

# **An osmotic laxative renders mice susceptible to prolonged *Clostridioides difficile* colonization and hinders clearance**

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**1 Abstract**

2 Antibiotics are a major risk factor for *Clostridioides difficile* infections (CDIs) because of their  
3 impact on the microbiota. However, non-antibiotic medications such as the ubiquitous osmotic  
4 laxative polyethylene glycol (PEG) 3350, also alter the microbiota, but whether PEG impacts  
5 CDI susceptibility and clearance is unclear. To examine how PEG impacts susceptibility, we  
6 treated C57Bl/6 mice with 5-day and 1-day doses of 15% PEG in the drinking water and then  
7 challenged the mice with *C. difficile* 630. We used clindamycin-treated mice as a control because  
8 they consistently clear *C. difficile* within 10 days post-challenge (dpc). PEG treatment alone  
9 was sufficient to render mice susceptible and 5-day PEG-treated mice remain colonized for up  
10 to 30 dpc. In contrast, 1-day PEG treated mice were transiently colonized, clearing *C. difficile*  
11 within 7 dpc. To examine how PEG treatment impacts clearance, we administered a 1-day PEG  
12 treatment to clindamycin-treated, *C. difficile*-challenged mice. Administering PEG to mice after *C.*  
13 *difficile* challenge prolonged colonization up to 30 dpc. When we trained a random forest model  
14 with community data from 5 dpc, we were able to predict which mice would exhibit prolonged  
15 colonization (AUROC = 0.90). Five of the top ten bacterial features important for predicting  
16 prolonged colonization had high relative abundances in the community. Examining the dynamics of  
17 these bacterial during the post-challenge period revealed patterns in the relative abundances of  
18 *Bacteroides*, *Enterobacteriaceae*, *Porphyromonadaceae*, *Lachnospiraceae*, and *Akkermansia* that  
19 were associated with prolonged *C. difficile* colonization in PEG-treated mice.

**20 Importance**

21 Diarrheal samples induced by medications such as laxatives are typically rejected for *Clostridioides*  
22 *difficile* testing. However, there are some microbiota similarities between diarrheal and *C. difficile*  
23 infection (CDI) communities such as lower diversity compared to healthy controls, which lead us  
24 to hypothesize that diarrhea may be an indicator of *C. difficile* risk. We explored how osmotic  
25 laxatives disrupt the microbiota's colonization resistance to *C. difficile* by administering a laxative to  
26 mice either before or after *C. difficile* challenge Our findings suggest the osmotic laxative disrupts  
27 colonization resistance to *C. difficile*, as well as clearance in mice already colonized with *C. difficile*.  
28 Considering that most hospitals recommend not performing *C. difficile* testing on patients taking

- 29 laxatives and laxatives are used when administering fecal microbiota transplants via colonoscopy
- 30 to patients with recurrent CDIs, further studies are needed to evaluate if laxatives impact microbiota
- 31 colonization resistance in humans.

## 32 **Introduction**

33 Antibiotics are a major risk factor for *Clostridioides difficile* infections (CDIs) because they disrupt  
34 microbiota colonization resistance (1). However, antibiotics are not the only types of medications  
35 that disrupt the microbiota (2–4). Although, other medications (proton pump inhibitors, osmotic  
36 laxatives, antimotility agents, and opioids) have been implicated as risk or protective factors for CDIs  
37 through epidemiological studies, whether the association is due to their impact on the microbiota is  
38 still unclear (5–9).

39 Many of the non-antibiotic medications associated with CDIs are known to modulate gastrointestinal  
40 motility leading to either increased or decreased colonic transit time, which in turn also strongly  
41 impacts microbiota composition and function (10, 11). Stool consistency often serves as an  
42 approximation of intestinal motility (10). Our group has shown that when *C. difficile* negative  
43 controls are separated into two groups based on stool consistency, there are shared microbiota  
44 features such as lower alpha diversity in samples from CDI patients and control patients with  
45 diarrhea compared to control samples that were *C. difficile* negative with non-diarrheal consistency  
46 (12). These results led to a hypothesis that bacterial communities from patients experiencing  
47 diarrhea are susceptible to developing CDIs.

48 Osmotic laxatives can lead to diarrhea depending on the administered dose and temporarily disrupt  
49 the human intestinal microbiota (13). The ubiquitous osmotic laxative, polyethylene glycol (PEG)  
50 3350 is found in Miralax, Nulytely, and Golytely and is also commonly used as bowel preparation  
51 for colonoscopies. Interestingly, previous studies have shown that treating mice with PEG alone  
52 altered microbiota composition, reduced acetate and butyrate production, altered the mucus barrier,  
53 and rendered the mice susceptible to *C. difficile* colonization (14–17). The mucus barrier is thought  
54 to mediate protection from CDIs by protecting intestinal epithelial cells from the toxins produced by  
55 *C. difficile* (18, 19). Whether laxative administration results in more severe CDIs in mice and how  
56 long mice remain colonized with *C. difficile* after challenge is unclear.

57 Beyond susceptibility, PEG is also relevant in the context of treating recurrent CDIs via fecal  
58 microbiota transplant (FMT) where a healthy microbiota is administered to the patient to restore  
59 colonization resistance. For FMTs that are delivered via colonoscopy, patients typically undergo

60 bowel preparation by taking an osmotic laxative prior to the procedure. Many of the FMT studies to  
61 date rationalize the use of laxatives (20–22) based on a 1996 case study with 2 pediatric patients  
62 where the authors suggested in the discussion that the laxative may help flush *C. difficile* spores  
63 and toxins from the intestine (23).

64 Our group has used C57BL/6 mice to characterize how antibiotics disrupt the microbiota and  
65 influence *C. difficile* susceptibility and clearance (24–26). Although, two groups have now shown  
66 PEG treatment alone renders mice susceptible to *C. difficile* (15, 17), these studies have raised  
67 additional questions regarding the dynamics and severity of infection as well as the role of laxative  
68 treatment in *C. difficile* clearance. Addressing these questions will better inform how we think  
69 about laxatives in the context of CDIs. Here, we characterized how long PEG-treated mice remain  
70 susceptible, whether PEG treatment results in more sustained *C. difficile* colonization and severe  
71 CDI than mice treated with clindamycin, and if PEG treatment after challenge can promote *C.*  
72 *difficile* clearance.

