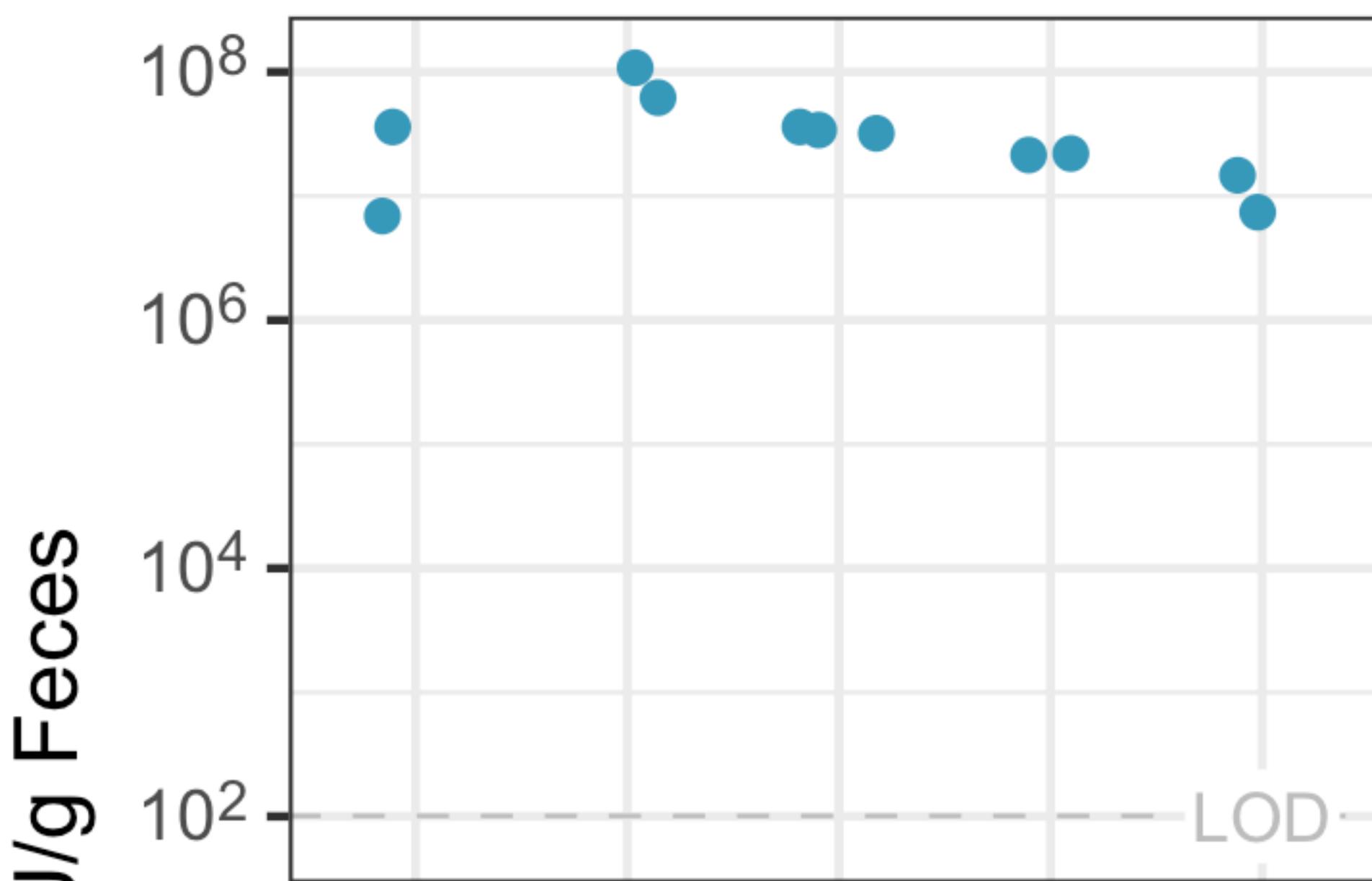
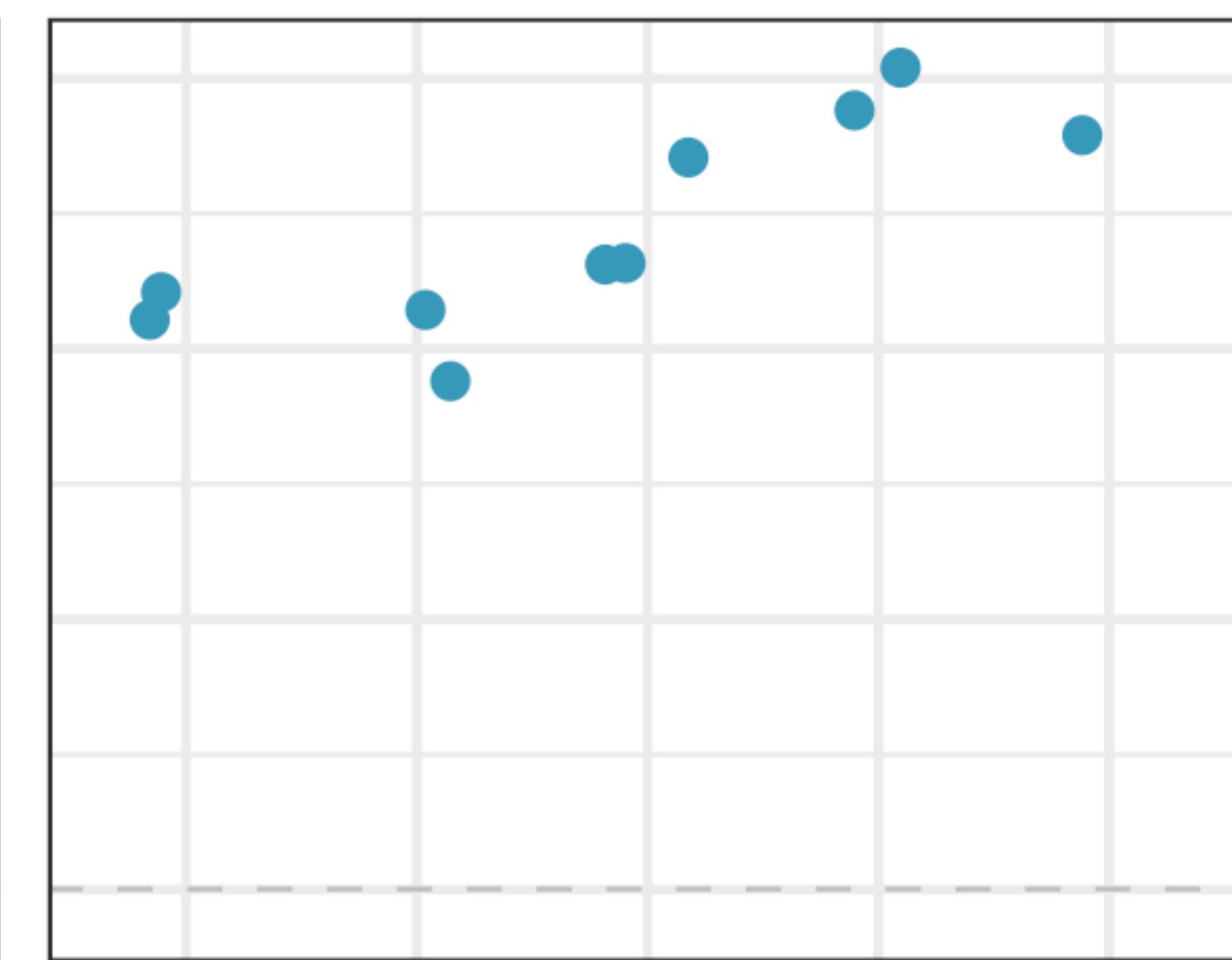


