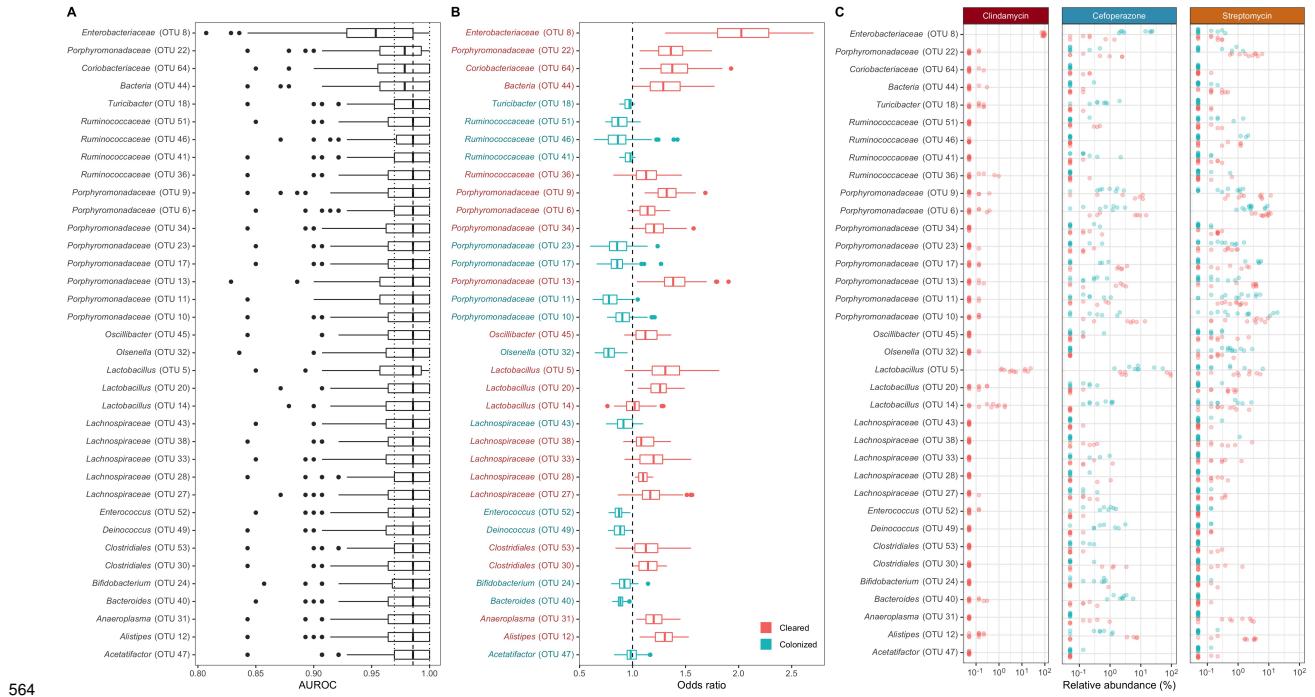


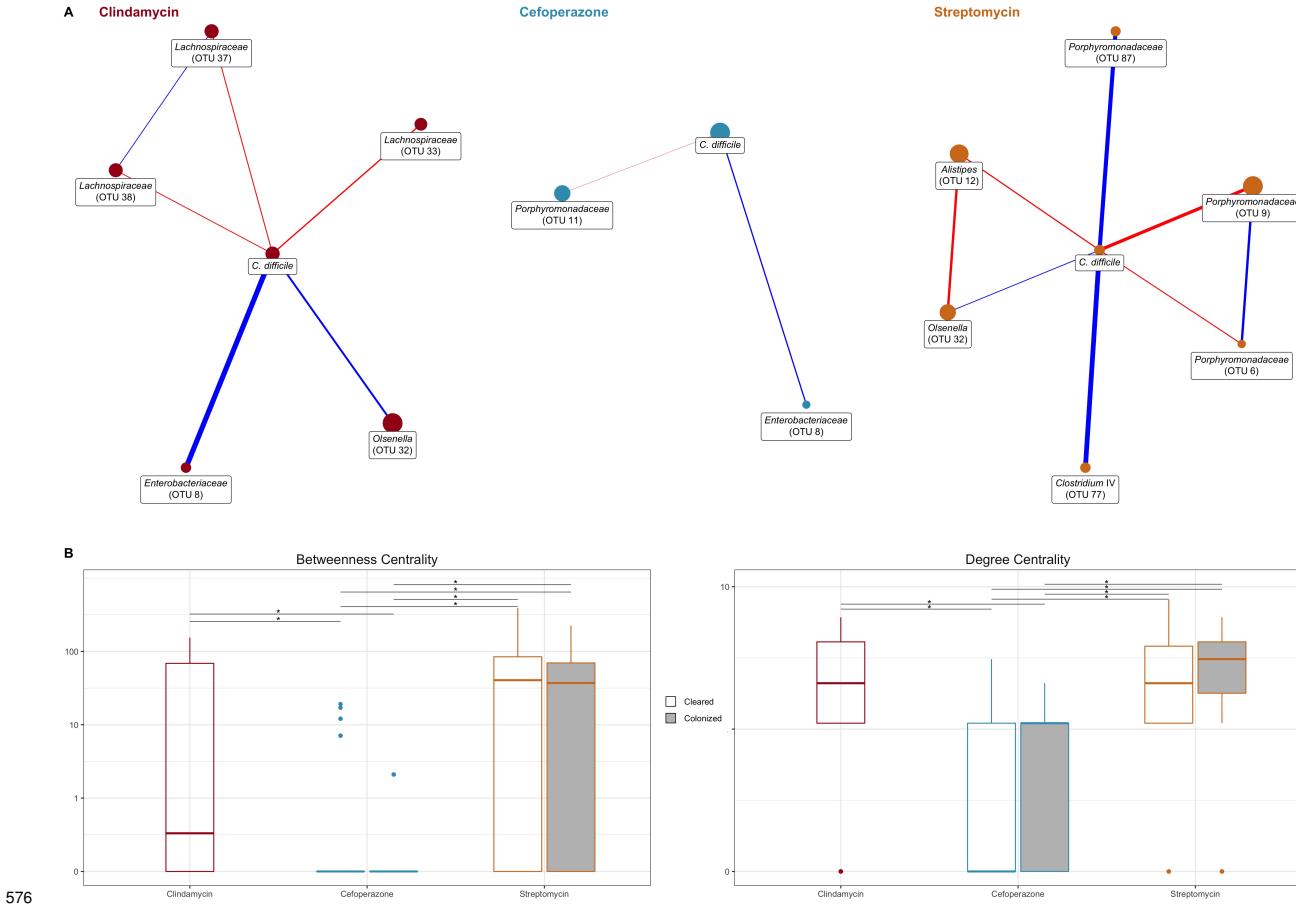
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552 **Figure 4. Each antibiotic had specific sets of temporal changes in OTU abundance**
 553 **associated with *C. difficile* colonization and clearance.