## 73 Results

74 **5-day laxative treatment led to prolonged *C. difficile* colonization in mice.** Building off of  
75 previous work that showed treating mice with the osmotic laxative, PEG 3350, rendered mice  
76 susceptible to *C. difficile* colonization (15, 17), we decided to test how long *C. difficile* colonization  
77 is sustained and how long PEG-treated mice remain susceptible to *C. difficile*. We compared three  
78 groups of mice treated with PEG 3350 to one group of mice treated with our standard 10 mg/kg  
79 clindamycin treatment, which temporarily renders the mice susceptible to *C. difficile* colonization,  
80 with mice typically clearing *C. difficile* within 10 days post-challenge (9, 26). All three groups of  
81 PEG-treated mice were administered a 15% PEG solution in the drinking water for 5-days: 1.  
82 received no additional treatment, 2. was also treated with clindamycin, and 3. was allowed to  
83 recover for 10 days prior to challenge (Fig. 1A). PEG treatment resulted in weight loss in all 3 groups  
84 of PEG-treated mice relative to their baseline weights, with the greatest change in weight observed  
85 on the fifth day of PEG treatment. The mice recovered most of the lost weight by five days after  
86 treatment (Fig. 1B). After either the PEG, clindamycin, or PEG and clindamycin treatment all mice  
87 were challenged with  $10^5$  *C. difficile* 630 spores (Fig. 1A). All treatments rendered mice susceptible

88 to *C. difficile* colonization. However, PEG-treated mice remained colonized with *C. difficile* at a  
89 high level through thirty days post-challenge (Fig. 1C). In contrast, the clindamycin-treated mice  
90 cleared *C. difficile* within ten days post-challenge (Fig. 1C). Therefore, PEG treatment led to  
91 sustained colonization in contrast to clindamycin mice that naturally cleared *C. difficile* within ten  
92 days post-challenge.

93 Notably, we also found PEG-treated mice were still susceptible to *C. difficile* colonization after a  
94 10-day recovery period, although *C. difficile* was not detectable in most of the group in the initial  
95 five days post-challenge (Fig. 1C, S1A). One mouse was found dead on the 6th day post-challenge,  
96 presumably due to *C. difficile* as the bacteria became detectable in stool samples from that mouse  
97 on the 4th day post-challenge (Fig. S1A, mouse 10). From 8 days post-challenge onward, the  
98 density of *C. difficile* stabilized in the 10-day recovery group and remained high through 20-30  
99 days post-challenge (Fig. 1C). Thus, osmotic laxative treatment alone was sufficient to render  
100 mice susceptible to prolonged *C. difficile* colonization and PEG-treated mice remained susceptible  
101 through ten days post-treatment.

102 **5-day laxative treatment differentially disrupted the fecal microbiota compared to**  
103 **clindamycin treatment.** Since osmotic laxatives and clindamycin have previously been  
104 shown to disrupt the murine microbiota (14–17), we hypothesized the different *C. difficile*  
105 colonization dynamics between mice treated with the osmotic laxative or clindamycin were due to  
106 the two drugs having differential effects on the microbiota. We profiled the stool microbiota over  
107 time by sequencing the V4 region of the 16S rRNA gene to compare changes across treatment  
108 groups. We found time and treatment group explained half of the observed variation between fecal  
109 communities with most of the remaining variation explained by interactions between treatment  
110 group and other experimental variables including time, cage effects, and sequencing preparation  
111 plate (PERMANOVA combined  $R^2 = 0.95$ ,  $P < 0.001$ , Fig. 2A, Data Set S1, Sheet X). Cage effects  
112 refer to the well-documented phenomenon that mice housed in the same cages have similar  
113 microbial communities due to coprophagy (27). We tried to minimize the impact of cage effects on  
114 our experiment by breaking up littermates when assigning mice to treatment groups. Importantly,  
115 although we conducted a total of 5 separate experiments, the experiment number and its interaction  
116 with treatment group did no significantly explain the observed variation in fecal communities (Data

117 Set S1, Sheet X). None of the treatment groups recovered to their baseline community structure  
118 either 10 or 30 days post-challenge suggesting other community features besides recovery to  
119 baseline were responsible for the prolonged *C. difficile* colonization in PEG-treated mice (Fig. 2B).

120 Since none of the communities completely recovered in the follow-up period after treatments, we  
121 next profiled community diversity and composition. We examined the alpha diversity dynamics by  
122 calculating the communities' Shannon diversity. Although both clindamycin and PEG treatments  
123 decreased diversity, Shannon diversity was lower in the groups of mice that received PEG treatment  
124 compared to those that received clindamycin through thirty days post-challenge (Fig. 2C). We next  
125 examined the bacterial genera that shifted after PEG treatment by comparing the baseline samples  
126 of mice treated with only PEG to samples from the same mice one day post-PEG-treatment. We  
127 found 18 genera that were altered by PEG treatment (Data Set S1, Sheet X). The majority of the  
128 bacterial relative abundances decreased after the PEG treatment, but the relative abundance among  
129 members of the *Enterobacteriaceae* and *Bacteroides* increased. The increase in *Bacteroides*  
130 relative abundance was unique to PEG treated mice, as the *Bacteroides* relative abundance  
131 actually decreased in clindamycin treated mice (Fig. 2D). Finally, we identified the genera whose  
132 relative abundance differed across treatment groups over multiple time points. Of the 33 genera  
133 that were different between treatment groups, 24 genera were different over multiple time points  
134 (Fig. 2E, Data Set S1, Sheet X). Thus, PEG had a significant impact on the fecal microbiota that  
135 was maintained over time and was distinct from clindamycin treatment.

136 Interestingly, *C. difficile* was not immediately detectable in the stools of the PEG-treated mice  
137 that were allowed to recover for 10 days prior to challenge. We decided to examine if there were  
138 genera that changed during the post-challenge period when the group median *C. difficile* shifted  
139 from undetectable at 1 day post-challenge to detectable in the stool samples with the density  
140 stabilizing around 8 days post-challenge (Fig. S1A). We found no bacteria with relative abundances  
141 that were significantly different over the two time points after multiple hypothesis correction (Data  
142 Set S1, Sheet X). However, there was also wide variation between individual mice regarding  
143 when *C. difficile* became detectable (Fig. S1A) as well as the relative abundances of bacterial  
144 genera in the communities (Fig. S1B). For example, two mice had a high relative abundance of  
145 *Enterobacteriaceae* throughout the post-challenge period and this corresponded to mouse 10,

146 which died on the sixth day post-challenge and mouse 11, where *C. difficile* was present at a high  
147 density from the 4th day post-challenge onward (Fig. S1B). While we did not identify a clear signal  
148 to explain the delayed appearance of *C. difficile* in the 5-day PEG mice that were allowed to recover  
149 for 10 days prior to challenge, the delay is striking and could reflect changes in microbial activity or  
150 metabolites that were not examined in this study.