A

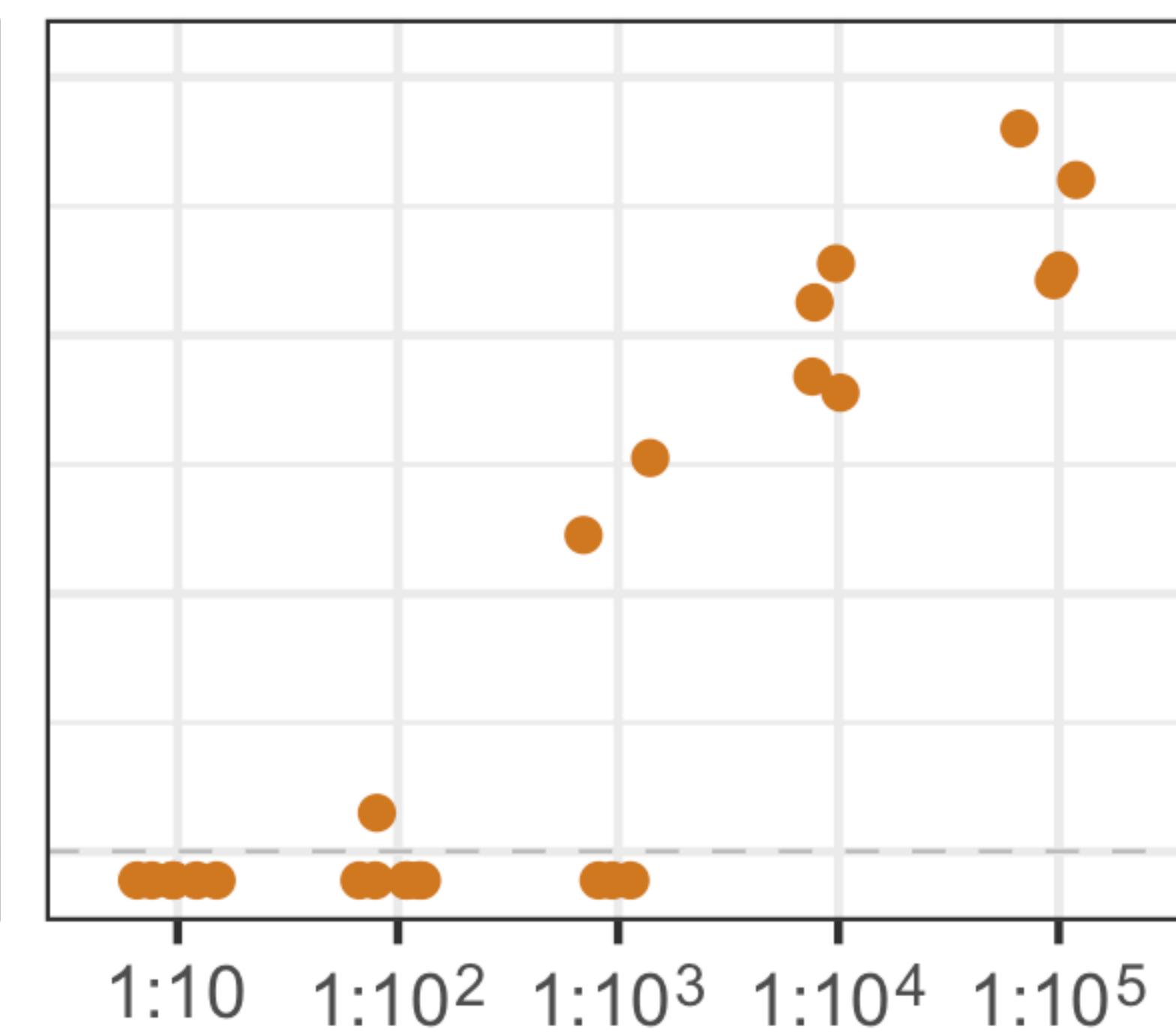
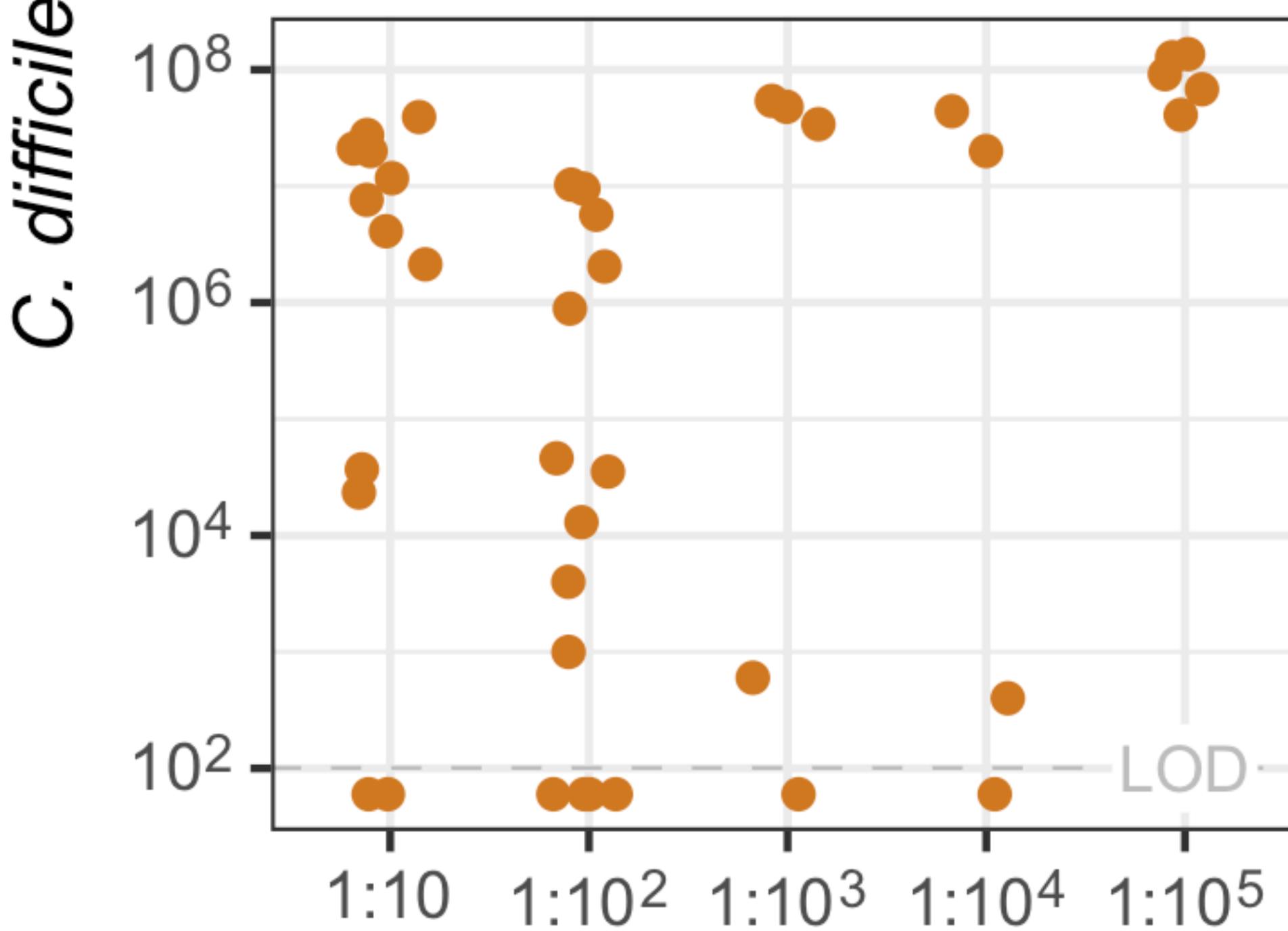
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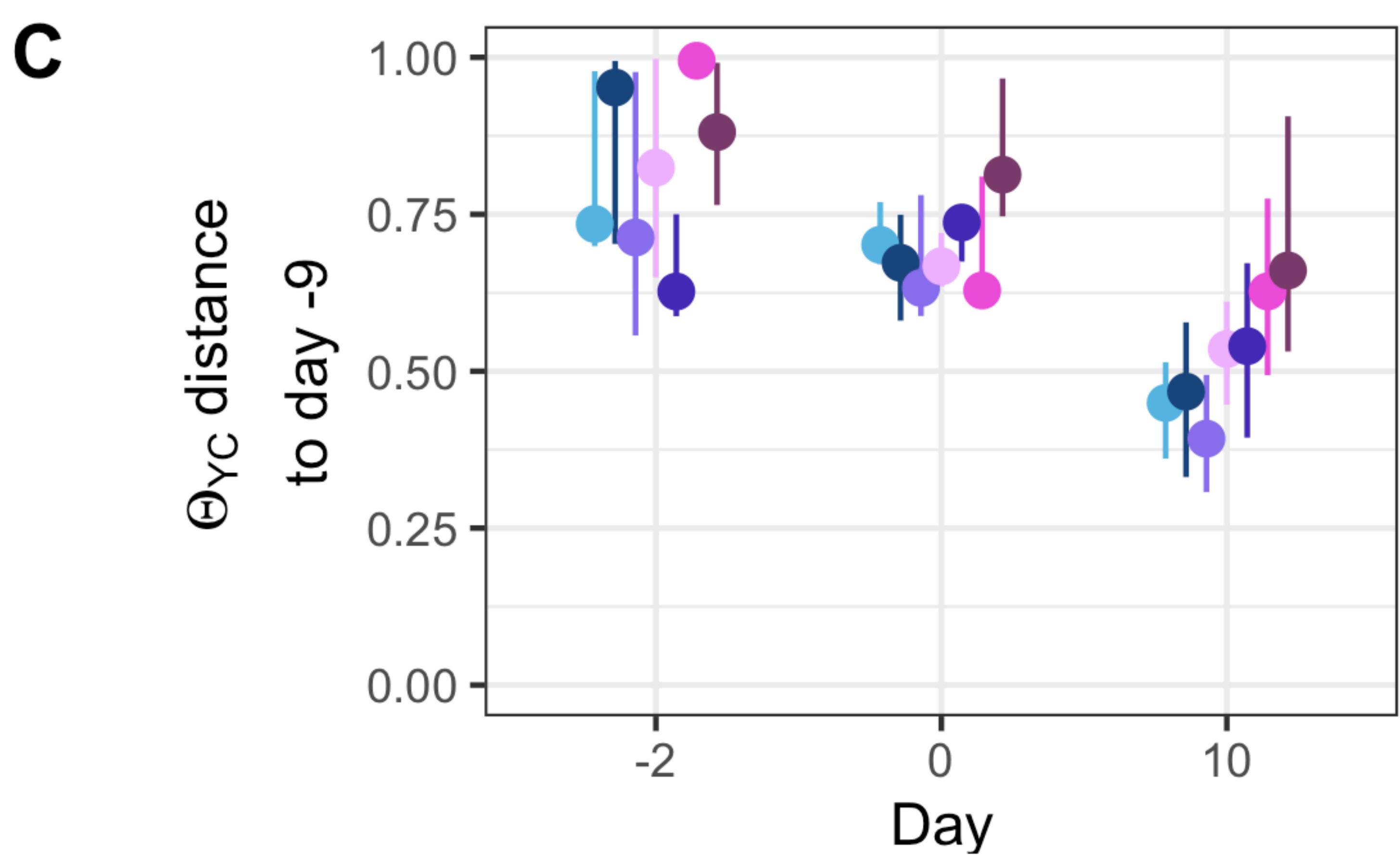
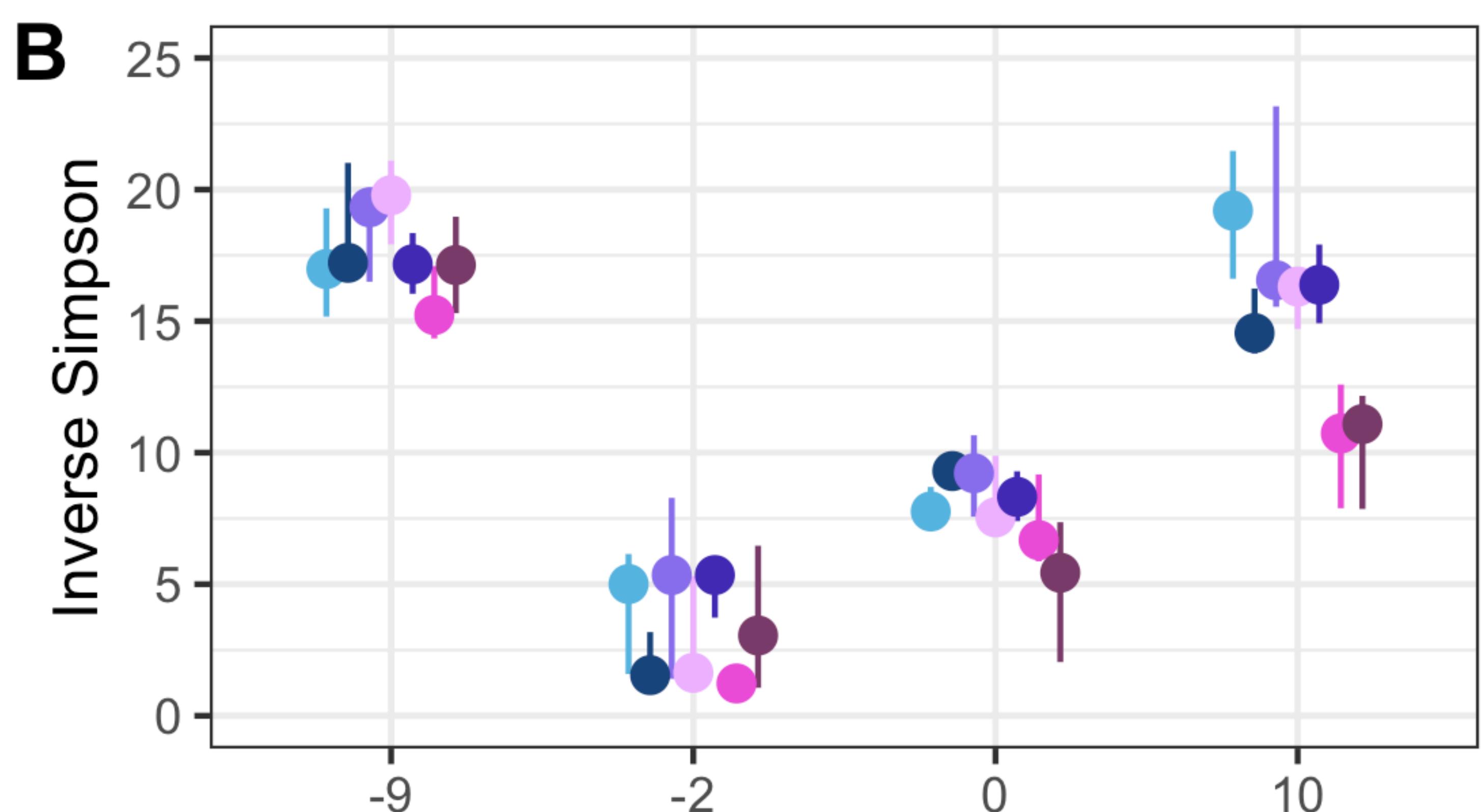
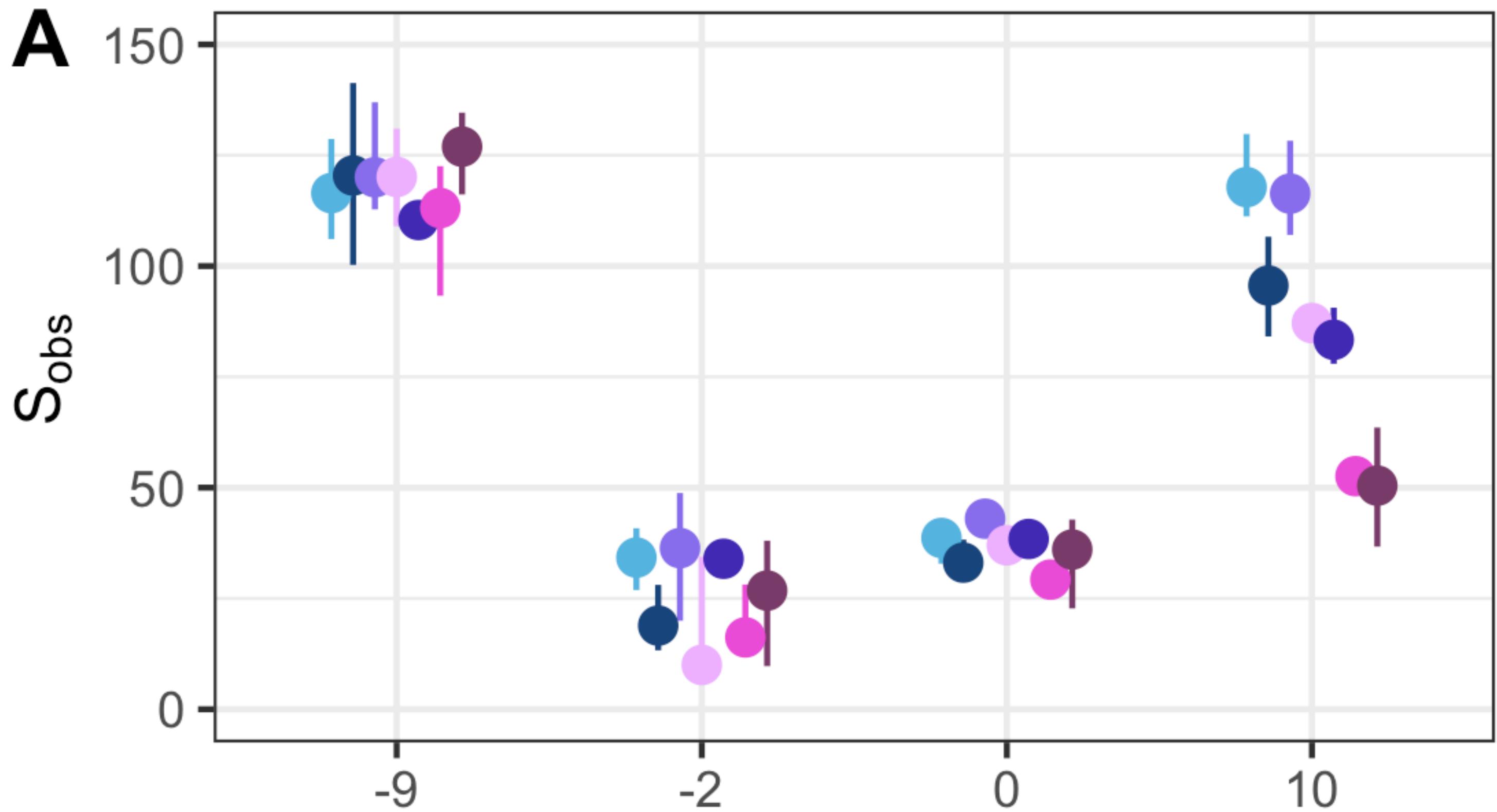
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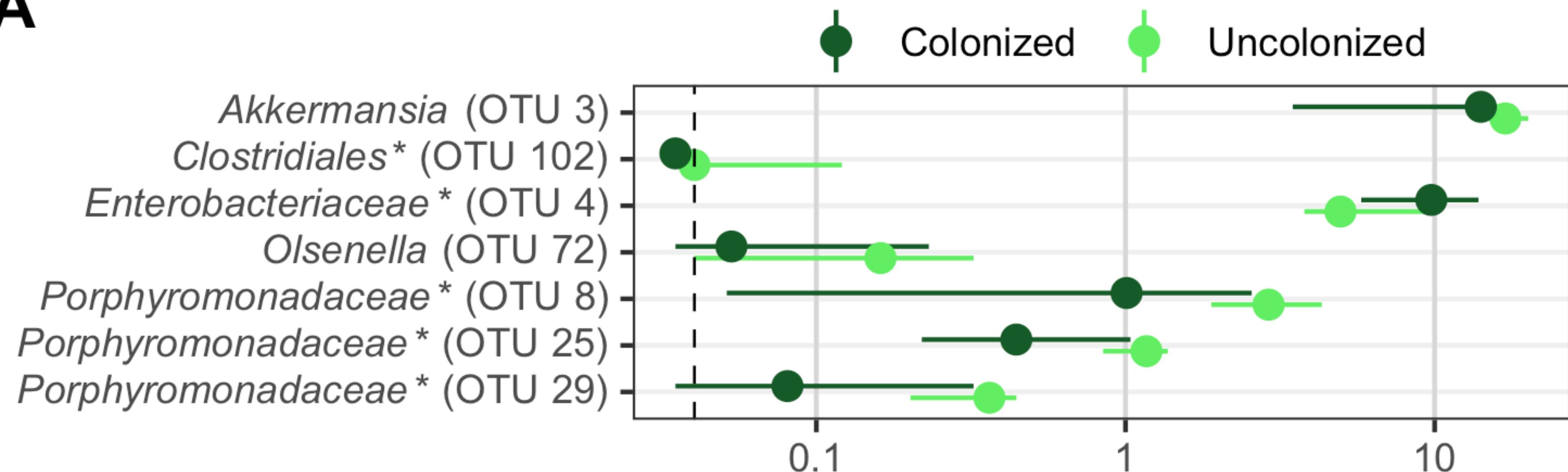
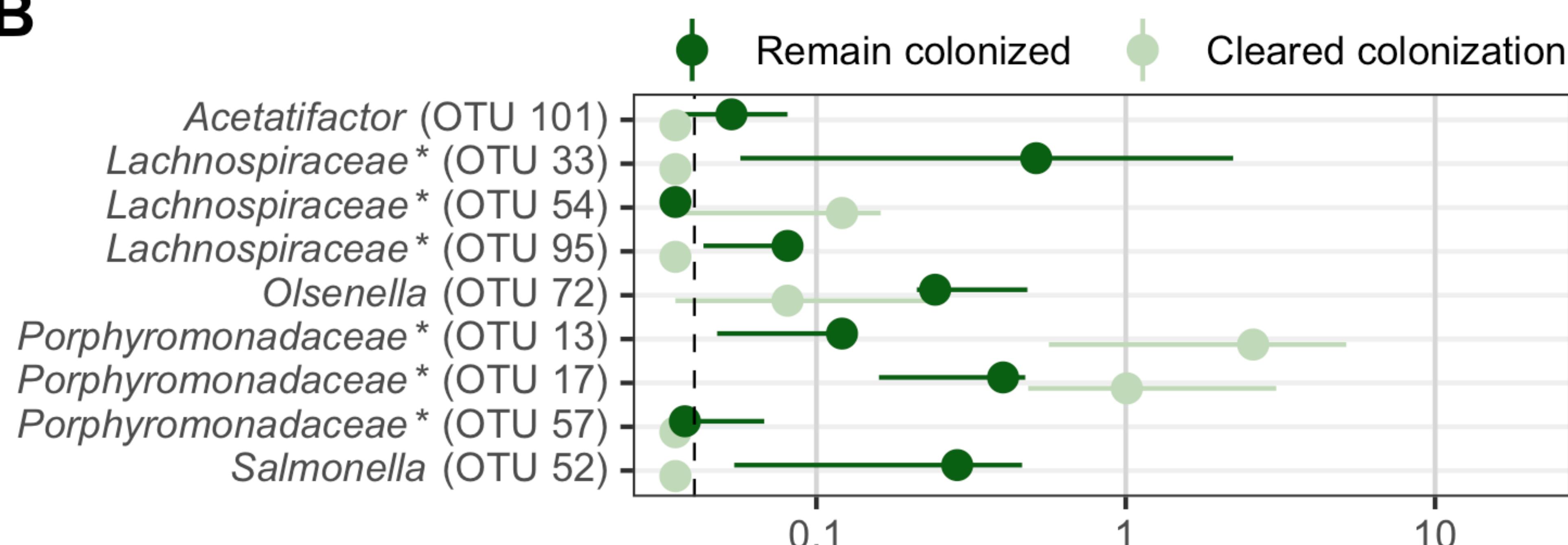
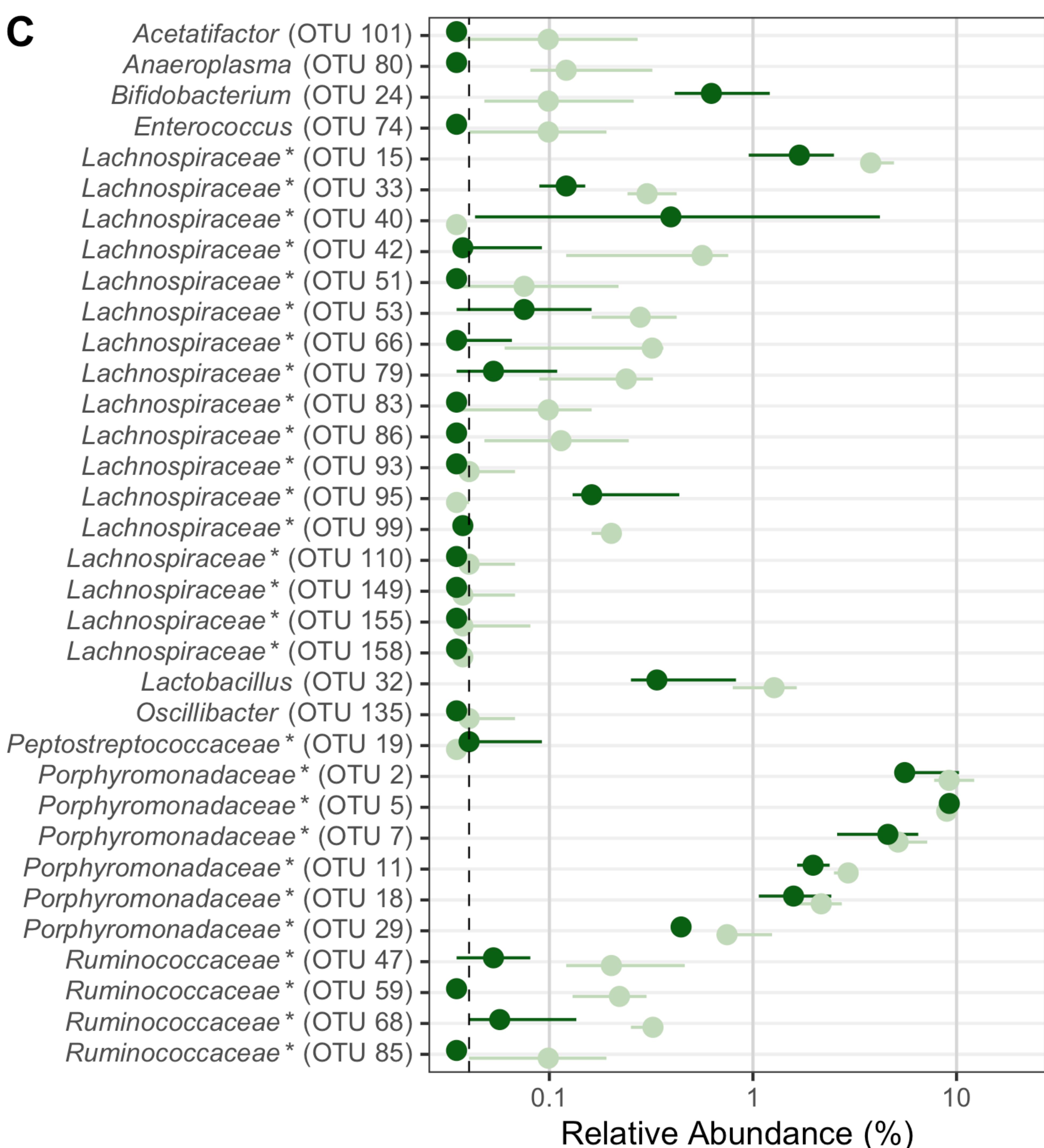
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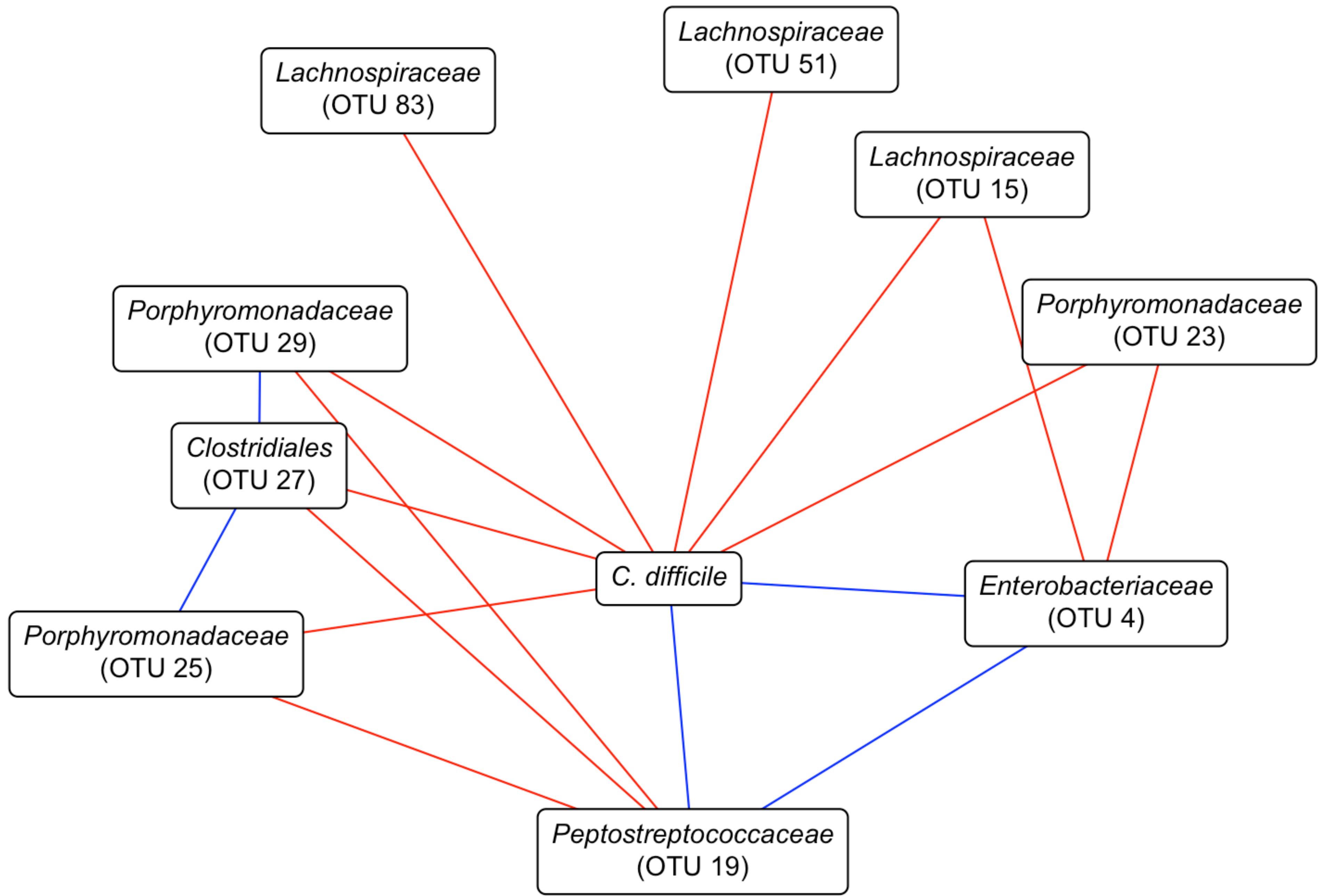
B