** For clindamycin (A), cefoperazone (C),
 554 and streptomycin (B, D), the difference in the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

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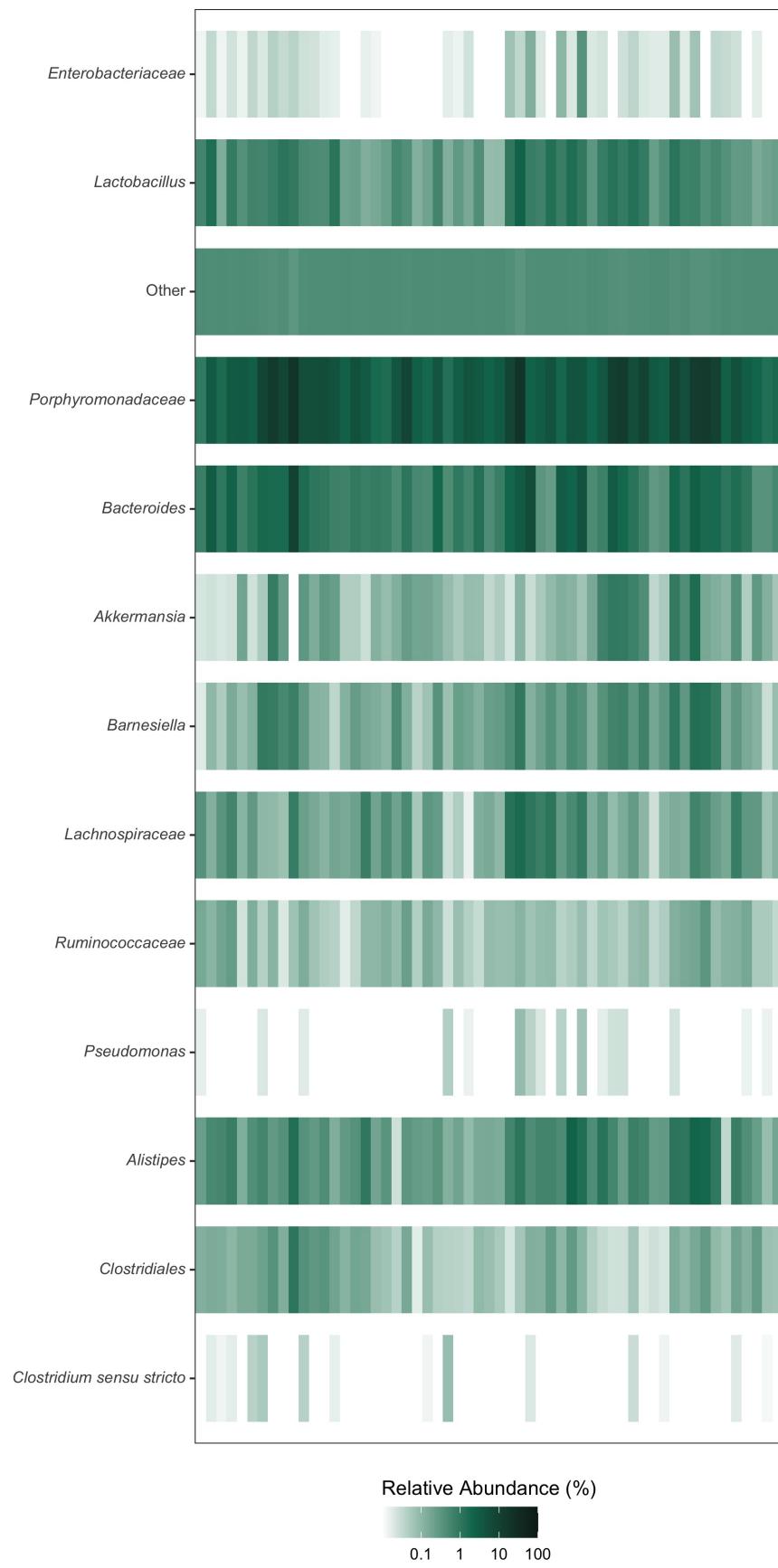