151 **5-day laxative treatment did not promote more severe CDIs despite altering the mucosal**  
152 **microbiota.** Given the findings from a previous study that demonstrated PEG treatment disrupts  
153 the mucus layer and alters the immune response in mice (16), we decided to examine the impact of  
154 PEG treatment on the mucosal microbiota and CDI severity. To evaluate the mucosal microbiota,  
155 we sequenced snips of tissue collected from the cecum, proximal colon, and distal colon. Similar  
156 to what was observed with the stool samples, alpha diversity was lower in the PEG-treated mice  
157 compared to clindamycin treated mice (Fig. 3A). Alpha diversity continued to increase over  
158 time based on the tissues from PEG-treated mice collected at 20 and 30 days post-challenge  
159 (Fig. 3A, Data Set S1, Sheet X). Group, time point, and their interactions with other variables  
160 (cage, experiment number, and sample type) explained the majority of the variation observed  
161 in mucosal communities (PERMANOVA combined  $R^2 = 0.83$ ,  $P < 0.05$ , Fig. 3B, Data Set S1,  
162 Sheet X). We saw the greatest difference in the relative abundance of the mucosal microbiota  
163 between treatment groups (clindamycin, 5-day PEG, and 5-day PEG plus clindamycin) at 6 days  
164 post-challenge with 10 genera that were significantly different ( $P < 0.05$ ) in all three of the tissue  
165 types we collected (cecum, proximal colon, and distal colon; Fig. S2A, Data Set S1, Sheet X).  
166 Interestingly, *Peptostreptococcaceae* (the family with a sequence that matches *C. difficile*) was one  
167 of the genera that had a significant difference in relative abundance between treatment groups  
168 at 6 days post-challenge. This population was primarily only present in the 5-day PEG treatment  
169 group of mice and decreased in the proximal and distal colon tissues over time (Fig. S2B). By 30  
170 days post-challenge, only the relative abundances of *Bacteroides*, *Clostridiales*, *Firmicutes*, and  
171 *Ruminococcaceae* were different between treatment groups and only in the cecum tissues (Fig.  
172 3C, Fig. 2E, Data Set S1, Sheet X). Thus, PEG treatment had a significant impact on the mucosal  
173 microbiota and we detected *C. difficile* sequences in the cecum, proximal colon, and distal colon  
174 tissue communities.

175 Because there were differences in the mucosal microbiota including detectable *C. difficile* sequences  
176 in tissues from PEG-treated mice relative to mice treated with clindamycin, we next examined the  
177 severity of *C. difficile* challenge by evaluating cecum and colon H&E stained histopathology (28).  
178 However, we found there was no difference in cecum and colon scores between clindamycin and  
179 PEG-treated mice that were challenged with *C. difficile* at 4 days post-challenge (Fig. 3D), the  
180 time point typically examined in *C. difficile* 630 challenged mice (29). We also looked at 6 days  
181 post-challenge because that was when there was a large difference in *C. difficile* density between  
182 PEG- and clindamycin-treated mice (Fig. 1C). Although, there was a slight difference in the colon  
183 between PEG and clindamycin-treated mice, there was not a significant difference in the cecum and  
184 the overall score was relatively low (1.5-2.5 out of 12, Fig. 3E). Therefore, although PEG treatment  
185 had a disruptive effect on the mucosal microbiota, the impact of *C. difficile* 630 challenge on the  
186 cecum and colon was similar between PEG and clindamycin treated mice.

187 ***C. difficile* challenge did not have a synergistic disruptive effect on the microbiota of**  
188 **PEG-treated mice.** Because *C. difficile* itself can have an impact on the microbiota (30), we also  
189 sequenced the tissue and stools of mock-challenged clindamycin and 5-day PEG treated mice.  
190 Examining the stools of the mock-challenged mice revealed similar bacterial disruptions as the *C.*  
191 *difficile* challenged mice (Fig. S3A-C). Similarly, there was no difference between the tissues of  
192 mock and *C. difficile* challenged mice (Fig. S3D-F). Thus, most of the microbiota alterations we  
193 observed in the PEG-treated mice were a result of the laxative and not an interaction between the  
194 laxative and *C. difficile*.

195 **1-day laxative treatment resulted in transient *C. difficile* colonization and minor microbiota**  
196 **disruption.** Next, we examined how a shorter osmotic laxative perturbation would impact the  
197 microbiome and susceptibility to *C. difficile*. We administered either a 1-day PEG treatment, a  
198 1-day PEG treatment with a 1-day recovery period, or clindamycin to mice before challenging  
199 them with *C. difficile* (Fig. 3A). In contrast to the 5-day PEG treated mice, the 1-day PEG treated  
200 mice were only transiently colonized and cleared *C. difficile* by 7 days post-challenge (Fig. 3B).  
201 The stool communities of PEG-treated mice were also only transiently disrupted, with Shannon  
202 diversity recovering by 7 days post-challenge (Fig. 3C-D). We found the relative abundances of 14  
203 genera were impacted by treatment, but recovered close to baseline levels by 7 days post-challenge

204 including *Enterobacteriaceae*, *Clostridiales*, *Porphyromonadaceae*, and *Ruminococcaceae* (Fig. 3E,  
205 Data Set S1, Sheet X). These findings suggest the duration of the PEG treatment was relevant,  
206 with shorter treatments resulting in a transient loss of *C. difficile* colonization resistance.

207 **Post-challenge laxative treatment disrupted clearance in clindamycin-treated mice**  
208 **regardless of whether an FMT was also administered.** Since a 1-day PEG treatment resulted  
209 in a more mild microbiota perturbation, we decided to use the 1-day treatment to examine the  
210 hypothesis that PEG helps to flush *C. difficile* spores from the intestine. To examine the impact of  
211 PEG treatment on *C. difficile* clearance, we treated 4 groups of mice with clindamycin and then  
212 challenged all mice with *C. difficile* before administering the following treatments: no additional  
213 treatment, 1-day PEG immediately after challenge, and 1-day PEG treatment 3 days after challenge  
214 followed by either administration of an FMT or PBS solution by oral gavage (Fig. 5A). Contrary to  
215 our hypothesis, all groups of mice that received PEG exhibited prolonged *C. difficile* colonization  
216 (Fig. 5B).