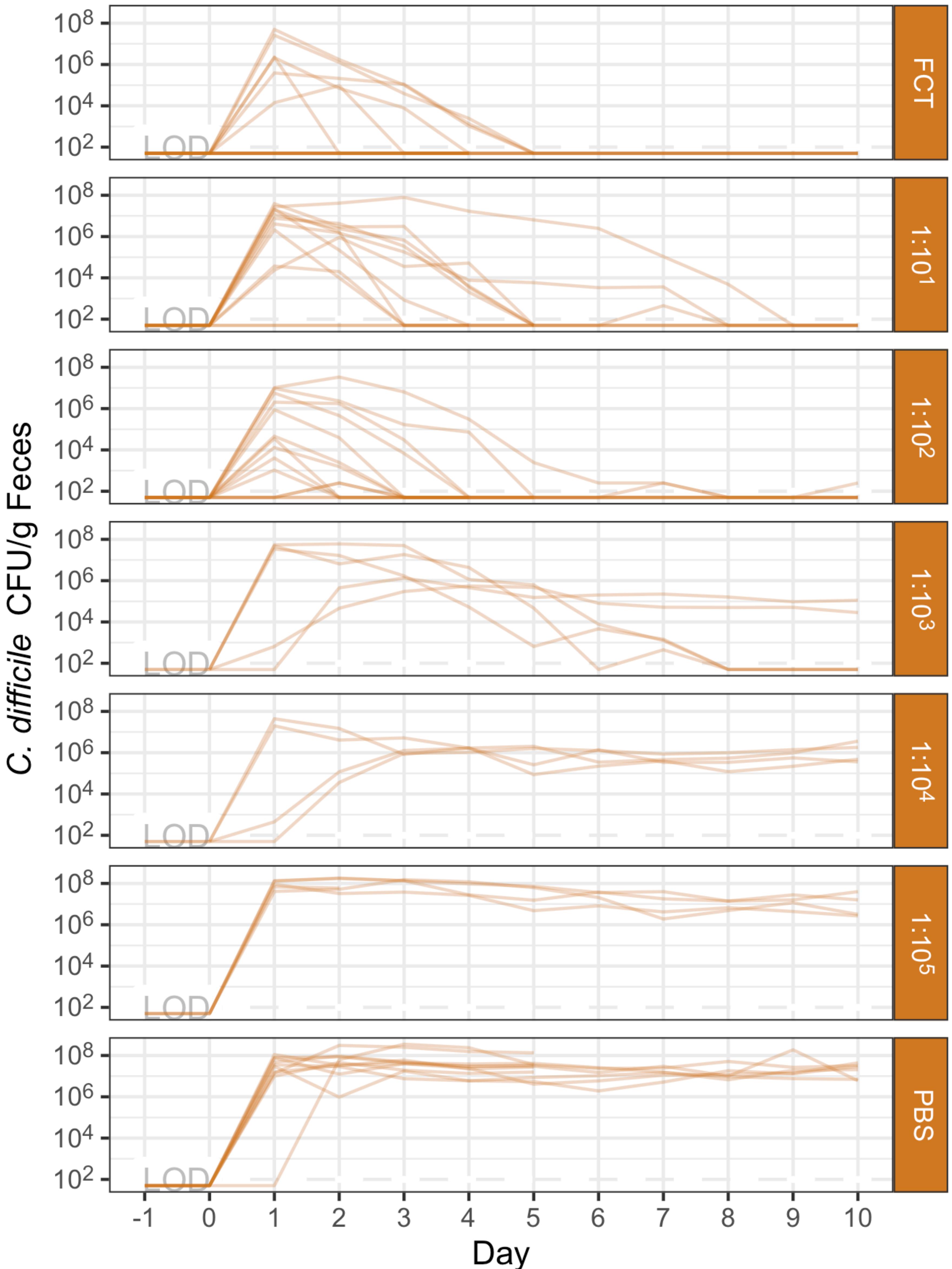
Streptomycin

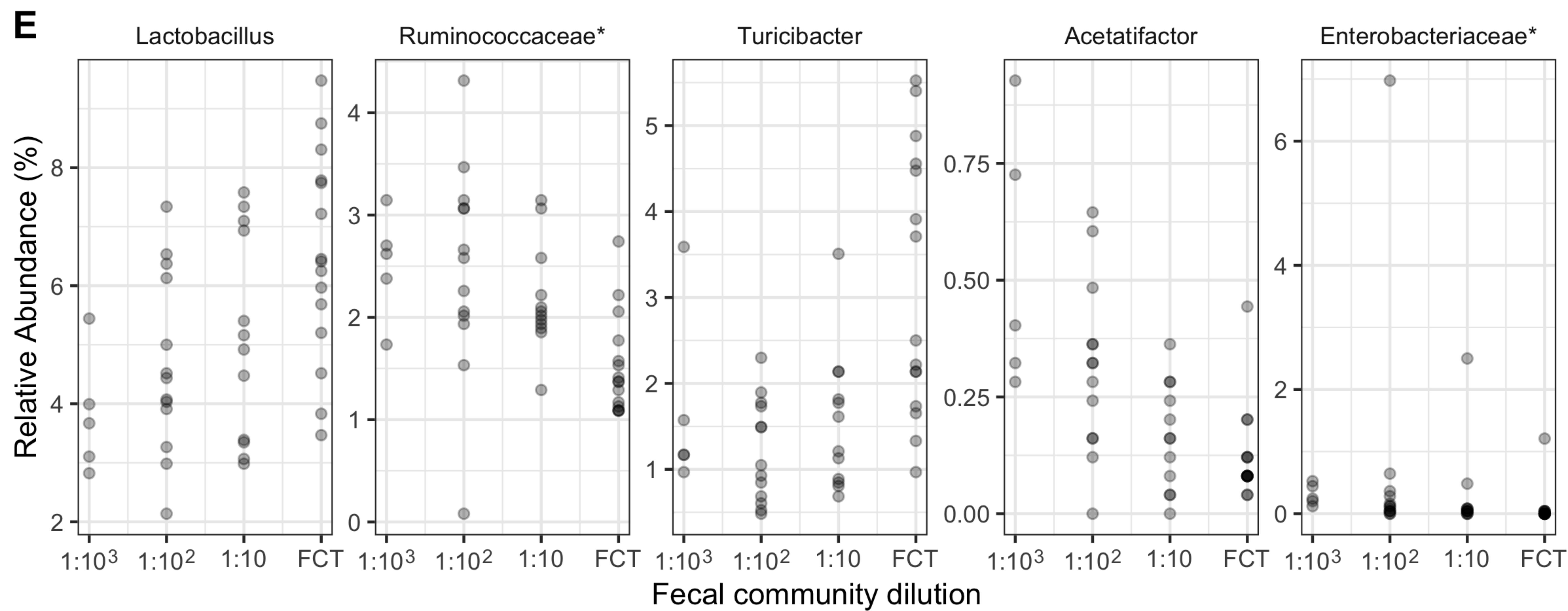
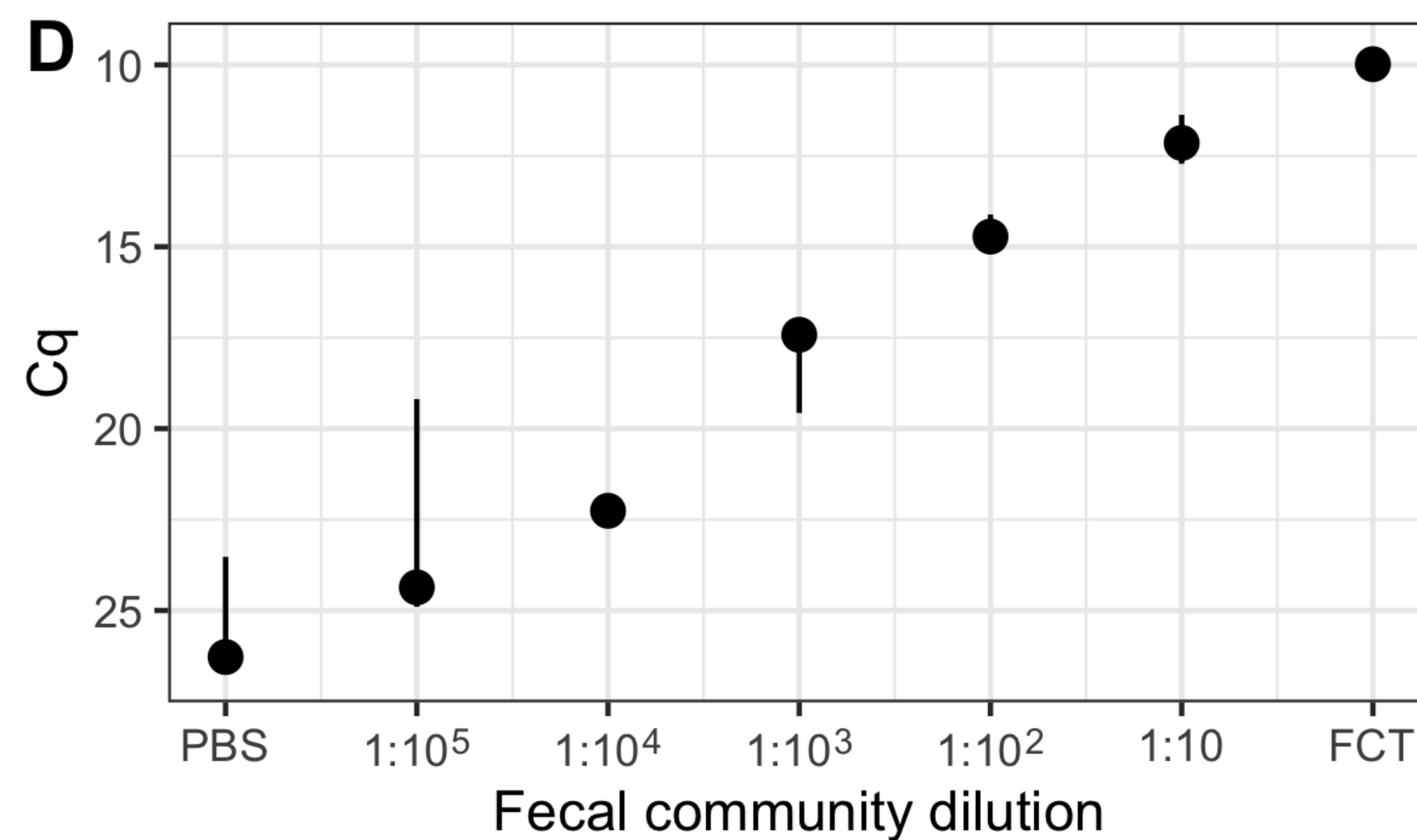
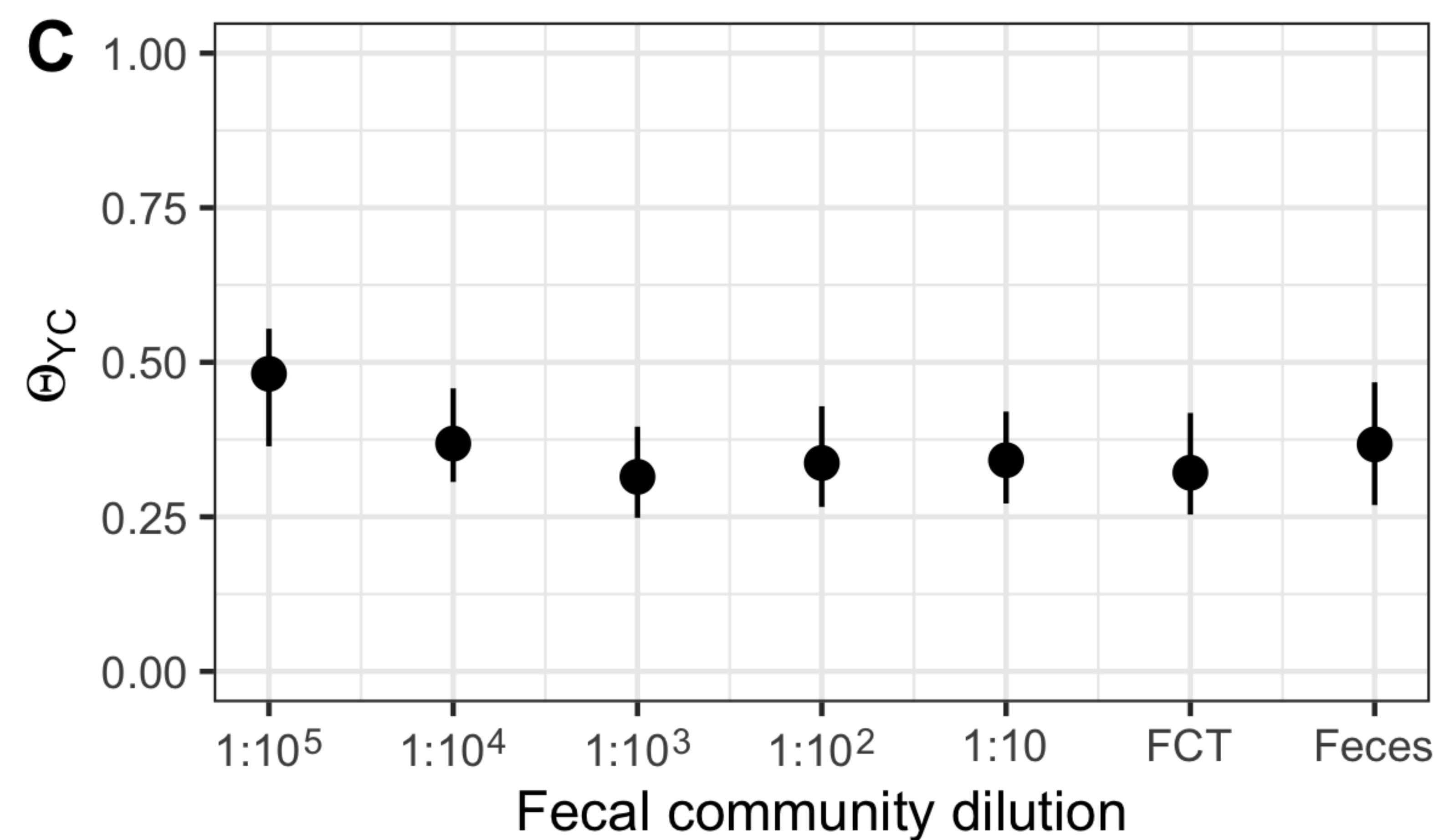
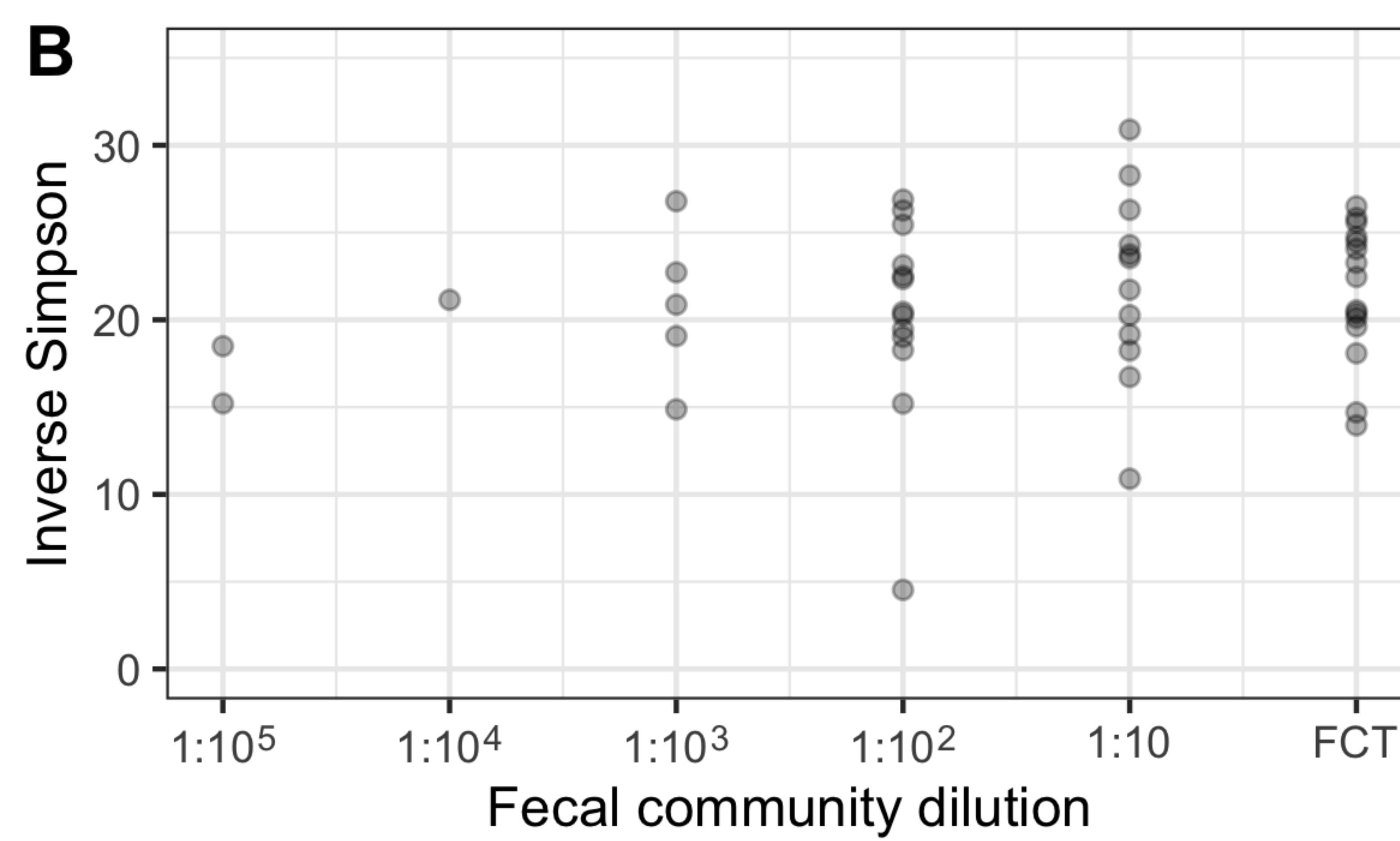
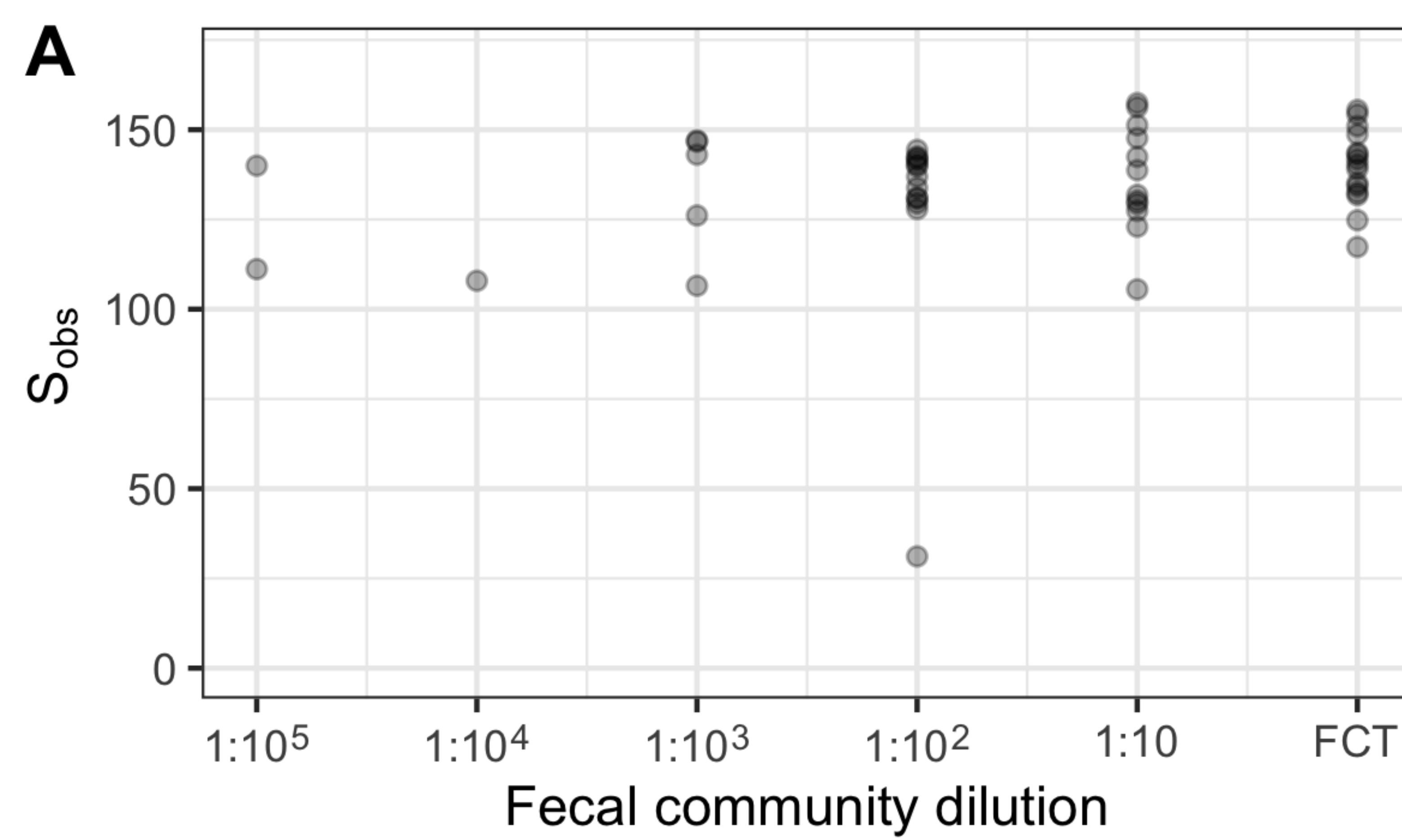