577 **Figure 6. Conditional independence networks reveal treatment-specific relationships**
578 **between the community and *C. difficile* during colonization clearance.** (A) SPIEC-EASI
579 (sparse inverse covariance estimation for ecological association inference) networks showing
580 conditionally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

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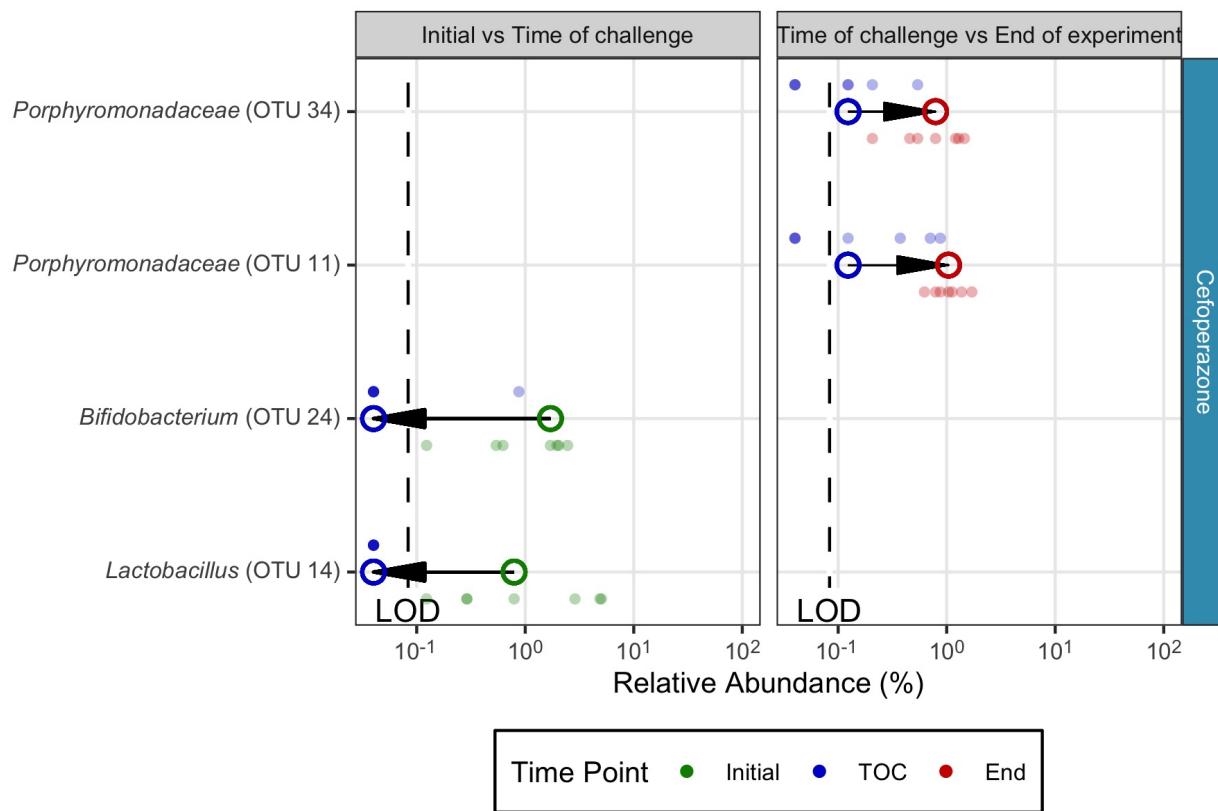