217 We were also interested in exploring whether PEG might help with engraftment in the context of  
218 FMTs. The FMT appeared to partially restore Shannon diversity but not richness (Fig. 5C-D).  
219 Similarly, we saw some overlap between the communities of mice that received FMT and the mice  
220 treated with only clindamycin after 5 days post-challenge (Fig. 6A). The increase in Shannon  
221 diversity suggests that the FMT did have an impact on the microbiota, despite seeing prolonged  
222 *C. difficile* colonization in the FMT treated mice. However, only the relative abundances of  
223 *Bacteroidales* and *Porphyromonadaceae* consistently differed between the mice received either  
224 an FMT or PBS gavage (Fig. 6B), suggesting the FMT only restored a couple of genera. Overall,  
225 we found the relative abundances of 24 genera were different between treatment groups over  
226 multiple timepoints. For example, the relative abundance of *Akkermansia* was increased and the  
227 relative abundances of *Ruminococcaceae*, *Clostridiales*, *Lachnospiraceae*, and *Oscillibacter* were  
228 decreased in mice that received PEG after *C. difficile* challenge relative to clindamycin treated mice  
229 (Fig. 6C). In sum, administering PEG actually prolonged *C. difficile* colonization, including in mice  
230 that received an FMT, which only restored 2 bacterial genera.

231 **Five-day post-challenge community data can predict which mice that will have prolonged**

232 **C. difficile colonization.** After identifying bacteria associated with the 5-day, 1-day and  
233 post-challenge 1-day PEG treatments, we decided to examine the bacteria that influenced  
234 prolonged *C. difficile* colonization. We trained 3 types of machine learning models (random  
235 forest, logistic regression, and support vector machine) with bacterial community data from 5  
236 days post-challenge to predict whether the mice were still colonized with *C. difficile* 10 days  
237 post-challenge. We chose 5 days post-challenge because that was the earliest time point where  
238 we would see a treatment effect in the post-challenge 1-day PEG experiments. The random  
239 forest model had the highest performance (median AUROC = 0.90, Data Set S1, Sheet X), so we  
240 next performed permutation importance to examine the bacteria that were the top contributors  
241 to the random forest model predicting prolonged *C. difficile* colonization. We selected the top 10  
242 bacteria contributing to our model's performance (Fig. 7A) and examined their relative abundance  
243 at 5 days post-challenge, the time point used to predict *C. difficile* colonization status on day 10  
244 (Fig. 7B). Next, we focused on the 5 genera that had a greater than 1 % relative abundance in  
245 either the cleared or colonized mice and examined how the bacteria changed over time. We found  
246 *Enterobacteriaceae* and *Bacteroides* tended to have a higher relative abundance, the relative  
247 abundance of *Akkermansia* was initially decreased and then increased, and *Porphyromonadaceae*  
248 and *Lachnospiraceae* had a lower relative abundance in the mice with prolonged colonization  
249 compared to the mice that cleared *C. difficile* (Fig. 7C). Together these results suggest a  
250 combination of low and high abundance bacterial genera influence the prolonged colonization  
251 observed in 5-day PEG and post-challenge 1-day PEG treated mice.

## 252 Discussion

253 While the disruptive effect of antibiotics on *C. difficile* colonization resistance is well established,  
254 the extent to which other drugs such as laxatives disrupt colonization resistance was unclear. By  
255 following osmotic laxative treated mice over time, we found 5-day PEG treatment before challenge  
256 resulted in prolonged *C. difficile* colonization, while a 1-day PEG treatment resulted in transient  
257 colonization. The differences in *C. difficile* colonization dynamics between the 5- and 1-day PEG  
258 treated mice were associated with differences in how much the treatments disrupted the microbiota.  
259 Additionally, the intestinal communities of 5-day PEG treated mice had not regained colonization

260 resistance after a 10-day recovery period. Although in contrast to the other 5-day PEG treatment  
261 groups, *C. difficile* was not immediately detectable in the stools of most of the mice in the 10-day  
262 recovery group. We also examined the impact of PEG treatment after *C. difficile* challenge and  
263 in opposition to the hypothesis suggested by the literature, found that PEG treatment prolonged  
264 colonization relative to mice that only received clindamycin treatment. We identified patterns in  
265 the relative abundances of *Bacteroides*, *Enterobacteriaceae*, *Akkermansia*, *Porphyromonadaceae*,  
266 and *Lachnospiraceae* that were associated with prolonged *C. difficile* colonization (Fig. 8). Overall,  
267 our results demonstrated that osmotic laxative treatment alone rendered mice susceptible to *C.*  
268 *difficile* colonization and the duration of colonization depended on the length of PEG treatment and  
269 whether treatment was administered before or after challenge.

270 In addition to altering composition, laxative treatment may alter microbiota-produced metabolites. A  
271 previous study demonstrated that a 5-day treatment of 10% PEG depleted acetate and butyrate  
272 and increased succinate compared to untreated mice (15). While we did not perform metabolomic  
273 analysis, we did see bacteria known to produce beneficial metabolites were depleted in mice  
274 that cleared *C. difficile* compared to mice with prolonged colonization. For example, *Oscillibacter*  
275 *valericigenes* can produce the SCFA valerate (31), and separate studies demonstrated valerate  
276 is depleted after clindamycin treatment and inhibited *C. difficile* growth based on *in vitro* data  
277 and an experiment with C57BL/6 mice (32, 33). Similarly, *Acetatifactor* can produce acetate and  
278 butyrate (34), SCFAs that are decreased in mice with prolonged *C. difficile* infection after antibiotic  
279 treatment (35). Thus protective bacteria and their metabolites could be depleted by osmotive  
280 laxative treatment depending on the timing and duration of treatment.