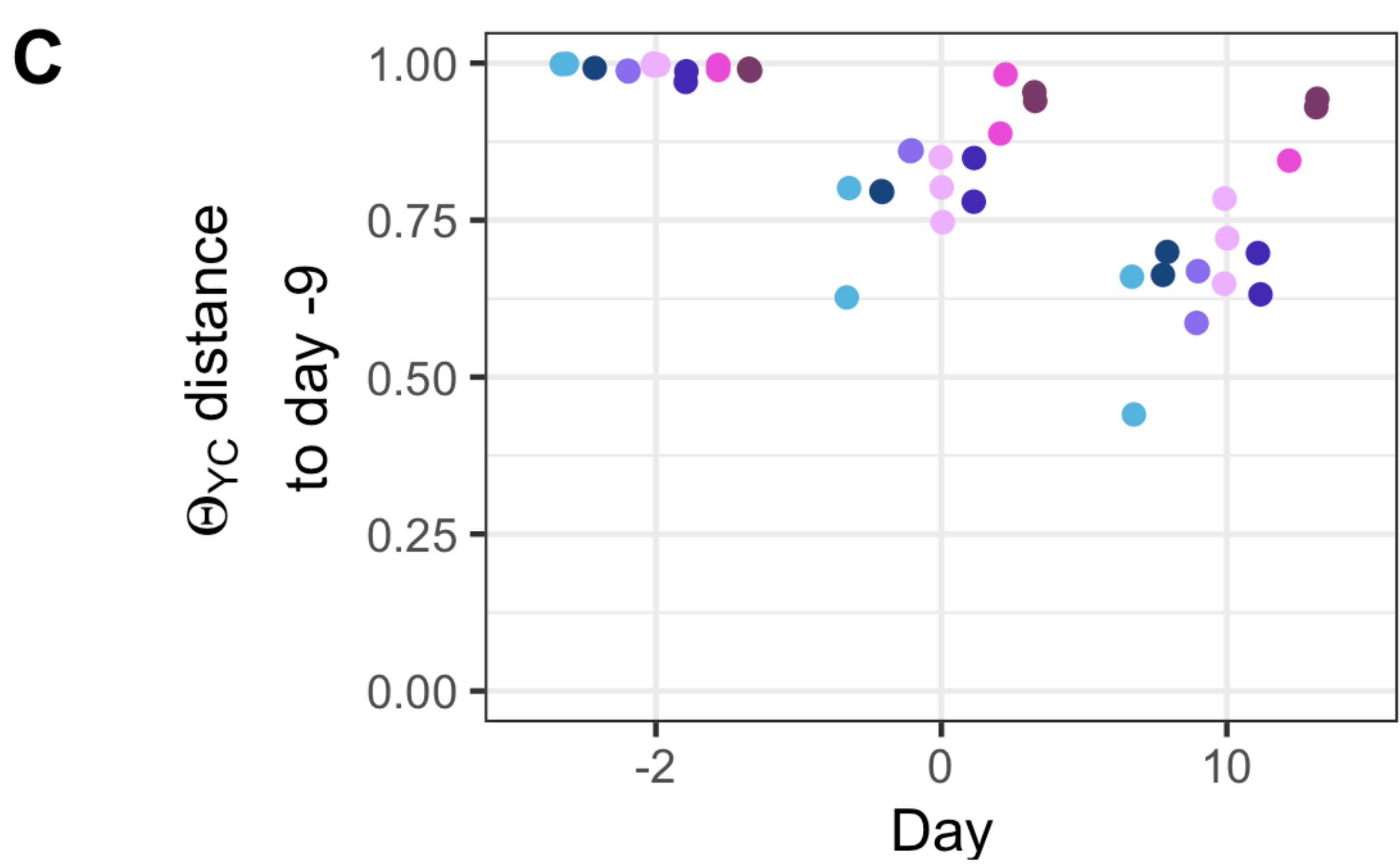
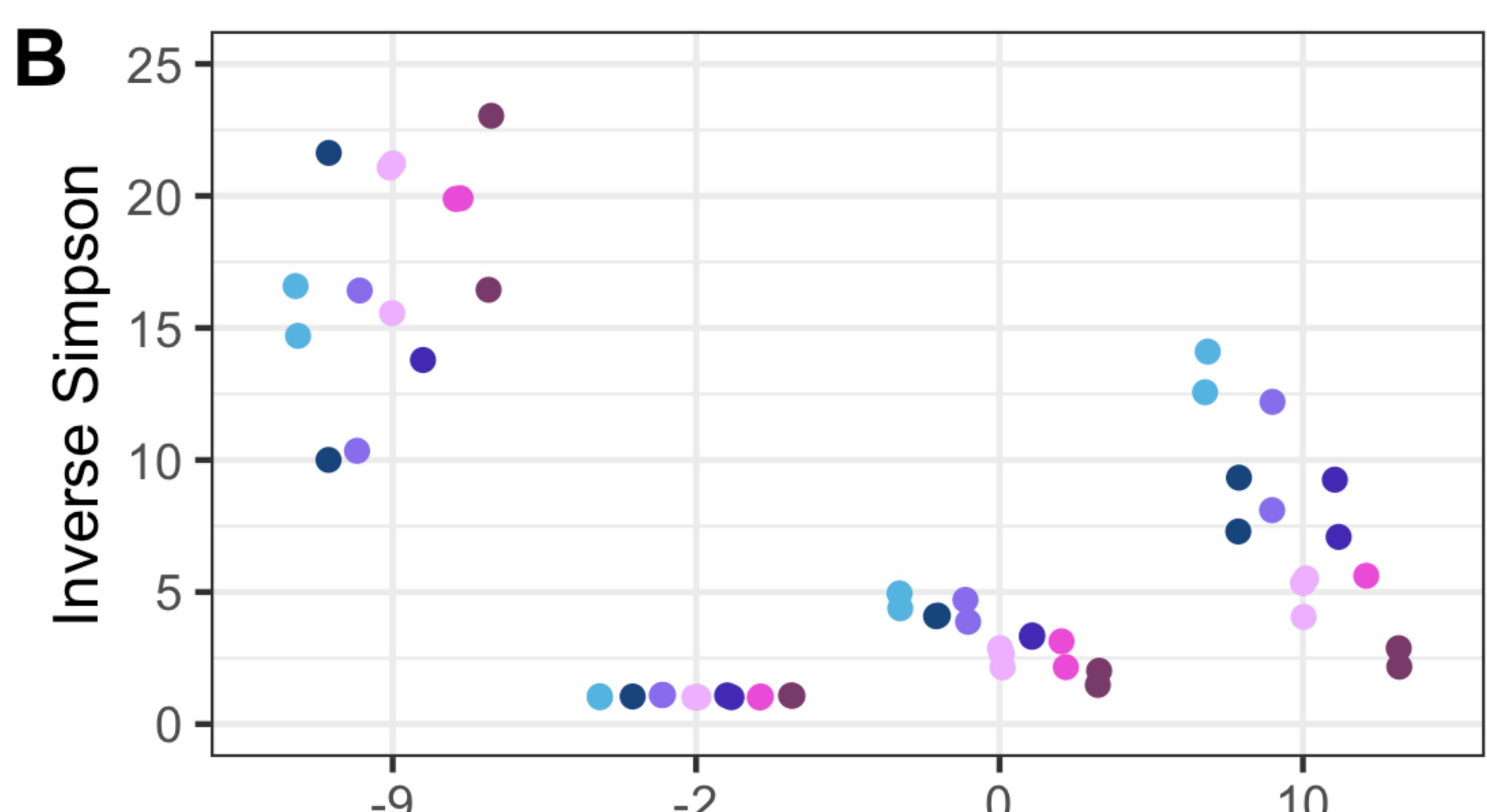
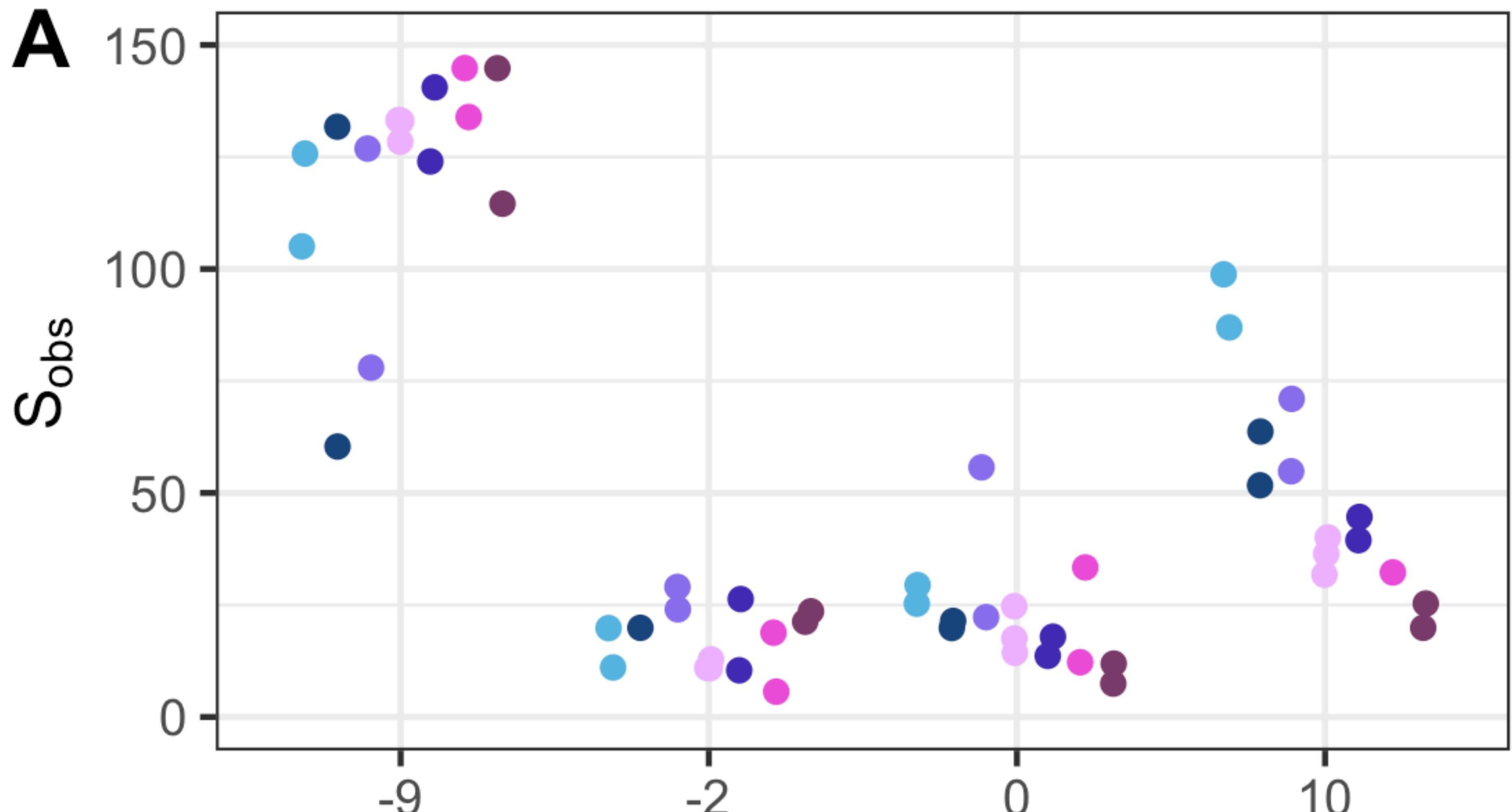
● FCT ● 1:10¹ ● 1:10² ● 1:10³ ● 1:10⁴ ● 1:10⁵ ● PBS