590 **Figure S1. Initial microbiota relative abundance of mice prior to antibiotic treatment.**

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

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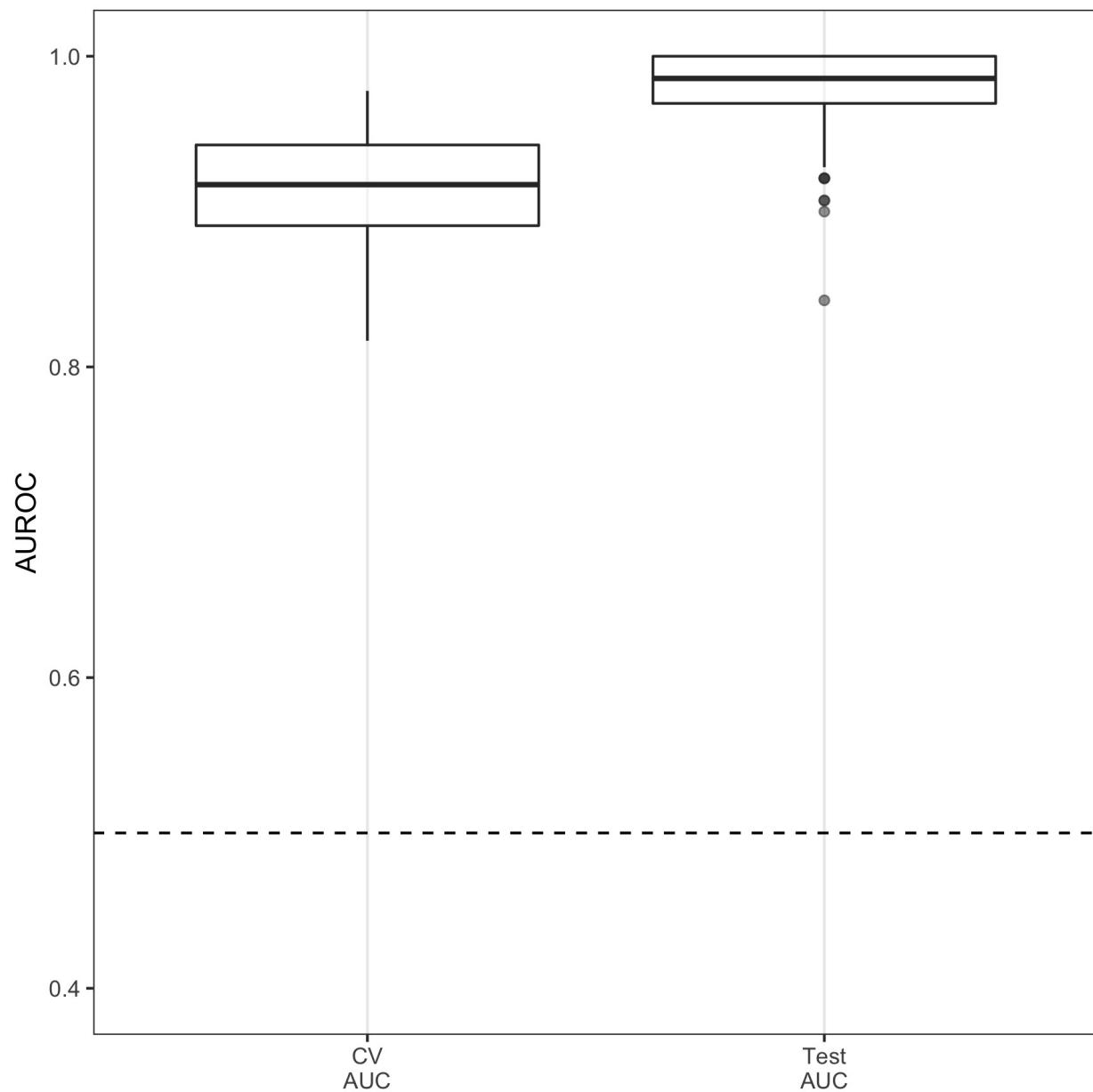
597

598 **Figure S2. Temporally differing OTU for cefoperazone-treated mice that cleared *C. difficile***

599 **colonization.** Bold points are median relative abundance and transparent points are relative
 600 abundance of individual mice. Lines connect points within each comparison to show difference
 601 in medians. Arrows point in the direction of the temporal change of the relative abundance. Only
 602 OTUs at time points with statistically significant differences, $P < 0.05$, were plotted (calculated by
 603 Wilcoxon rank sum test with Benjamini-Hochberg correction). Limit of detection (LOD).

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

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