281 One possible explanation for the prolonged *C. difficile* colonization in 5-day PEG treated mice,  
282 might be due to the bacteria's persistence in the mucosal compartment. In fact, it has been  
283 hypothesized that *C. difficile* biofilms may serve as reservoirs for recurrent infections (36) and *C.*  
284 *difficile* biofilms were recently identified in patients and aggregates with *Fusobacterium nucleatum*,  
285 another species capable of forming biofilms (37). There was an interesting pattern of increased  
286 *Enterobacteriaceae*, *Bacteroides*, and *C. difficile* in both the stool and mucosal communities of  
287 PEG-treated mice suggesting a potential synergy. *Bacteroides* has the potential to degrade mucus  
288 and the osmotic laxative may have allowed *Bacteroides* to colonize the mucosal niche by degrading

289 mucin glycans with glycosyl hydrolases that are absent in *C. difficile* (38). *Bacteroides* persistent  
290 in the mucosal tissue might also have helped *Enterobacteriaceae* to colonize the region, as a  
291 synergy between mucus-degrading *B. fragilis* and *E. coli* has previously been described (39). A  
292 separate study demonstrated *C. difficile* was present in the outer mucus layer and associated with  
293 *Enterobacteriaceae* and *Bacteroidaceae* using fluorescent in situ hybridization (FISH) staining (40).  
294 However, *B. fragilis* prevented CDI morbidity in a mouse model and inhibited *C. difficile* adherence  
295 *in vitro* (41). In coculture experiments *B. longum* decreased *C. difficile* biofilm formation while *B.*  
296 *thetaiotamicron* enhanced biofilm formation (42). Therefore, whether *Bacterodes* is detrimental or  
297 beneficial in the context of *C. difficile* infection or colonization is still unclear.

298 *Akkermansia* is also a mucin degrader with potentially beneficial or detrimental roles depending  
299 on context in other diseases (43, 44). In our study the relative abundance of *Akkermansia* shifted  
300 over time between groups. In the stool it was initially increased in mice that cleared *C. difficile*, but  
301 shifted after 5-days post-challenge so that it was increased in mice that had prolonged colonization.  
302 In the context of CDIs, some studies suggest a protective role (45, 46), while others suggest  
303 detrimental (47–49). Because the relative abundance of *Akkermansia* was dynamic in our study so  
304 it is unclear whether *Akkermansia* helps with clearance of *C. difficile* or allows it to persist. A better  
305 understanding how *C. difficile* interacts with the mucosal microbiota may lead to insights into CDIs,  
306 asymptomatic *C. difficile* colonization, and resistance.

307 Despite identifying an altered compositional profile that included higher relative abundance of  
308 the *C. difficile* sequence in the mucosal tissues of mice treated with 5-day PEG compared to the  
309 clindamycin group, we did not see a difference in histopathology scores between the groups. One  
310 reason there was no difference could be the *C. difficile* strain used, *C. difficile* 630 results in mild  
311 histopathology summary scores in mice compared to VPI 10463 despite both strains producing  
312 toxin in mice (50). Part of our hypothesis for why there could have been increased histopathology  
313 scores in PEG-treated mice was because PEG was previously shown to disrupt the mucus layer in  
314 mice. However, recent studies demonstrated that broad spectrum antibiotics can also disrupt the  
315 host mucosal barrier in mice (51, 52). Future research is needed to tease out the interplay between  
316 medications that influence the mucus layer and different strains of *C. difficile* in the context of CDIs.

317 It is more difficult what are findings mean in the context of *C. difficile* colonization resistance mean  
318 for human patients. Considering that most hospitals recommend not performing *C. difficile* testing  
319 on patients taking laxatives and laxatives are used when administering fecal microbiota transplants  
320 via colonoscopy to patients with recurrent CDIs. further studies are needed to evaluate if laxatives  
321 impact human microbiota colonization resistance. Further studies are needed to understand the  
322 impact of osmotic laxatives on *C. difficile* colonization resistance and clearance in human patients.

323 + What's known regarding laxatives and susceptibility to CDIs + Clinical trial of PEG, results never  
324 posted (53)

325 Relevance to human FMTs? Unclear what the best administration route is because there have  
326 been no studies designed to evaluate the best administration route for FMTs. However, results  
327 from the FMT National Registry where 85% of FMTs were delivered by colonoscopy demonstrate  
328 FMTs are highly effective treatment for recurrent CDIs with 90% achieving resolution in the 1 month  
329 follow-up window (54).

330 We have demonstrated that osmotic laxative treatment alone has a substantial impact on the  
331 microbiota and rendered mice susceptible to prolonged *C. difficile* colonization in contrast to  
332 clindamycin-treated mice. The duration and timing of the laxative treatment impacted the duration of  
333 *C. difficile* colonization, with only 5-day PEG and post-challenge 1-day PEG treatments prolonging  
334 colonization compared to clindamycin treated mice. Further studies are warranted to ascertain  
335 whether laxatives have a similar impact on *C. difficile* colonization resistance on the human  
336 microbiota.

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348 **Materials and Methods**

349 **Animals.** All experiments were approved by the University of Michigan Animal Care and Use  
350 Committee IACUC (protocol numbers PRO00006983 and PRO00008975). All mice were C57Bl/6  
351 and part of the Schloss lab colony which was established in 2010 with mice donated from Vincent  
352 Young's lab colony (established with mice purchased from The Jackson Laboratory in 2002). We  
353 used 7-19 week old female mice for all experiments, which allowed us to break up littermates prior  
354 to starting an experiment and distribute them as evenly as possible across treatment groups in  
355 order to minimize microbiota differences between experiment groups prior to starting treatments  
356 with medications. During the experiment, mice were housed at a density of 2-3 mice per cage, with  
357 the majority of cages limited to two mice.

358 **Drug treatments.** For PEG treatment groups, fifteen percent PEG 3350 (Miralax) was administered  
359 in the drinking water for either 5 or 1-day periods depending on the experiment. PEG solution  
360 was prepared fresh every 2 days in distilled water and administered to the mice in water bottles.  
361 Clindamycin treatment groups received distilled water in water bottles during the PEG-treatment  
362 periods, with the water being changed at the same frequency. For clindamycin treatment, groups of  
363 mice received 10 mg/kg clindamycin (Sigma-Aldrich) via intraperitoneal injection. All PEG treatment  
364 groups received a sham intraperitoneal injection containing filter sterilized saline.