A**B****C**





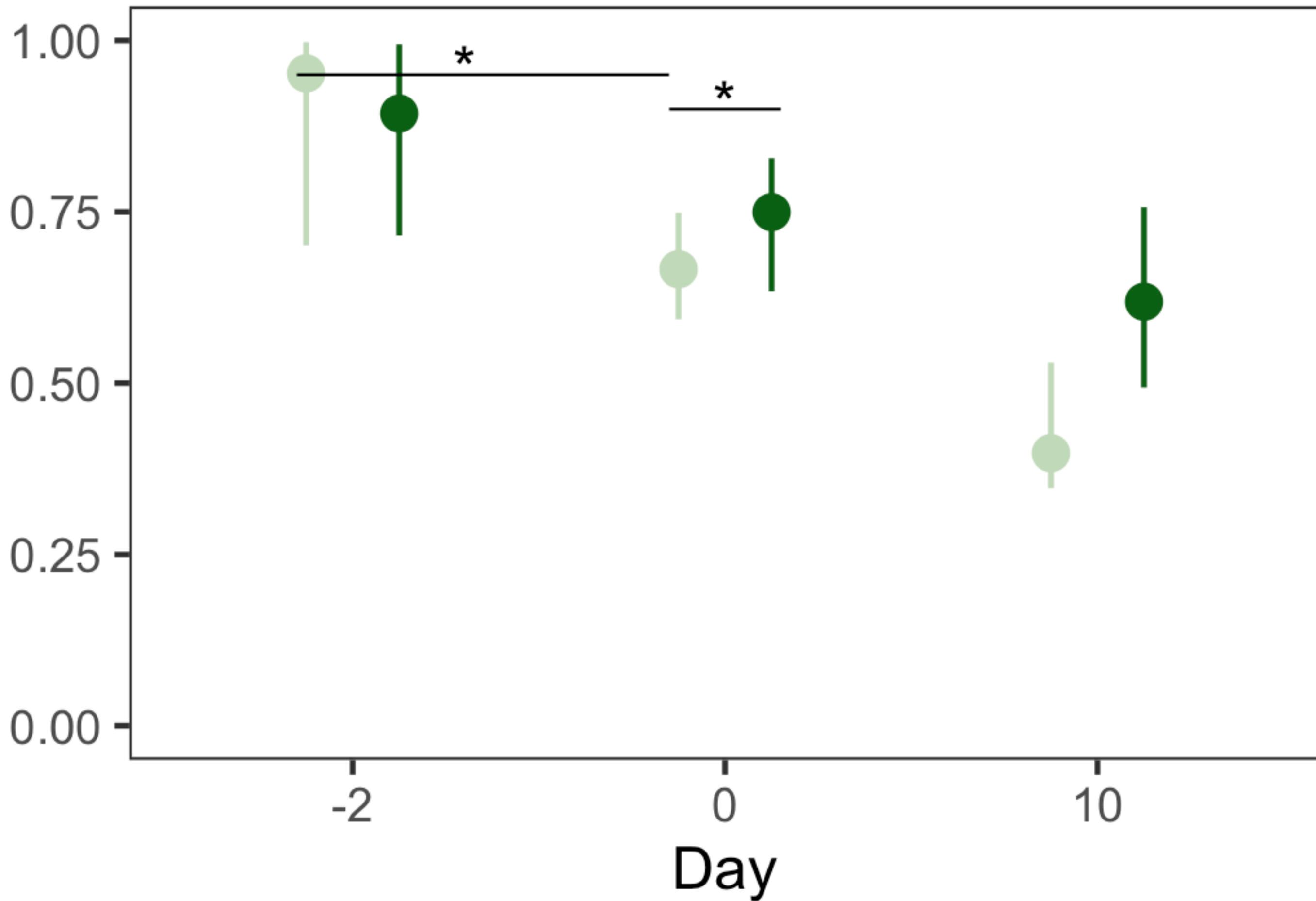





● FCT ● 1: 10^2 ● 1: 10^4 ● PBS
● 1: 10^1 ● 1: 10^3 ● 1: 10^5

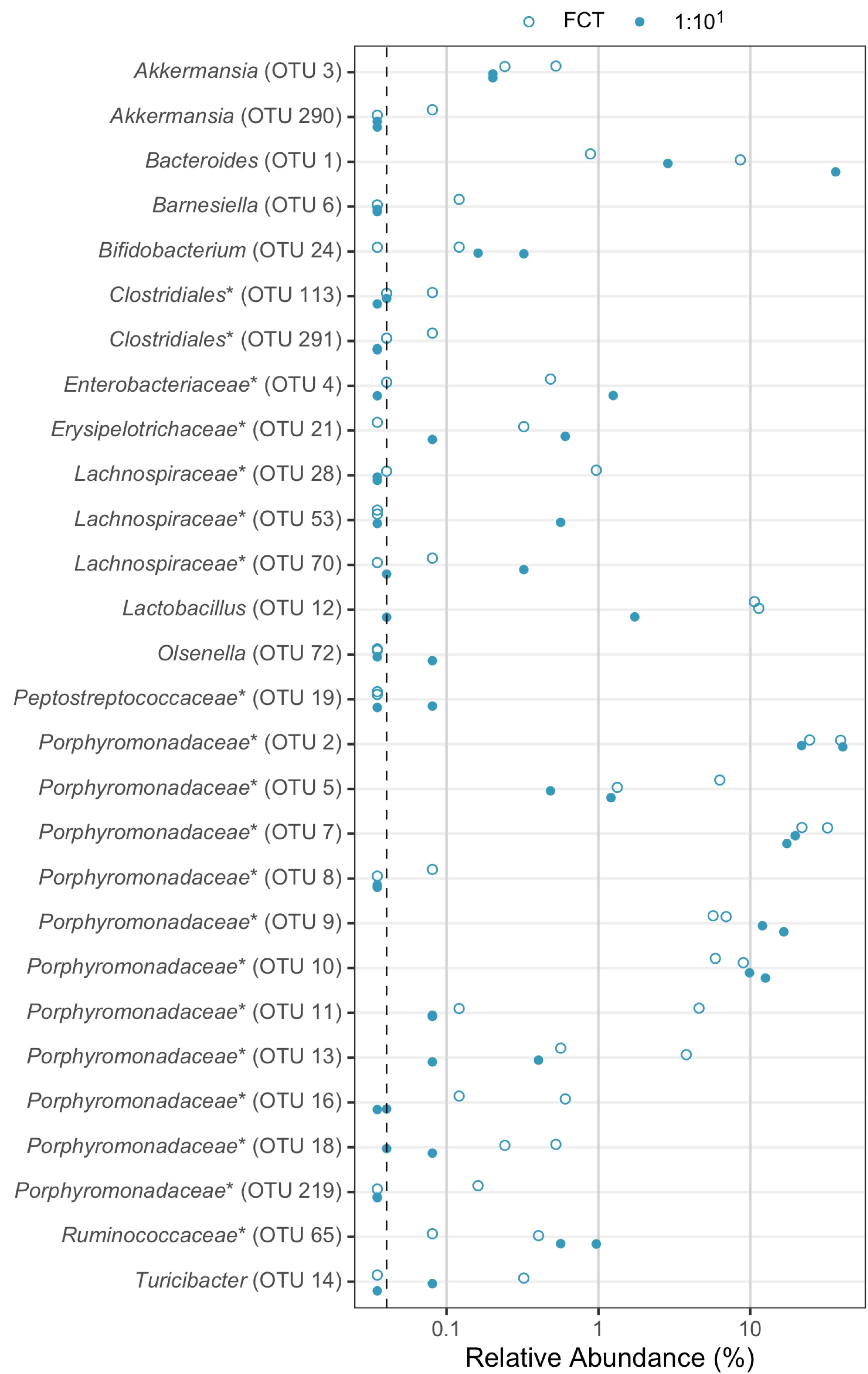
Θ_{YC} distance

to day -9

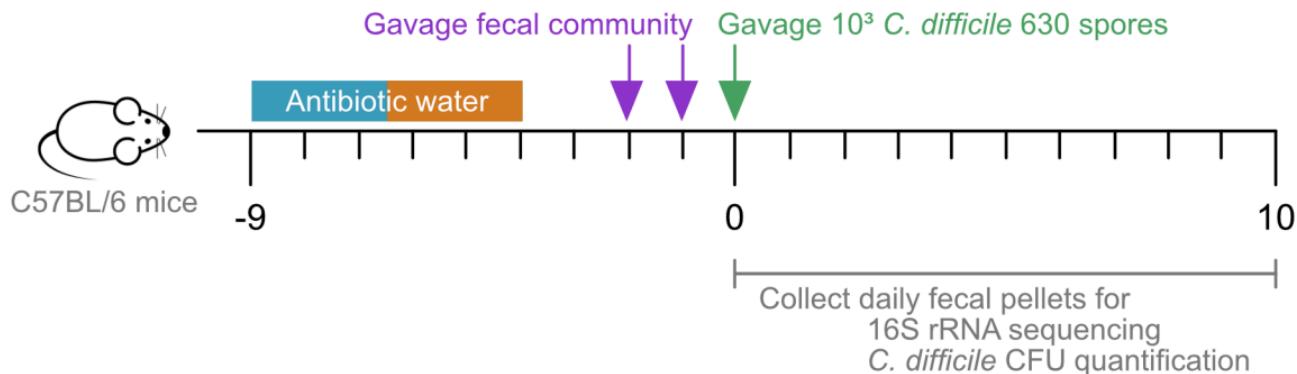


● Cleared colonization ● Remain colonized

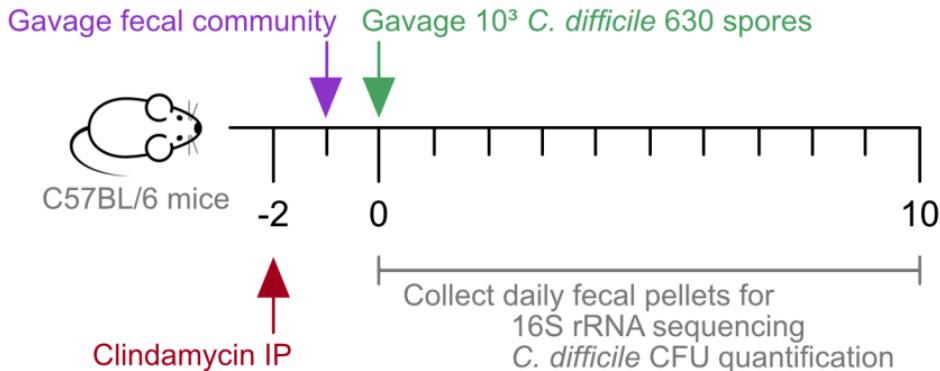




Cefoperazone/Streptomycin



Clindamycin



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

4 Running title: Diluted fecal community transplant inhibits CDI

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10 **Abstract**

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

31 **Importance**

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

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

45 **Introduction**

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

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

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

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

85 Results

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

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

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

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

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

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

challenge but were sufficient to affect *C. difficile* colonization. This would suggest the effect was driven by the most abundant populations.

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

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

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

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

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

204 **Discussion**

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

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

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

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

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

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

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

282 Further investigation into the heterogeneity of CDI will help to elucidate the niche range *C.*
283 *difficile* and the interventions to eliminate them. Here we were limited by our experimental
284 design and methods to refining our understanding of colonization resistance restoration
285 in streptomycin-treated mice. Future studies can expand beyond the presence and
286 abundance of the bacterial groups and investigate the metabolites and host immune
287 response. A refined understanding of the bacteria, metabolites and host response can help
288 develop more targeted therapies to restore *C. difficile* colonization resistance. Additionally,
289 building up experimental design to incorporate more FCT treatment variations or inoculation
290 regimens could expand our understanding of the necessary components for colonization
291 resistance for each antibiotic treatment. We designed our experiments to closely match
292 previous mouse models for CDI and added days prior to *C. difficile* challenge for the FCT
293 treatment (14, 31, 32). It may be possible to restore colonization resistance to clindamycin
294 or cefoperazone if the antibiotic treatment, recovery period, and FCT treatment were
295 modified to allow the FCT to have an effect. Other methods could be used to make the
296 mice susceptible to CDI and then tested for the effectiveness of the FCT treatment (18,
297 33). Further modification and characterization of the fecal communities could reduce
298 the necessary community members and metabolites to promote colonization resistance.
299 The results from these additional studies could expand upon our limitations and reveal
300 specific bacterial communities that could restore *C. difficile* colonization resistance for each
301 susceptibility.

302 We have demonstrated that a reduced bacterial community can restore colonization
303 resistance but the effect of the community and the bacteria that colonized was dependent
304 on the specific changes to the community that were caused by each antibiotic. When
305 transplanting the fecal community into antibiotic-induced susceptible mice, only mice
306 treated with streptomycin were able to restore colonization resistance. Previous studies

307 that have identified reduced communities in a murine model using a homogeneous gut
308 microbiota with a bottom-up approach (7, 19). Treatments supplementing the gut microbiota
309 would benefit from being tested in different communities susceptible to CDI. Further
310 research is necessary to characterize the specific niche spaces *C. difficile* of susceptibilities
311 communities and the specific requirements fill those spaces. Then it may be possible
312 to identify people with gut microbiota that are susceptible to CDI and develop targeted
313 reduced bacterial communities to recover colonization resistance and reduce the risk of
314 CDI.

315 Materials and Methods

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

321 **Antibiotic administration.** Antibiotics were chosen and administered based on previous
322 studies (14, 31, 32). Cefoperazone, clindamycin, and streptomycin treatment produced
323 diverse communities and responses to CDI. Mice were given either cefoperazone,
324 clindamycin, or streptomycin. Cefoperazone (0.5 mg/ml) and streptomycin (5 mg/ml)
325 were administered via drinking water *ad libitum* for 5 days, beginning 9 days prior to *C.*
326 *difficile* challenge. Antibiotic water was replaced every two days. Clindamycin (10 mg/kg)
327 was injected into the intraperitoneal space, 2 days prior to challenge with *C. difficile*. All
328 antibiotics were filter sterilized with a 0.22 μm syringe filter prior to use.

329 **Fecal community transplants.** Fecal pellets were collected from similar aged C57BL/6
330 mice not being used in an experiment the day of the fecal community transplants. 15-20
331 pellets were collected and weighed. The fecal pellets were homogenized weight per weight

332 in phosphate-saline buffer (PBS) containing 15% glycerol (fecal community transplant,
333 FCT) in anaerobic conditions. The FCT was serially diluted in PBS containing 15% glycerol
334 down to 1:10⁵ fecal dilution and aliquoted into tubes for gavaging into mice. One set
335 of aliquots were frozen at -80°C to be used the following day for the cefoperazone and
336 streptomycin experiments. Frozen aliquots were thawed at 37°C for 5 minutes prior to being
337 used. All fecal community dilutions were centrifuged at 7500 RPM for 60 seconds and the
338 supernatant was used for inoculation. Mice were inoculated with 100 uL of the fecal dilution
339 oral via a 21 gauge gavage needle. Fecal community transplants were administered from
340 most dilute to least, which began with mice receiving PBS and finished with mice receiving
341 FCT. Aliquots were frozen at -80°C after use for sequencing. These experiments were
342 repeated 8 times with a different starting source each time. This method was adapted from
343 our previous study (18).

344 **16S rRNA quantitative real-time PCR.** Quantitative analysis of 16S rRNA in fecal
345 community dilutions used for FCT was carried out using quantitative real-time PCR using
346 primers and cycler conditions specified previously (34). Reaction volumes were prepared
347 using 6 uL of SYBRTM Green PCR Master Mix (Applied Biosciences Ref 4344463), 1 uL
348 each forward and reverse primer, and 2 uL sample DNA template. All qPCR reactions
349 were run on a LightCycler® 96 (Roche Ref 05815916001) using instrument-specific plates
350 and seals.

351 **C. difficile challenge.** For experiments using streptomycin or cefoperazone, mice were
352 given untreated drinking water for 96 hours before challenging with *C. difficile* strain
353 630Δerm spores. For experiments using clindamycin, mice were given untreated drinking
354 water for 48 hours the time of the intraperitoneal injection and being challenged with
355 *C. difficile* strain 630Δerm spores. This time frame was designed to closely replicate
356 the previous mouse model (14, 31, 32), with the insertion of a day (clindamycin) or two
357 (cefoperazone and streptomycin) for inoculating the mice with the fecal communities. *C.*

358 *C. difficile* spores were aliquoted from a single spore stock stored at 4°C. Spore concentration
359 was determined two days prior to the day of challenge (35). Mice were inoculated with 10³
360 *C. difficile* spores via oral gavage. After inoculating the mice, remaining spore solution was
361 serially diluted and plated to confirm the spore concentration.

362 **Sample collection.** Fecal samples were collected prior to administering antibiotics, after
363 antibiotics were removed, prior to *C. difficile* challenge and on each of the 10 days post
364 infection. Approximately 15 mg of each fecal sample was collected and weighed for plating
365 *C. difficile* colony forming units (CFU) and the remaining sample was frozen at -80°C for
366 later sequencing. The weighed fecal samples were anaerobically serially diluted in PBS,
367 plated on TCCFA plates, and incubated at 37°C for 24 hours. The resultant colonies were
368 enumerated to determine the *C. difficile* CFUs (36).

369 **DNA sequencing.** Total bacterial DNA was extracted from the frozen samples by the
370 MOBIO PowerSoil-htp 96-well soil DNA isolation kit. We amplified the 16S rRNA gene V4
371 region and the amplicons were sequenced on an Illumina MiSeq as described previously
372 (37).

373 **Sequence curation.** Sequences were processed using mothur (v.1.44.1) (37, 38). We
374 used a 3% dissimilarity cutoff to group sequences into operational taxonomic units (OTUs)
375 and a naive Bayesian classifier with the Ribosomal Database Project training set (version
376 16) to assign taxonomic classifications to OTUs (39). We sequenced a mock community of
377 a known 16S rRNA gene sequences and composition. We processed this mock community
378 in parallel with our samples to determine the error rate for our sequence curation, which
379 resulted in an error rate of 0.029%.

380 **Statistical analysis and modeling.** We calculated diversity metrics in mothur. For
381 α-diversity comparisons, we calculated the number of OTUs (S_{obs}) and the Inverse Simpson
382 diversity index. For β-diversity comparisons, we calculated dissimilarity matrices based on

metric of Yue and Clayton (θ_{YC}) (40). We averaged 1000 sub-samples of 2,480 counts per sample, or rarified, to limit uneven sampling biases. We tested for differences in relative abundance between outcomes with LEfSe in mothur (41). All other statistical analyses and data visualization was completed in R (v4.0.5) with the tidyverse package (v1.3.1). Pairwise comparisons of α -diversity (S_{obs} and Inverse Simpson), β -diversity (θ_{YC}), were calculated by pairwise Wilcoxon rank sum test. Correlations between bacterial genera and fecal community dilution were calculated using the Spearman correlation. *P* values were corrected for multiple comparisons with a Benjamini and Hochberg adjustment for a type I error rate of 0.05 (42). For streptomycin experiments, conditional independence networks were calculated from the day 1 through 5 samples of all mice using SPIEC-EASI (sparse inverse covariance estimation for ecological association inference) methods from the SpiecEasi R package after optimizing lambda to 0.001 with a network stability between 0.045 and 0.05 (v1.0.7) (43).

Code availability. Scripts necessary to reproduce our analysis and this paper are available in an online repository (https://github.com/SchlossLab/Lesniak_restoreCR_mBio_2022).

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

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410 N.A.L., P.D.S.; Writing - original draft: NAL; Writing - review & editing: N.A.L., S.T., A.H.,
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574 **Figure 1. Mouse experiment timeline.** Mice were given water with cefoperazone (0.5
575 mg/ml) or streptomycin (5 mg/ml) for 5 days. The mice were returned to untreated water
576 for the remainder of the experiment. Two days after the antibiotic water was removed,
577 mice were orally gavaged 100 μ l of PBS or fecal community, once a day for two days.
578 The following day, the mice were challenged with 10^3 *C. difficile* 630 spores. Alternatively,
579 mice were given an intraperitoneal injection of clindamycin (10 mg/kg) 2 days prior to *C.*
580 *difficile* infection. 24 hours later, mice were orally gavaged with 100 μ l of PBS or fecal
581 community. The following day, the mice were challenged with 10^3 *C. difficile* 630 spores.
582 Fecal pellets were collected prior to treatment (day -9 for cefoperazone/streptomycin, day
583 -2 for clindamycin), cessation of antibiotics (day -2 for cefoperazone/streptomycin, day -1
584 for clindamycin), prior to *C. difficile* infection (day 0), and each of the following 10 days.

585

586

587 **Figure 2. Fecal community transplant inhibited *C. difficile* colonization for mice**
588 **treated with cefoperazone or streptomycin.** *C. difficile* CFU per gram of feces for mice
589 treated with clindamycin (red points), cefoperazone (blue points), or streptomycin (orange
590 points). Mice were orally gavaged either PBS (open circles) or FCT (fecal community
591 transplant, filled circles) prior to the *C. difficile* infection. Each point represents an individual
592 mouse (Clindamycin - PBS n = 4, FCT n = 7; Cefoperazone - PBS n = 2, FCT n = 2;
593 Streptomycin - PBS n = 10, FCT n = 14). LOD = limit of detection.

594

595

596 **Figure 3. Diluted FCT inhibited *C. difficile* colonization for mice treated with**
597 **streptomycin.** *C. difficile* CFU per gram of feces for mice treated with cefoperazone
598 (blue points) or streptomycin (orange points). Mice were orally gavaged with a dilution of

599 FCT (1:10 to 1:10⁵) prior to the *C. difficile* infection at (A) one day post *C. difficile* infection
600 (dpi) and (B) 10 dpi. Each point represents an individual mouse (Cefoperazone - 1:10 n =
601 2, 1:10² n = 2, 1:10³ n = 3, 1:10⁴ n = 2, 1:10⁵ n = 2; Streptomycin - 1:10 n = 12, 1:10² n =
602 14, 1:10³ n = 5, 1:10⁴ n = 4, 1:10⁵ n = 5). LOD = limit of detection.

603

604

605 **Figure 4. Diversity of murine gut bacterial community had not recovered at the time**
606 **of *C. difficile* infection in streptomycin-treated mice.** α -diversity, measured by S_{obs}
607 (A) and Inverse Simpson (B), prior to beginning antibiotic treatment (day -9), before fecal
608 community transplant (day -2), after fecal community transplant on the day of *C. difficile*
609 challenge (day 0) and at the end of the experiment (day 10). (C) β -diversity, measured by
610 θ_{YC} , distance between community structures on day 0 or 10 compared to the community
611 prior to antibiotic treatment (day -9) community of that individual. Data are grouped
612 by the transplant received, undiluted fecal community (FCT), diluted fecal community
613 (1:10¹-1:10⁵), or PBS. Points are median values and lines represent the interquartile range.

614

615

616 **Figure 5. Bacterial community OTUs differentially abundant in streptomycin-treated**
617 **mice which resisted or cleared colonization.** Murine gut bacterial community OTUs
618 that were significantly different by LEfSe analysis. OTUs from streptomycin-treated mice
619 at the time of *C. difficile* challenge (day 0) which were differentially abundant between
620 (A) mice that were colonized (dark green) and those that were not (no detectable CFU
621 throughout the experiment, bright green) or (B) mice that remained colonized (dark green)
622 and those that cleared colonization (CFU reduced to below the limit of detection by the
623 end of the experiment, faint green). (C) OTUs from streptomycin-treated mice at the end of

the experiment (day 10) which were differentially abundant between mice that remained colonized (dark green) and those that cleared colonization (CFU reduced to below the limit of detection by the end of the experiment, faint green). Points are median values and lines represent the interquartile range. Dashed vertical line is the limit of detection. OTUs ordered alphabetically. * indicates that the OTU was unclassified at lower classification rank.

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Figure 6. Streptomycin-treated murine fecal community associations with *C. difficile*.
Network constructed with SpiecEasi from the OTU relative abundances and *C. difficile* CFU data from 1 through 5 days post *C. difficile* infection. Red lines represent negative associations and blue lines indicate positive associations. *C. difficile* is based on CFU counts and *Peptostreptococcaceae* (OTU 19), the OTU most closely related to *C. difficile*, is based on sequence counts. Only *C. difficile* subnetwork shown.

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Figure S1. *C. difficile* colonization dynamics in streptomycin-treated mice across all prophylactic transplant treatments. *C. difficile* CFU per gram of feces for streptomycin-treated mice orally gavaged PBS, fecal community transplant (FCT), or diluted FCT (1:10-1:10⁵) prior to the *C. difficile* infection. Each semi-transparent line represents an individual mouse. Mice challenged with 10³ *C. difficile* spores on day 0. Lines grouped by the transplant treatment received. LOD = limit of detection.

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648 **Figure S2. Diversity and quantification of fecal community dilutions used for**
649 **prophylactic transplants in antibiotic-treated mice.** (A-C) Diversity of fecal community
650 dilutions. α -diversity, measured by (A) S_{obs} and (B) Inverse Simpson for undiluted fecal
651 community (FCT) and diluted fecal communities (1:10-1:10⁵). Points are individual
652 samples. (C) β -diversity, measured by θ_{YC} , community structure of feces collected
653 from untreated mice, undiluted fecal community (FCT), and diluted fecal communities
654 (1:10-1:10⁵) compared to untreated feces. Points are median values and lines represent
655 the interquartile range. (D) Cq values for qPCR of FCT and its dilutions for eubacterial
656 16S rRNA gene. Points are median values and lines represent the interquartile range. (E)
657 Relative abundance of bacterial taxonomic groups that significantly correlate with fecal
658 community dilutions (FCT-1:10³) by Spearman correlation. Points are individual mice. *
659 indicates that the bacterial taxonomic group was unclassified at lower classification rank.
660
661

662 **Figure S3. Diversity of murine gut bacterial community was not recovered at the**
663 **time of *C. difficile* infection in cefoperazone-treated mice.** Diversity changes through
664 experiments with cefoperazone-treated mice. α -diversity, measured by (A) S_{obs} and (B)
665 Inverse Simpson, prior to beginning antibiotic treatment (day -9), before fecal community
666 transplant (day -2), after fecal community transplant on the day of *C. difficile* infection
667 (day 0) and at the end of the experiment (day 10). (C) β -diversity, measured by θ_{YC} ,
668 distance between community structures on day 0 or 10 compared to the community
669 prior to antibiotic treatment (day -9) community of that individual. Data are grouped
670 by the transplant received, undiluted fecal community (FCT), diluted fecal community
671 (1:10¹-1:10⁵), or PBS. Points are individual mice.
672
673

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

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

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696 **Figure S6. Murine gut bacterial community OTUs of cefoperazone-treated mice at**
697 **the time of challenge.** Murine gut bacterial community OTUs that were present in at
698 least one sample at the time of *C. difficile* challenge (day 0). Mice were pre-treated with

⁶⁹⁹ either fecal community transplant (FCT, open circles) or FCT diluted 1:10 (filled circles).
⁷⁰⁰ Points are individual samples. Dashed vertical line is the limit of detection. OTUs ordered
⁷⁰¹ alphabetically. * indicates that the OTU was unclassified at lower classification rank.

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

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resistance...

3 Running title: Diluted fecal community transplant inhibits CDI

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9

14 **Abstract**

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

35 **Importance**

36 Antibiotic use, ubiquitous with the healthcare environment, is a major risk factor for
37 *Clostridioides difficile* infection (CDI), the most common nosocomial infection. When *C*
38 *difficile* becomes resistant to antibiotics, a fecal microbiota transplant from a healthy
39 individual can effectively restore the gut bacterial community and eliminate the infection.
40 While this relationship between the gut bacteria and CDI is well established, there are no
41 therapies to treat a perturbed gut community to prevent CDI. This study explored the
42 potential of restoring colonization resistance to antibiotic-induced susceptible gut
43 communities. We described the effect gut bacteria community variation has on the
44 effectiveness of a fecal community transplant for inhibiting CDI. These data demonstrated
45 that communities susceptible to CDI can be supplemented with fecal communities but the
46 effectiveness depended on the structure of the community following the perturbation.
47 Thus, a reduced bacterial community may be able to recover colonization resistance to
48 patients treated with antibiotics.

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51 **Introduction**

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

65 The benefits and risks of the FMT has led to the development of reduced bacterial
66 communities to treat CDI. Synthetic communities are more defined than an FMT, making
67 them easier to regulate as a drug. Tvede and Rask-Madsen were the first to successfully
68 treat CDI with a community of isolates cultured from human feces (6). More recently,
69 Lawley *et al.* analyzed murine experiments and the fecal communities from patients with
70 CDI to develop a synthetic community of six isolates to inhibit *C. difficile* colonization (7).

71 Reduced communities derived from human fecal communities, by methods such as
72 selective isolation of spores or culturing bacteria, have cured recurrent CDI in their initial

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75 application (8–10). Although, a recent phase 2 trial of SER-109, a spore-based treatment,
76 failed its phase 2 clinical trial (11), these therapies have the potential to offer the benefits
77 of the FMT without the associated risks but are only used once a patient has had multiple
78 CDIs. For these to be successful, we need a better approach to identify candidate bacterial
79 populations. Recently, an autologous FMT was shown to be effective at restoring the gut
80 microbiota in allogeneic hematopoietic stem cell transplantation patients and prevented
81 future complications, such as systemic infections (12). It is unclear whether an treatment
82 similar to an autologous FMT or reduced bacterial communities could be used to restore
83 susceptible communities and prevent CDI (13).

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

93 Results

94 Effect of fecal transplant on *C. difficile* colonization was not consistent across
95 antibiotic treatments. Our previous research demonstrated that when mice were
96 perturbed with different antibiotics, there were antibiotic-specific changes to the microbial

98 community that resulted in different levels of colonization and clearance of *Clostridioides*
99 *difficile* infection (14). Because each of these treatments opened different niche spaces that
100 *C. difficile* could fill, we hypothesized that the resulting community varied in the types of
101 bacteria required to recover colonization resistance. To test the ability of the murine
102 communities to recover colonization resistance, we treated conventionally raised SPF
103 C57BL/6 mice with either clindamycin, cefoperazone, or streptomycin. After a short
104 recovery period, the mice were given either phosphate-saline buffer (PBS) or a fecal
105 community transplant via oral gavage (Figure 1). The fecal community was obtained from
106 untreated mice. One day after receiving the FCT, the mice were challenged with 10^3 *C.*
107 *difficile* 630 spores. One day after the challenge, mice that were treated with either
108 clindamycin or cefoperazone and received the FCT pre-treatment had similar amounts of *C.*
109 *difficile* colony forming units (CFU) as those which received PBS. Among the clindamycin-
110 treated mice, *C. difficile* colonization was cleared at similar rates regardless of whether they
111 received the FCT or PBS pre-treatments (Figure 2). For cefoperazone-treated mice, *C.*
112 *difficile* colonized all of the mice, but the mice that received the FCT pre-treatment cleared
113 the infection (Figure 2). For the streptomycin-treated mice, the FCT pre-treatment resulted
114 in either no detectable *C. difficile* colonization (8 of 14) or an infection that the community
115 cleared within 5 days (Figure 2). For mice that would have normally had a persistent
116 infection, the FCT enabled them to clear the infection and in the streptomycin-treated mice
117 it was able to prevent infection entirely for some mice.

118 **Diluted fecal communities prevented colonization and promoted clearance for**
119 **streptomycin-treated mice.** Next, we sought to test whether mice that received a diluted
120 FCT pre-treatment could still benefit. To identify the minimally effective dilution, we

121 repeated the same experimental design (Figure 1) with the FCT diluted serially down to
122 1:10⁵. Since the FCT pre-treatment had no detected effect in clindamycin-treated mice, we
123 did not study those mice further. Cefoperazone-treated mice pre-treated with diluted FCT,
124 1:10 and lower, were not affected and were colonized throughout the experiment (Figure
125 3). Streptomycin-treated mice pre-treated with diluted FCT either regained colonization
126 resistance or were enabled to clear *C. difficile*. The streptomycin-treated mice pre-treated
127 with FCT as dilute as 1:10³ cleared *C. difficile*. Some streptomycin-treated mice pre-treated
128 with FCT as dilute as 1:10² had no *C. difficile* CFU detected throughout the length of the
129 experiment. While more mice pre-treated with lower FCT dilutions were colonized (FCT 6
130 of 14 were colonized, 1:10-10 of 12 were colonized, 1:10²-10 of 14 were colonized), the
131 colonized mice that received the lower dilutions were still able to clear *C. difficile* (Figure
132 S1). Thus, the reduced fecal communities from the diluted FCT were able to restore
133 colonization resistance and promote clearance of *C. difficile* in streptomycin-treated mice.

134 The reduced fecal communities of the diluted FCT may have reduced abundance and
135 membership. We compared the FCT communities to determine the differences between the
136 dilutions. The most significant difference between the communities of the FCT and its
137 dilutions was the quantity of the 16S rRNA gene, which decreased monotonically (Figure
138 S2D). The FCT dilutions of 1:10³ to 1:10⁵ of the had few samples with sufficient sequencing
139 depth to provide bacterial community information. The FCT and its dilutions were not
140 significantly different in either α -diversity (number of operational taxonomic units (OTUs)
141 (S_{obs}) or Inverse Simpson) or β -diversity (θ_{ve}) (Figure S2A-C). Populations of *Acetatitofactor*,
142 *Enterobacteriaceae*, *Lactobacillus*, *Ruminococcaceae*, and *Turicibacter* correlated with the

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146 FCT dilution factor (Figure S2E). Overall, the abundance of the bacteria appeared to be
147 largest difference between FCT and its dilutions.

148 **Murine gut bacterial communities had not recovered their diversity by the time of *C.***
149 ***difficile* challenge.** To elucidate the effects of the fecal community dilution on the murine
150 gut bacterial community and *C. difficile* infection, we sequenced the V4 region of the 16S
151 rRNA gene from the fecal community. For the gut communities, in comparison to the initial
152 community (day -9), FCT pre-treatment did not result in a significant recovery of diversity
153 at the time of *C. difficile* challenge (day 0) for cefoperazone-treated mice (Figure S3) or
154 streptomycin-treated mice (Figure 4). At the end of the experiment (day 10), the gut
155 bacterial communities were more similar to their initial community in α -diversity (number
156 of OTUs (S_{obs}) and Inverse Simpson diversity index) and β -diversity (θ_{YC}) diversity. The
157 mice pre-treated with less dilute FCT were most similar to the initial community structure,
158 whereas, the mice pre-treated with more dilute FCT resulted in little recovery of diversity,
159 similar to the mice given PBS. Thus, the ~~less dilute FCT treatments did not result in~~
160 ~~restoration of pre-antibiotic treatment community diversity~~ at the time of *C. difficile*
161 challenge but ~~were~~ sufficient to affect *C. difficile* colonization. This would suggest the effect
162 was driven by the most abundant populations.

163 **Gut bacterial community members are differentially abundant in streptomycin-
164 treated mice resistant to colonization.** Although there were no significant differences in
165 diversity at the time of challenge, we next investigated how the individual bacterial
166 populations were different in the uncolonized streptomycin-treated mice pre-treated with
167 FCT. We used linear discriminant analysis (LDA) effect size (LEfSe) analysis to identify

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172 OTUs within the fecal bacterial communities from the streptomycin-treated mice that were
173 differentially abundant between uncolonized and colonized mice. The antibiotic treatment
174 significantly altered 99 OTUs (Figure S5), but on the day of *C. difficile* challenge only 7 OTUs
175 were differentially abundant between colonized and uncolonized communities (Figure 5A).
176 Communities resistant to *C. difficile* colonization had more abundant populations of OTUs
177 related to *Akkermansia*, *Clostridiales*, *Olsenella*, and *Porphyromonadaceae* and less
178 abundant populations of an OTU related to *Enterobacteriaceae*. Thus, a small portion of
179 OTUs, relative to the changes due to streptomycin treatment, were differentially abundant
180 in mice that resisted *C. difficile* colonization compared to those that were colonized.