365 **C. difficile challenge model.** Mice were challenged with 25 microliters off *C. difficile* 630 spores  
366 at  $10^5$  concentration, except for 1 experiment (Fig. 5A) where the concentration was  $10^3$ . All mock  
367 challenged mice received 25 ul vehicle solution (Ultrapure water). A Dymax stepper pipette was  
368 used to administer the same challenge dose to mice via oral gavage. Mice were weighed daily  
369 throughout the experiment and stool was collected for quantifying *C. difficile* CFU and 16S rRNA  
370 gene sequencing. There were two groups of mice that received either a PBS or fecal microbiota

371 transplant (FMT) gavage post-PEG treatment. The fecal microbiota transplant was prepared with  
372 stool samples collected from the mice in the experiment prior to the start of any treatments. The  
373 stool samples were transferred to an anaerobic chamber and diluted 1:10 in reduced PBS and  
374 glycerol was added to make a 15% glycerol solution. The solution was then aliquoted into tubes and  
375 stored at -80 °C until the day of the gavage. An aliquot of both the FMT and PBS solutions were also  
376 set aside in the -80 °C for 16S rRNA gene sequencing. The day of the gavage, aliquots were thawed  
377 and centrifuged at 7500 RPM for 1 minute. The supernatant was then transferred to a separate  
378 tube to prevent the gavage needle from clogging with debris during gavage. The PBS solution that  
379 was administered to the other group was also 15% glycerol. Each mouse was administered 100  
380 microliters of either the FMT or PBS solution via gavage. When we refer to mice that cleared *C.*  
381 *difficile*, we mean that no *C. difficile* was detected in the first serial dilution (limit of detection: 100  
382 CFU). In some experiments, we collected tissues for 16SrRNA gene sequencing, histopathology,  
383 or both. For 16S rRNA gene sequencing, we collected small snips of cecum, proximal colon,  
384 and distal colon tissues in microcentrifuge tubes, snap froze in liquid nitrogen, and stored at  
385 -80 °C. For histopathology, cecum and colon tissues were placed into separate cassettes, fixed, and  
386 then submitted to McClinchey Histology Labs (Stockbridge, MI) for processing, embedding, and  
387 hematoxylin and eosin (H&E) staining.

388 ***C. difficile* quantification.** Stool samples from mice were transferred to an anaerobic chamber and  
389 serially diluted in reduced PBS. Serial dilutions were plated onto taurocholate-cycloserine-cefoxitin-fructose  
390 agar (TCCFA) plates plates and counted after 24 hours of incubation at 37 °C. Stool samples  
391 collected from the mice on day 0 post-challenge were also plated onto TCCFA plates to ensure  
392 mice were not already colonized with *C. difficile* prior to challenge.

393 **16S rRNA gene sequencing.** Stool samples that were stored in the -80 °C were placed into 96-well  
394 plates for DNA extractions and library preparation. DNA was extracted using the DNeasy Powersoil  
395 HTP 96 kit (Qiagen) and an EpMotion 5075 automated pipetting system (Eppendorf). For library  
396 preparation, each plate had a mock community control (ZymoBIOMICS microbial community DNA  
397 standards) and a negative control (water). The V4 region of the 16S rRNA gene was amplified with  
398 the AccuPrime Pfx DNA polymerase (Thermo Fisher Scientific) using custom barcoded primers, as  
399 previously described (55). The PCR amplicons were normalized (SequalPrep normalizatin plate kit

400 from Thermo Fisher Scientific), pooled and quantified (KAPA library quantification kit from KAPA  
401 Biosystems), and sequenced with the MiSeq system (Illumina).

402 **16S rRNA gene sequence analysis.** All sequences were processed with mothur (v. 1.43)  
403 using a previously published protocol (55, 56). Paired sequencing reads were combined and  
404 aligned with the SILVA (v. 132) reference database (57) and taxonomy was assigned with a  
405 modified version (v. 16) of the Ribosomal Database Project (v. 11.5) (58). The error rate for  
406 sequencing data was 0.0559% based on the 17 mock communities we ran with the samples.  
407 Samples were rarefied to 1,000 sequences, 1,000 times for alpha and beta diversity analyses  
408 in order to account for uneven sequencing across samples. All but 3 out of 17 water controls  
409 had less than 1000 sequences. PCoAs were generated based on Bray-Curtis Index distance  
410 matrices. Permutational multivariate analysis of variance (PERMANOVA) tests were performed on  
411 mothur-generated Bray-Curtis distance matrices with the adonis function from the vegan R package  
412 (59).

413 **Histopathology.** H&E stained sections of cecum and colon tissues collected at either 0, 4, or 6  
414 days post-challenge were coded to be scored in a blinded manner by a board-certified veterinary  
415 pathologist (ILB). Slides were evaluated using a scoring system developed for mouse models of *C.*  
416 *difficile* infection (50). Each slide was evaluated for edema, cellular infiltration, and inflammation  
417 and given a score ranging from 0-4. The summary score was calculated by combining the scores  
418 from the 3 categories and ranged from 0-12.

419 **Classification model training and evaluation.** We used the mikropml package to train and  
420 evaluate models to predict *C. difficile* colonization status 10 days post-challenge where mice were  
421 categorized as either cleared or colonized (60, 61). We removed the *C. difficile* genus relative  
422 abundance data prior to training the model. Input community relative abundance data at the  
423 genus level from 5 days post-challenge was used to generate random forest, logistic regression,  
424 and support vector machine classification models to predict *C. difficile* colonization status 10 days  
425 post-challenge. To accommodate the small number of samples in our data set we used 50% training  
426 and 50% testing splits with repeated 2-fold cross-validation of the training data for hyperparameter  
427 tuning. Permutation importance was performed as described previously (62) using mikropml (60,

428 61) with the random forest model because it had the highest AUROC value.

429 **Statistical analysis.** R (v. 4.0.2) and the tidyverse package (v. 1.3.0) were used for statistical  
430 analysis (63, 64). Kruskal-Wallis tests with Benjamini-Hochberg correction for testing multiple time  
431 points were used to analyze differences in *C. difficile* CFU, mouse weight change, and alpha  
432 diversity between treatment groups. Paired Wilcoxon rank signed rank tests were used to identify  
433 genera impacted by treatments on matched pairs of samples from 2 time points. Bacterial relative  
434 abundances that varied between treatment groups at the genus level were identified with the  
435 Kruskal-Wallis test with Benjamini-Hochberg correction for testing all identified OTUs, followed by  
436 pairwise Wilcoxon comparisons with Benjamini-Hochberg correction.

437 **Code availability.** Code for data analysis and generating this paper with accompanying figures is  
438 available at [https://github.com/SchlossLab/Tomkovich\\_PEG3350\\_XXXX\\_2021](https://github.com/SchlossLab/Tomkovich_PEG3350_XXXX_2021).

439 **Data availability.** The 16S rRNA sequencing data have been deposited in the National Center for  
440 Biotechnology Information Sequence Read Archive (BioProject Accession no. PRJNA727293).