181 **Murine gut bacterial communities that cleared *C. difficile* colonization were more**
182 **similar to the initial community.** To better understand the differences in streptomycin-
183 treated murine fecal community that contributed to *C. difficile* clearance, we compared the
184 communities that cleared *C. difficile* to those that did not at the time of challenge and 10
185 days post infection. Communities from mice that cleared colonization were more similar to
186 their initial community at the end of the experiment than the mice that remained colonized
187 (Figure S4). At the time of *C. difficile* challenge, 9 OTUs were differentially abundant
188 between communities that remained colonized to those that cleared colonization (Figure
189 S8). Communities that cleared *C. difficile* colonization had more abundant populations of
190 OTUs related to *Porphyromonadaceae* and *Lachnospiraceae* and less abundant populations
191 of OTUs related to *Acetotifactor*, *Lachnospiraceae*, *Olsenella*, *Porphyromonadaceae*, and
192 *Salmonella*. At the end of the experiment, 29 of the 34 differentially abundant OTUs were
193 more abundant in the mice that were able to clear the colonization (Figure S9). The relative
194 abundance of OTUs related to *Acetotifactor*, *Anaeroplasm*, *Enterococcus*, *Lachnospiraceae*,

195 *Lactobacillus*, *Porphyromonadaceae*, and *Ruminococcaceae* were higher in communities that
196 cleared, recovering in abundance from the streptomycin treatment. Multiple OTUs related
197 to *Lachnospiraceae* and *Porphyromonadaceae* ($N = 14$ and $N = 5$, respectively) were
198 significant and accounted for greater portions of the community (more than 10%).

199 However one *Porphyromonadaceae* population (OTU 5) and two *Lachnospiraceae* related
200 populations (OTU 40 and 95) were more abundant in the mice that remain colonized. Thus,

201 ~~as more of the gut bacterial members returned to their initial abundance, there was a~~ Deleted: the
202 greater likelihood of clearing *C. difficile*.

203 **Negative associations dominated the interactions between the gut bacterial**
204 **community and *C. difficile* in streptomycin-treated mice.** In streptomycin-treated mice,
205 pre-treatment with FCT and its dilutions had different effects on the bacterial community
206 members which resulted in different community relative abundance and *C. difficile*
207 colonization dynamics. We quantified the relationships occurring throughout this
208 experiment using SPIEC-EASI (sparse inverse covariance estimation for ecological
209 association inference) to construct a conditional independence network. Here, we focused
210 on the associations of the *C. difficile* subnetwork (Figure 6). *C. difficile* CRU had positive
211 associations with populations of OTUs related to *Enterobacteriaceae* (OTU 4) and
212 *Peptostreptococcaceae* (OTU 19). OTUs related to *Clostridiales* (OTU 27), *Lachnospiraceae*
213 (OTUs 15, 51, and 83), and *Porphyromonadaceae* (OTUs 23, 25, and 29) had negative
214 associations with *C. difficile*, as well as the OTUs related to *Enterobacteriaceae*, and
215 *Peptostreptococcaceae*. Overall, the majority of the associations between *C. difficile* and the
216 gut bacterial community in streptomycin-treated mice were negative. This suggests this

218 subset of the community may be driving the inhibition of *C. difficile* in streptomycin-treated
219 communities.

220 **Discussion**

221 Transplanting the fecal community from untreated mice to antibiotic-treated mice prior to
222 being challenged with *C. difficile* varied in effectiveness based on the antibiotic treatment.
223 This indicated that FCT pre-treatment can prevent *C. difficile* colonization in an antibiotic-
224 specific manner. Additionally, by diluting the FCT we were able to narrow the community
225 changes responsible for the effect to the most abundant OTUs. Overall, these results show
226 that a reduced fecal community can assist a perturbed microbiota in preventing or resisting
227 *C. difficile* colonization but the effect was dependent on the antibiotic that was given.

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228 By diluting the FCT we were able to narrow the definition of the minimal community
229 features that restored colonization resistance. Bacterial interactions with *C. difficile* were
230 associated with the identity, abundance, and functions of adjacent bacteria. Ghimire *et al.*
231 recently showed individual species that inhibited *C. difficile* in co-culture but when other
232 inhibitory species were added the overall effect on *C. difficile* was changed, in some cases to
233 increase *C. difficile* growth (15). Based on these observations from their bottom-up

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234 approach, it is unclear how more complex combinations would affect the inhibition of *C.*
235 *difficile*. So instead, we sought to find an inhibitory community using a top-down approach
236 and begin with an inhibitory community. In a recent top-down approach, Auchtung *et al.*
237 recently developed a set of reduced communities from human fecal communities that were
238 grown in mini bioreactor arrays and tested for inhibition first *in vitro* then in a mouse
239 model (16). They found four reduced communities that were able to reduce *C. difficile*

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244 colonization but with varied effect in a mouse model with the same gut microbiota. One
245 way they reduced the community was through diluting the initial fecal sample. In our
246 experiments, we began with a fresh whole fecal community to first determine if inhibition
247 was possible. In the conditions which *C. difficile* was inhibited, with cefoperazone and
248 streptomycin, we diluted the FCT to determine the minimal community which maintained
249 inhibition. Cefoperazone-treated mice were unable to maintain inhibition of *C. difficile* with
250 diluted FCT pre-treatments and *C. difficile* remained colonized. Streptomycin-treated mice
251 were able to maintain inhibition with diluted FCT pre-treatment. While the diluted FCTs
252 had similar diversity and bacterial abundances, the differences in effect on *C. difficile*
253 revealed the minimal changes associated with either colonization resistance or clearance.

254 We previously hypothesized that mice treated with either clindamycin, cefoperazone, or
255 streptomycin would not have the same bacterial community changes associated with *C.*
256 *difficile* clearance (14). In that set of experiments, the dose of the antibiotic was varied to
257 titrate changes to the community and determine what changes allow *C. difficile* to colonize
258 and then be spontaneously cleared. We observed antibiotic-specific changes associated
259 with *C. difficile* clearance. The data presented here complement those observations. For
260 clindamycin-treated mice, there was no difference in colonization, clearance or relative
261 abundance between PBS and FCT pre-treatment. *C. difficile* had similar colonization
262 dynamics. It is possible that there was insufficient time for the FCT to engraft. However,
263 when we added more time between clindamycin treatment and *C. difficile* challenge, *C.*
264 *difficile* was unable to colonize (data not shown). Therefore, clindamycin-treated mice
265 appeared to have been naturally recovering inhibition to *C. difficile*, which was unaffected
266 by the FCT pre-treatment. For cefoperazone-treated mice, the FCT pre-treatment enabled

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268 the gut microbiota to eliminate the colonization but only in its most concentrated dose.
269 This observation supports our previous discussion (14), indicating that the cefoperazone-
270 treated community is more sensitive to the amount of FCT it receives since cefoperazone
271 reduced many bacterial groups and associations (Figure S6). As we previously
272 hypothesized, streptomycin-treated mice were enabled to clear with a subset of the
273 community, with the FCT pre-treatment diluted 1:10¹. Since we titrated the FCT dilutions,
274 we could compare the bacterial communities of the mice which gained the ability to clear *C.*
275 *difficile* to the mice that received the next dilution which could not to elucidate the minimal
276 relative abundance differences. In agreement with previous studies, OTUs related to
277 *Lachnospiraceae*, *Porphyromonadaceae*, and *Ruminococcaceae* increased with the clearance
278 of *C. difficile* in the streptomycin-treated mice (14, 17–21). These data agree with our
279 previous hypothesis that a reduced fecal community would only be able to promote
280 clearance of *C. difficile* in streptomycin-treated mice.

281 In addition to clearing *C. difficile*, a reduced fecal community restored colonization
282 resistance to streptomycin-treated mice. Mice that received FCT pre-treatment as dilute as
283 1:10² were not colonized to a detectable level. While restoring colonization resistance is
284 not novel (22), here we have shown that the restoration of colonization resistance is
285 dependent on the community perturbation and the fecal community being transplanted. As
286 we identified community members associated with clearance, OTUs related to
287 *Akkermansia*, *Olsenella*, and *Porphyromonadaceae* were more abundant and an OTU related
288 to *Enterobacteriaceae* was less abundant at the time of *C. difficile* challenge.
289 *Enterobacteriaceae* has been associated with *C. difficile* colonization and inflammation (14,
290 23, 24). Larger populations of *Akkermansia* were associated with preventing colonization,

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295 which we had previously observed, potentially indicating the maintenance of a protective
296 mucus layer (14, 25–27). Increased populations of a select set of OTUs related to
297 *Porphyromonadaceae* were also more abundant in mice that were resistant to colonization.
298 *Porphyromonadaceae* may be inhibiting *C. difficile* via butyrate and acetate production,
299 which has been associated with successful FMT treatments (28–30). Different populations
300 of OTUs associated with *Porphyromonadaceae* were associated with colonization resistance
301 than with colonization clearance. These colonization resistance-associated OTUs (OTUs 8,
302 25 and 29) may have OTU-specific functions or dependent abundances of members of the
303 community, such as *Akkermansia* and *Enterobacteriaceae*. With our top-down approach, we
304 reduced the number of gut bacterial community members that were associated with
305 colonization resistance in streptomycin-treated mice.

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306 Further investigation into the heterogeneity of CDI will help to elucidate the niche range *C.*
307 *difficile* and the interventions to eliminate them. Here we were limited by our experimental
308 design and methods to refining our understanding of colonization resistance restoration in
309 streptomycin-treated mice. Future studies can expand beyond the presence and abundance
310 of the bacterial groups and investigate the metabolites and host immune response. A
311 refined understanding of the bacteria, metabolites and host response can help develop
312 more targeted therapies to restore *C. difficile* colonization resistance. Additionally, building
313 up experimental design to incorporate more FGT treatment variations or inoculation
314 regimens could expand our understanding of the necessary components for colonization
315 resistance for each antibiotic treatment. We designed our experiments to closely match
316 previous mouse models for CDI and added days prior to *C. difficile* challenge for the FGT
317 treatment (14, 31, 32). It may be possible to restore colonization resistance to clindamycin

319 once ~~to~~ perazone if the antibiotic treatment, recovery period, and FCT treatment were
320 modified to allow the FCT to have an effect. Other methods could be used to make the mice
321 susceptible to CDI and then tested for the effectiveness of the FCT treatment (18, 33).
322 Further modification and characterization of the fecal communities could reduce the
323 necessary community members and metabolites to promote colonization resistance. The
324 results from these additional studies could expand upon our limitations and reveal specific
325 bacterial communities that could restore *C. difficile* colonization resistance for each
326 susceptibility.

327 We have demonstrated that a ~~reduced~~ bacterial community can restore colonization
328 resistance but the effect of the community and the bacteria that colonized was dependent
329 on the specific changes to the community that were caused by each antibiotic. When
330 transplanting the fecal community into antibiotic-induced susceptible mice, only mice
331 treated with streptomycin were able to restore colonization resistance. Previous studies
332 that have identified ~~reduced~~ communities in a murine model using a homogeneous gut
333 microbiota with a bottom-up approach (7, 19). Treatments supplementing the gut
334 microbiota would benefit from being tested in different communities susceptible to CDI.
335 Further research is necessary to characterize the specific niche spaces *C. difficile* of
336 susceptibility communities and the specific requirements fill those spaces. Then it may be
337 possible to identify people with gut microbiota that are susceptible to CDI and develop
338 targeted ~~reduced~~ bacterial communities to recover colonization resistance and reduce the
339 risk of CDI.

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343 Materials and Methods

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

349 **Antibiotic administration.** Antibiotics were chosen and administered based on previous
350 studies (14, 31, 32). Cefoperazone, clindamycin, and streptomycin treatment produced
351 diverse communities and responses to CDI. Mice were given either cefoperazone,
352 clindamycin, or streptomycin. Cefoperazone (0.5 mg/ml) and streptomycin (5 mg/ml)
353 were administered via drinking water ad libitum for 5 days, beginning 9 days prior to *C.*
354 *difficile* challenge. Antibiotic water was replaced every two days. Clindamycin (10 mg/kg)
355 was injected into the intraperitoneal space, 2 days prior to challenge with *C. difficile*. All
356 antibiotics were filter sterilized with a 0.22 μ m syringe filter prior to use.

357 **Fecal community transplants.** Fecal pellets were collected from similar aged C57BL/6
358 mice not being used in an experiment the day of the fecal community transplants. 15-20
359 pellets were collected and weighed. The fecal pellets were homogenized weight per weight
360 in phosphate-saline buffer (PBS) containing 15% glycerol (fecal community transplant,
361 FCT) in anaerobic conditions. The FCT was serially diluted in PBS containing 15% glycerol
362 down to 1:10⁶ fecal dilution and aliquoted into tubes for gavaging into mice. One set of
363 aliquots were frozen at -80°C to be used the following day for the cefoperazone and
364 streptomycin experiments. Frozen aliquots were thawed at 37°C for 5 minutes prior to

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366 being used. All fecal community dilutions were centrifuged at 7500 RPM for 60 seconds ~~and~~
367 ~~the supernatant was used for inoculation.~~ Mice were inoculated with 100 uL of the fecal
368 dilution oral via a 21 gauge gavage needle. Fecal community transplants were administered
369 from most dilute to least, which began with mice receiving PBS and finished with mice
370 receiving FCT. Aliquots were frozen at -80°C after use for sequencing. These experiments
371 were repeated 8 times with a different starting source each time. ~~This method was adapted~~
372 ~~from our previous study (18).~~

373 **16S rRNA quantitative real-time PCR.** Quantitative analysis of 16S rRNA in fecal
374 community dilutions used for FCT was carried out using quantitative real-time PCR using
375 primers and cycler conditions specified previously (34). Reaction volumes were prepared
376 using 6 uL of SYBRTM Green PCR Master Mix (Applied Biosciences Ref 4344463), 1 uL each
377 forward and reverse primer, and 2 uL sample DNA template. All qPCR reactions were run
378 on a LightCycler 96 (Roche Ref 05815916001) using instrument-specific plates and seals.

379 **C. difficile challenge.** For experiments using streptomycin or cefoperazone, mice were
380 given untreated drinking water for 96 hours before challenging with *C. difficile* strain
381 630Δrm spores. For experiments using clindamycin, mice were given untreated drinking
382 water for 48 hours the time of the intraperitoneal injection and being challenged with *C.*
383 *difficile* strain 630Δrm spores. ~~This time frame was designed to closely replicate the~~
384 ~~previous mouse model (14, 31, 32), with the insertion of a day (clindamycin) or two~~
385 ~~(cefoperazone and streptomycin) for inoculating the mice with the fecal communities.~~ *C.*
386 *difficile* spores were aliquoted from a single spore stock stored at 4°C. Spore concentration
387 was determined two days prior to the day of challenge (35). Mice were inoculated with 10³

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391 *C. difficile* spores via oral gavage. After inoculating the mice, remaining spore solution was
392 serially diluted and plated to confirm the spore concentration.

393 **Sample collection.** Fecal samples were collected prior to administering antibiotics, after
394 antibiotics were removed, prior to *C. difficile* challenge and one each of the 10 days post
395 infection. Approximately 15 mg of each fecal sample was collected and weighed for plating
396 *C. difficile* colony forming units (CFU) and the remaining sample was frozen at -80°C for
397 later sequencing. The weighed fecal samples were anaerobically serially diluted in PBS,
398 plated on TCCFA plates, and incubated at 37°C for 24 hours. The resultant colonies were
399 enumerated to determine the *C. difficile* CFUs (36).

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400 **DNA sequencing.** Total bacterial DNA was extracted from the frozen samples by the
401 MOBIO PowerSoil-htp 96-well soil DNA isolation kit. We amplified the 16S rRNA gene V4
402 region and the amplicons were sequenced on an Illumina MiSeq as described previously
403 (37).

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404 **Sequence curation.** Sequences were processed using mothur (v.1.44.1) (37, 38). We used
405 a 3% dissimilarity cutoff to group sequences into operational taxonomic units (OTUs) and a
406 naïve Bayesian classifier with the Ribosomal Database Project training set (version 16) to
407 assign taxonomic classifications to OTUs (39). We sequenced a mock community of a
408 known 16S rRNA gene sequences and composition. We processed this mock community in
409 parallel with our samples to determine the error rate for our sequence curation, which
410 resulted in an error rate of 0.029%.

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

417 diversity index. For β -diversity comparisons, we calculated dissimilarity matrices based on
418 metric of Yue and Clayton (θ_{YC}) (40). We averaged 1,000 sub-samples of 2,480 counts per
419 sample, ~~arrarified~~, to limit uneven sampling biases. We tested for differences in relative
420 abundance between outcomes with LEfSe in mothur (41). All other statistical analyses and
421 data visualization was completed in R (v4.0.5) with the tidyverse package (v1.3.1).

422 Pairwise comparisons of α -diversity (S_{obs} and Inverse Simpson), β -diversity (θ_{YC}), were
423 calculated by pairwise Wilcoxon rank sum test. Correlations between bacterial genera and
424 fecal community dilution were calculated using the Spearman correlation. *P* values were
425 corrected for multiple comparisons with a Benjamini and Hochberg adjustment for a type I
426 error rate of 0.05 (42). For streptomycin experiments, conditional independence networks
427 were calculated from the day 1 through 5 samples of all mice using SPIEC-EASI (sparse
428 inverse covariance estimation for ecological association inference) methods from the
429 SpiecEasi R package after optimizing lambda to 0.001 with a network stability between
430 0.045 and 0.05 (v1.0.7) (43).

431 **Code availability.** Scripts necessary to reproduce our analysis and this paper are available
432 in an online repository (https://github.com/SchlossLab/Lesniak_restoreCR_mBio_2022).

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

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451 decision to publish, or preparation of the manuscript.

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453 Conceptualization: N.A.L., S.T., P.D.S.; Data curation: N.A.L., L.B., K.M.; Formal analysis:
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456 Writing - original draft: NAL; Writing - review & editing: N.A.L., S.T., A.H., A.T., J.C., L.B., K.B.,
457 P.D.S.; Funding acquisition: P.D.S.; Project administration: P.D.S.; Supervision: P.D.S.

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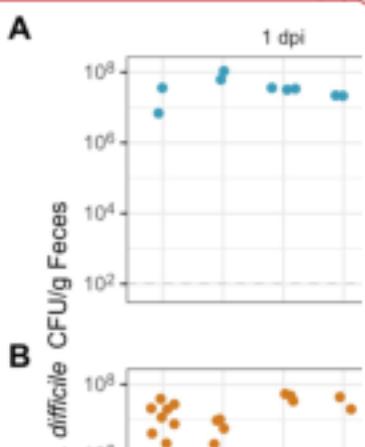
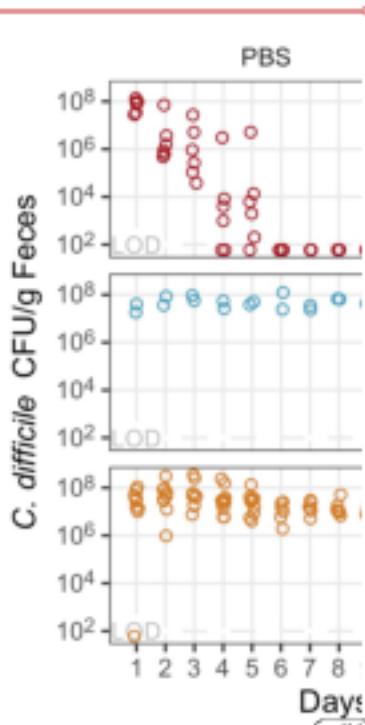
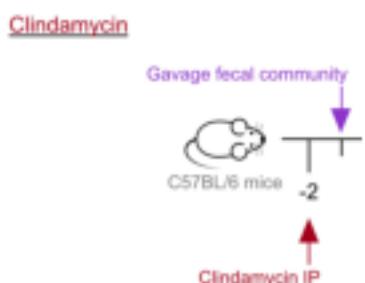
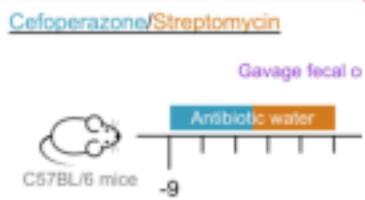
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637 **Figure 1. Mouse experiment timeline.** Mice were given water with cefoperazone (0.5
 638 mg/ml) or streptomycin (5 mg/ml) for 5 days. The mice were returned to untreated water
 639 for the remainder of the experiment. Two days after the antibiotic water was removed,
 640 mice were orally gavaged 100 μ l of PBS or fecal community, once a day for two days. The
 641 following day, the mice were challenged with 10^3 *C. difficile* 630 spores. Alternatively, mice
 642 were given an intraperitoneal injection of clindamycin (10 mg/kg) 2 days prior to *C. difficile*
 643 infection. 24 hours later, mice were orally gavaged with 100 μ l of PBS or fecal community.
 644 The following day, the mice were challenged with 10^3 *C. difficile* 630 spores. Fecal pellets
 645 were collected prior to treatment (day -9 for cefoperazone/streptomycin, day -2 for
 646 clindamycin), cessation of antibiotics (day -2 for cefoperazone/streptomycin, day -1 for
 647 clindamycin), prior to *C. difficile* infection (day 0), and each of the following 10 days.

648 **Figure 2. Fecal community transplant inhibited *C. difficile* colonization for mice**
 649 treated with cefoperazone or streptomycin. *C. difficile* CFU per gram of feces for mice
 650 treated with clindamycin (red points), cefoperazone (blue points), or streptomycin (orange
 651 points). Mice were orally gavaged either PBS (open circles) or FCT (fecal community
 652 transplant, filled circles) prior to the *C. difficile* infection. Each point represents an
 653 individual mouse. (Clindamycin - PBS n = 4, FCT n = 7; Cefoperazone - PBS n = 2, FCT n = 2;
 654 Streptomycin - PBS n = 10, FCT n = 14). LOD = limit of detection.

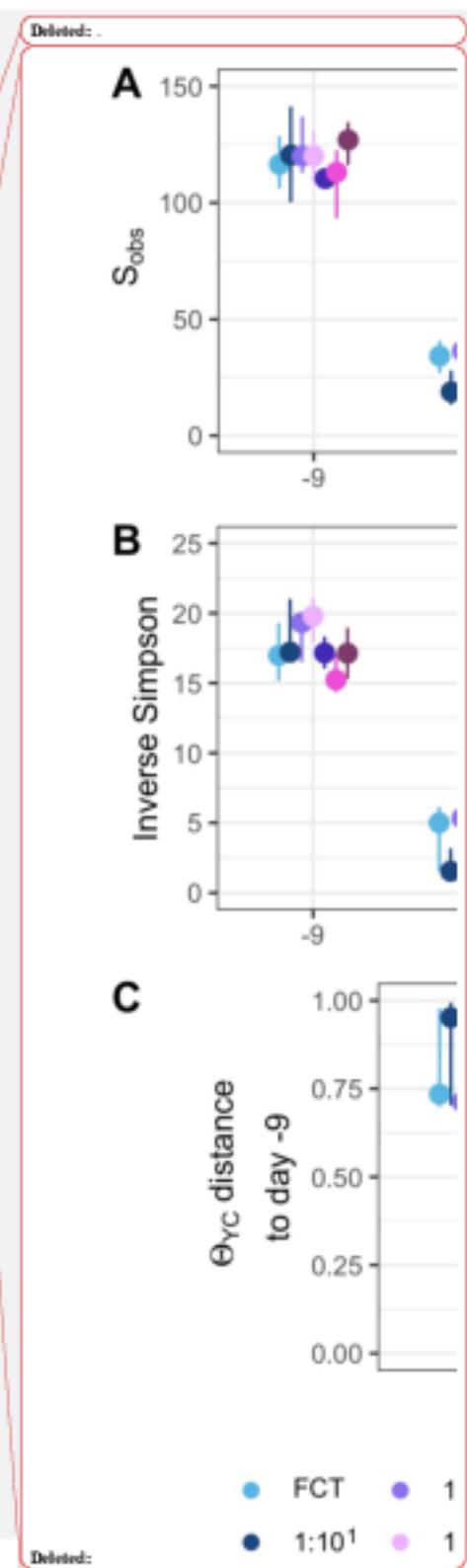
655 **Figure 3. Diluted FCT inhibited *C. difficile* colonization for mice treated with**
 656 streptomycin. *C. difficile* CFU per gram of feces for mice treated with cefoperazone (blue
 657 points) or streptomycin (orange points). Mice were orally gavaged with a dilution of FCT
 658 (1:10 to 1:10⁵) prior to the *C. difficile* infection at (A) one day post *C. difficile* infection (dpi)



676 and (B) 10 dpi. Each point represents an individual mouse. *Cefoperazone* - 1:10 $n = 2$, 1:10 2
 677 $n = 2$, 1:10 3 $n = 3$, 1:10 4 $n = 2$, 1:10 5 $n = 2$; *Streptomycin* - 1:10 $n = 12$, 1:10 2 $n = 14$, 1:10 3 $n =$
 678 5, 1:10 4 $n = 4$, 1:10 5 $n = 5$. LOD = limit of detection.

679 **Figure 4.** Diversity of murine gut bacterial community had not recovered at the time
 680 of *C. difficile* infection in streptomycin-treated mice. α -diversity, measured by S_{obs} (A)
 681 and Inverse Simpson (B), prior to beginning antibiotic treatment (day -9), before fecal
 682 community transplant (day -2), after fecal community transplant on the day of *C. difficile*
 683 challenge (day 0) and at the end of the experiment (day 10). (C) β -diversity, measured by
 684 θ_{YC} , distance between community structures on day 0 or 10 compared to the community
 685 prior to antibiotic treatment (day -9) community of that individual. Data are grouped by
 686 the transplant received, undiluted fecal community (FCT), diluted fecal community (1:10 1 -
 687 1:10 5), or PBS. Points are median values and lines represent the interquartile range.

688 **Figure 5.** Bacterial community OTUs differentially abundant in streptomycin-treated
 689 mice which resisted or cleared colonization. Murine gut bacterial community OTUs that
 690 were significantly different by LEfSe analysis. OTUs from streptomycin-treated mice at the
 691 time of *C. difficile* challenge (day 0) which were differentially abundant between (A) mice
 692 that were colonized (dark green) and those that were not (no detectable CFU throughout
 693 the experiment, bright green) or (B) mice that remained colonized (dark green) and those
 694 that cleared colonization (CFU reduced to below the limit of detection by the end of the
 695 experiment, faint green). (C) OTUs from streptomycin-treated mice at the end of the
 696 experiment (day 10) which were differentially abundant between mice that remained
 697 colonized (dark green) and those that cleared colonization (CFU reduced to below the limit



A

Akkermansia (OTU 3)
 Clostridiales* (OTU 102)
 Enterobacteriaceae* (OTU 4)
 Olsenella (OTU 72)
 Porphyromonadaceae* (OTU 8)
 Porphyromonadaceae* (OTU 25)
 Porphyromonadaceae* (OTU 29)

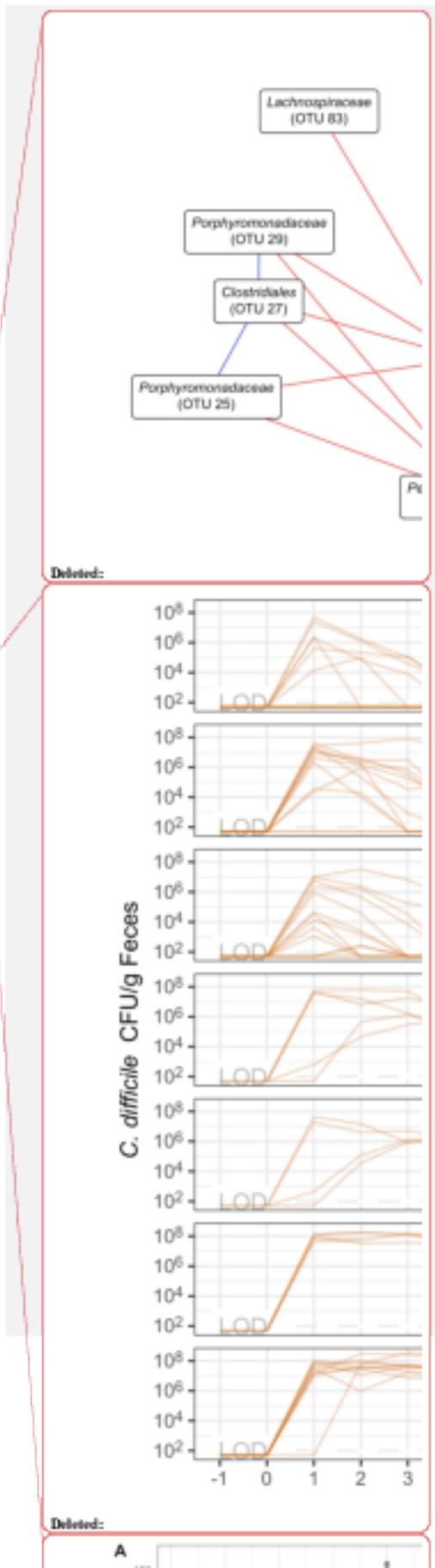
704 of detection by the end of the experiment, faint green). Points are median values and lines
705 represent the interquartile range. Dashed vertical line is the limit of detection. OTUs
706 ordered alphabetically. * indicates that the OTU was unclassified at lower classification
707 rank.

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

709 Network constructed with SpecEasi from the OTU relative abundances and *C. difficile* CFU
710 data from 1 through 5 days post *C. difficile* infection. Red lines represent negative
711 associations and blue lines indicate positive associations. *C. difficile* is based on CFU counts
712 and *Pectostreptococcaceae* (OTU 19), the OTU most closely related to *C. difficile*, is based on
713 sequence counts. Only *C. difficile* subnetwork shown.

714 **Figure S1. *C. difficile* colonization dynamics in streptomycin-treated mice across all**
715 **prophylactic transplant treatments.** *C. difficile* CFU per gram of feces for streptomycin-
716 treated mice orally gavaged PBS, fecal community transplant (FCT), or diluted FCT (1:10-
717 1:10⁵) prior to the *C. difficile* infection. Each semi-transparent line represents an individual
718 mouse. Mice challenged with 10³ *C. difficile* 630 spores on day 0. Lines grouped by the
719 transplant treatment received. LOD = limit of detection.

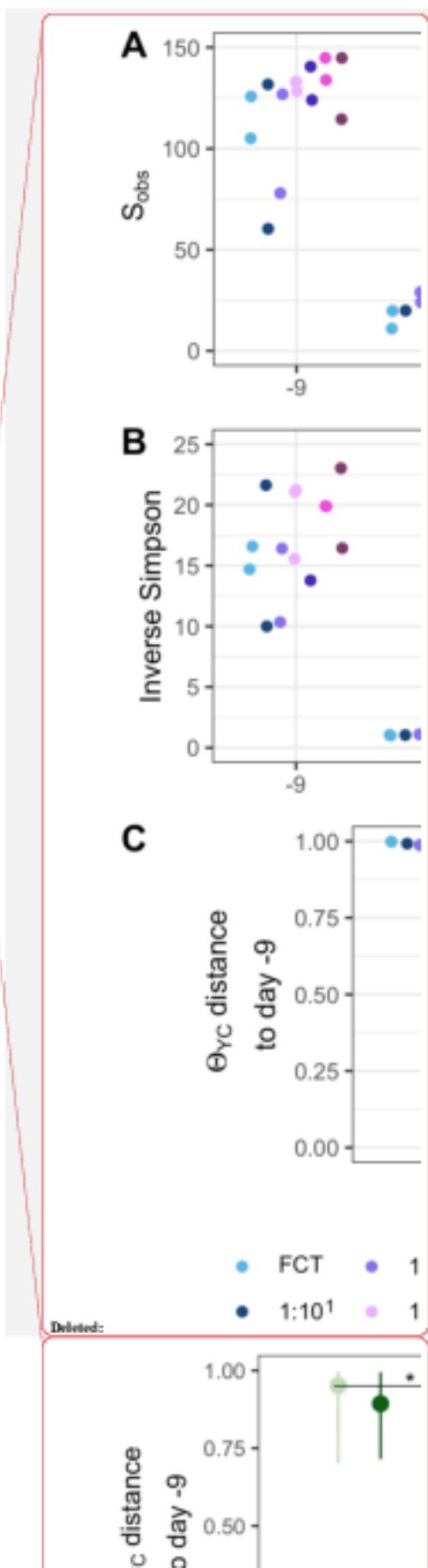
720 **Figure S2. Diversity and quantification of fecal community dilutions used for**
721 **prophylactic transplants in antibiotic-treated mice.** (A-C) Diversity of fecal community
722 dilutions. α -diversity, measured by (A) S_{obs} and (B) Inverse Simpson for undiluted fecal
723 community (FCT) and diluted fecal communities (1:10-1:10⁵). Points are individual
724 samples. (C) β -diversity, measured by θ_{VC} , community structure of feces collected from
725 untreated mice, undiluted fecal community (FCT), and diluted fecal communities (1:10-



732 1:10⁵) compared to untreated feces. Points are median values and lines represent the
 733 interquartile range. (D) Cq values for qPCR of FCT and its dilutions for eubacterial 16S
 734 rRNA gene. Points are median values and lines represent the interquartile range. (E)
 735 Relative abundance of bacterial taxonomic groups that significantly correlate with fecal
 736 community dilutions (FCT-1:10³) by Spearman correlation. Points are individual mice. *
 737 indicates that the bacterial taxonomic group was unclassified at lower classification rank.

738 **Figure S3.** Diversity of murine gut bacterial community was not recovered at the
 739 time of *C. difficile* infection in cefoperazone-treated mice. Diversity changes through
 740 experiments with cefoperazone-treated mice. α -diversity, measured by (A) S_{obs} and (B)
 741 Inverse Simpson, prior to beginning antibiotic treatment (day -9), before fecal community
 742 transplant [day -2], after fecal community transplant on the day of *C. difficile* infection (day
 743 0) and at the end of the experiment (day 10). (C) β -diversity, measured by θ_{YC} , distance
 744 between community structures on day 0 or 10 compared to the community prior to
 745 antibiotic treatment (day -9) community of that individual. Data are grouped by the
 746 transplant received, undiluted fecal community (FCT), diluted fecal community (1:10¹-
 747 1:10⁵), or PBS. Points are individual mice.

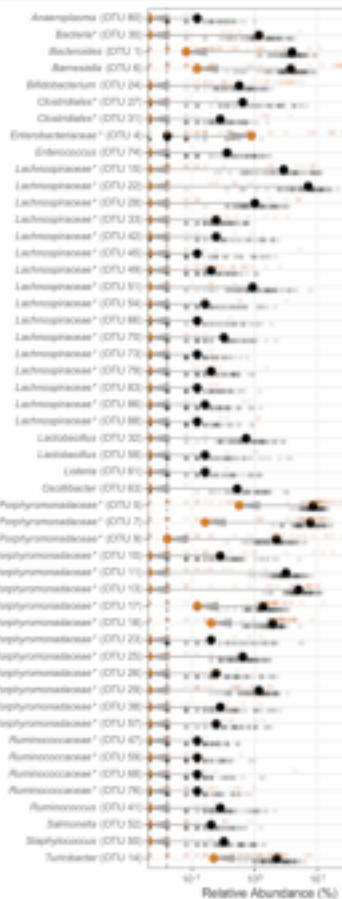
748 **Figure S4.** Gut bacterial community of streptomycin-treated mice that cleared
 749 colonization were more similar to their initial community. Diversity differences by
 750 outcome in streptomycin-treated mice. β -diversity, measured by θ_{YC} , distance between
 751 community structures on day 0 or 10 compared to the community prior to antibiotic
 752 treatment (day -9) community of that individual. Data are grouped by the outcome, cleared
 753 colonization (faint green) or remain colonized (dark green). Points are median values and



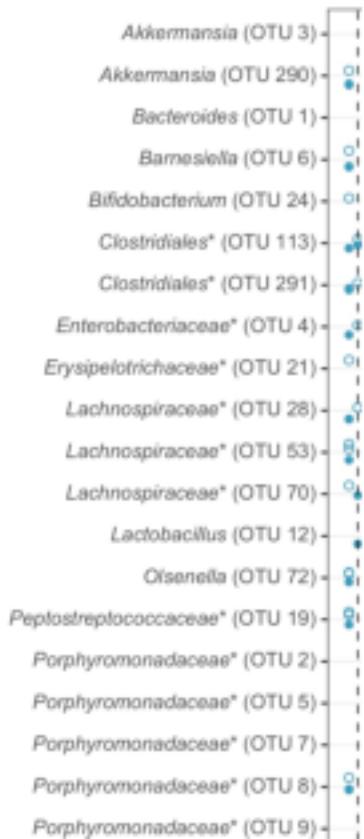
758 lines represent the interquartile range. * indicates significant difference by Wilcoxon rank
759 sum test with Bonferroni correction.

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

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



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