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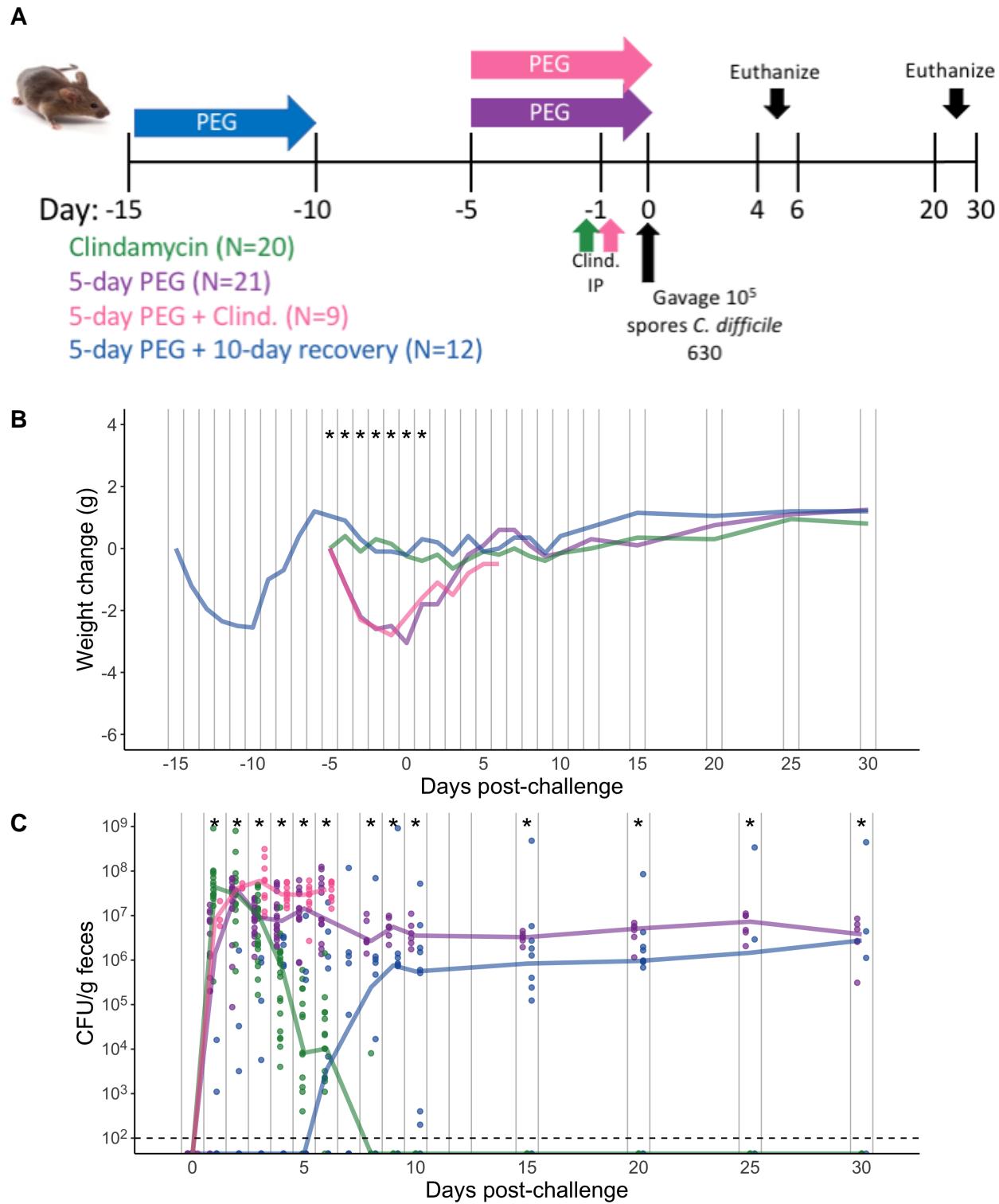
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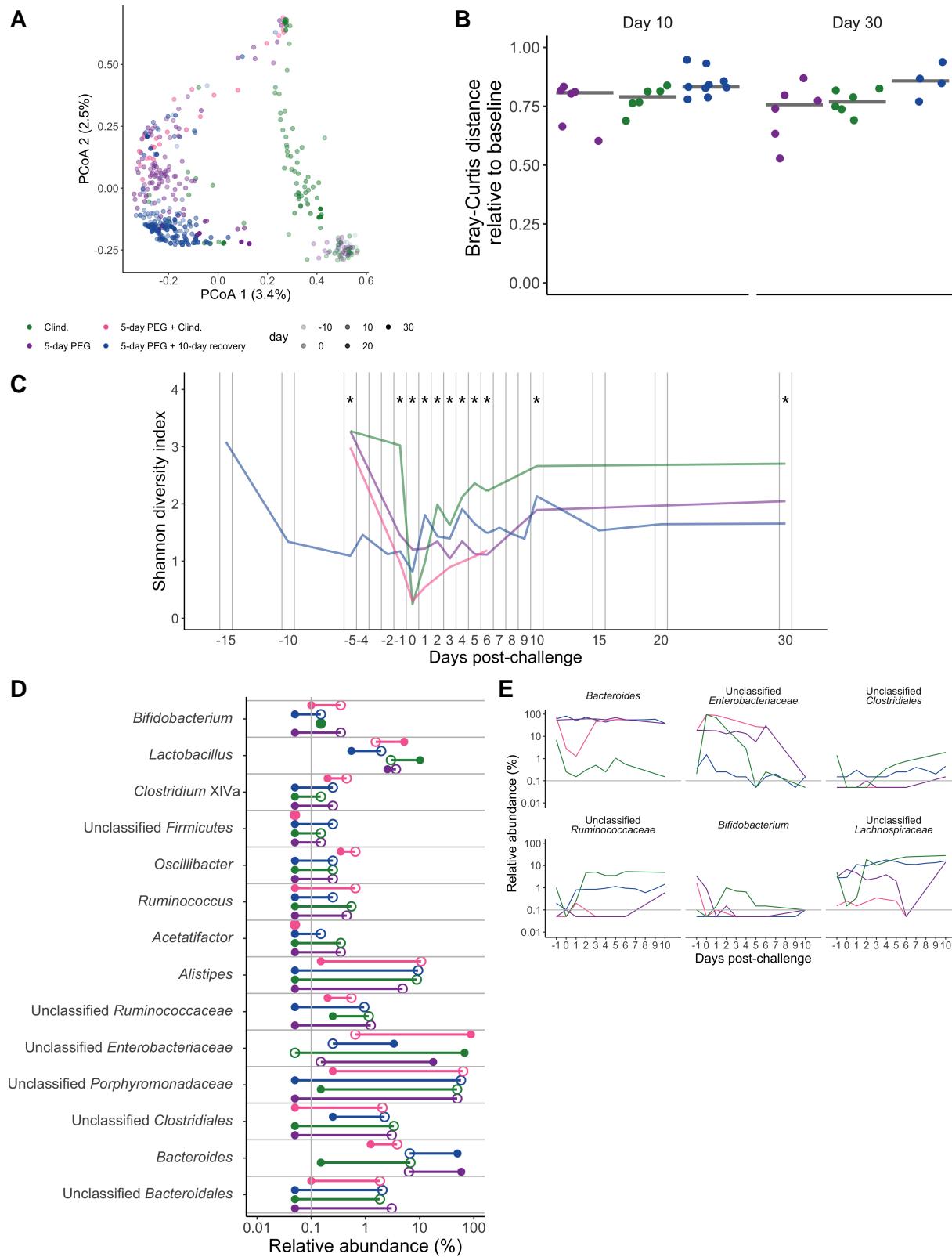
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658 **Figure 1. 5-day PEG treatment prolongs susceptibility and mice become persistently**  
 659 **colonized with *C. difficile*.** A. Setup of the experimental time line for experiments with 5-day PEG  
 660 treated mice consisting of 4 treatment groups. 1. Clindamycin was administered at 10 mg/kg by

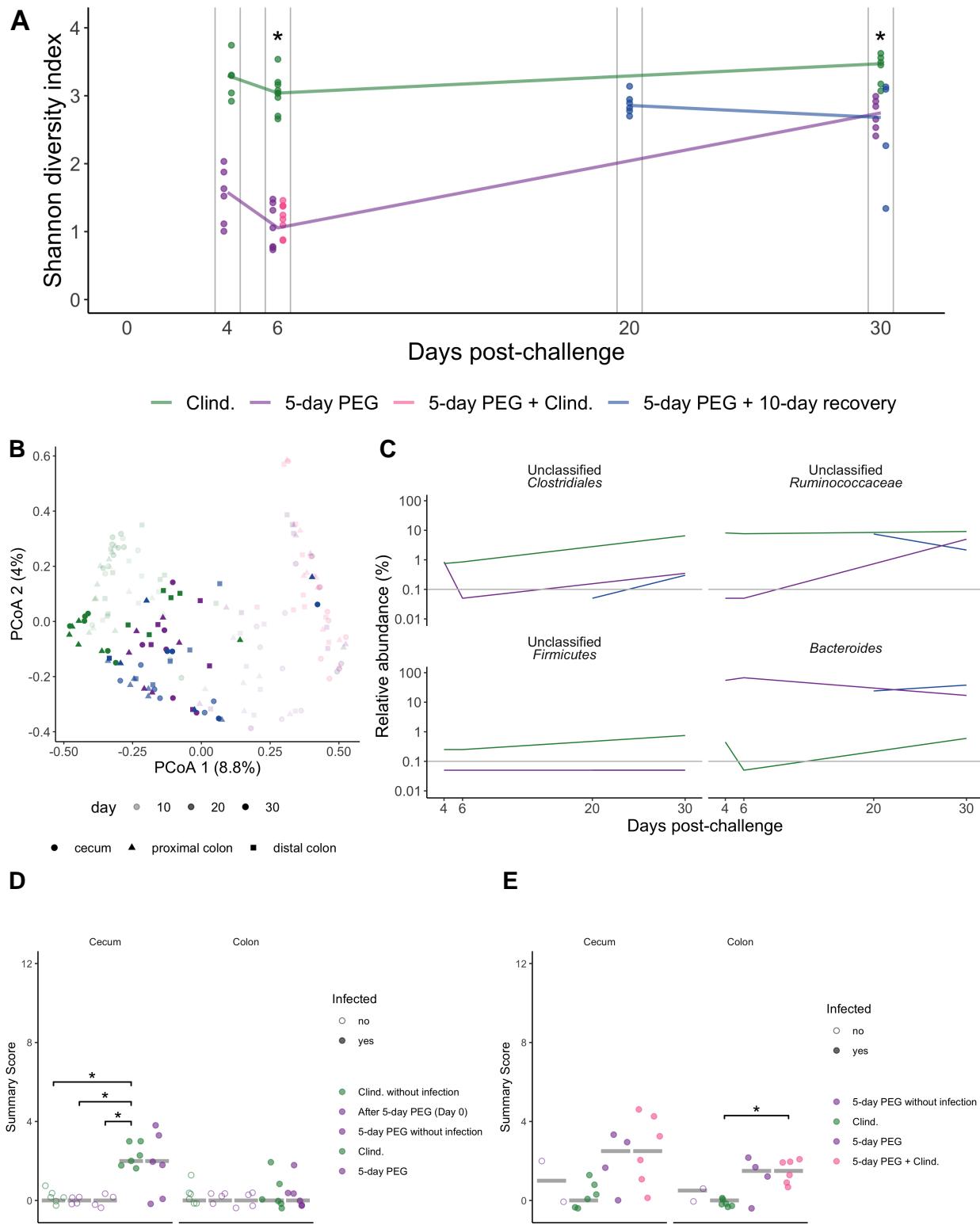
intraperitoneal injection. 2. 15% PEG 3350 was administered in the drinking water for five days. 3. 5-day PEG plus clindamycin treatment. 4. 5-day PEG plus 10-day recovery treatment. All treatment groups were then challenged with  $10^5$  *C. difficile* 630 spores. A subset of mice were euthanized on either 4 or 6 days post-challenge and tissues were collected for histopathology analysis, the remaining mice were followed through 20 or 30 days post-challenge. B. Weight change from baseline weight in groups after treatment with PEG and/or clindamycin, followed by *C. difficile* challenge. C. *C. difficile* CFU/gram stool measured over time via serial dilutions(N = 10-59 mice per time point). The black line represents the limit of detection for the first serial dilution. CFU quantification data was not available for each mouse due to stool sampling difficulties (particularly the day the mice came off of the PEG treatment) or early deaths. For B-C, lines represent the median for each treatment group and circles represent samples from individual mice. Asterisks indicate time points where the weight change or CFU/g was significantly different between groups by the Kruskal-Wallis test with Benjamini-Hochberg correction for testing multiple time points. The data presented are from a total of 5 separate experiments.



675

676 **Figure 2. 5-day PEG treatment disrupts the stool microbiota for a longer amount of time**

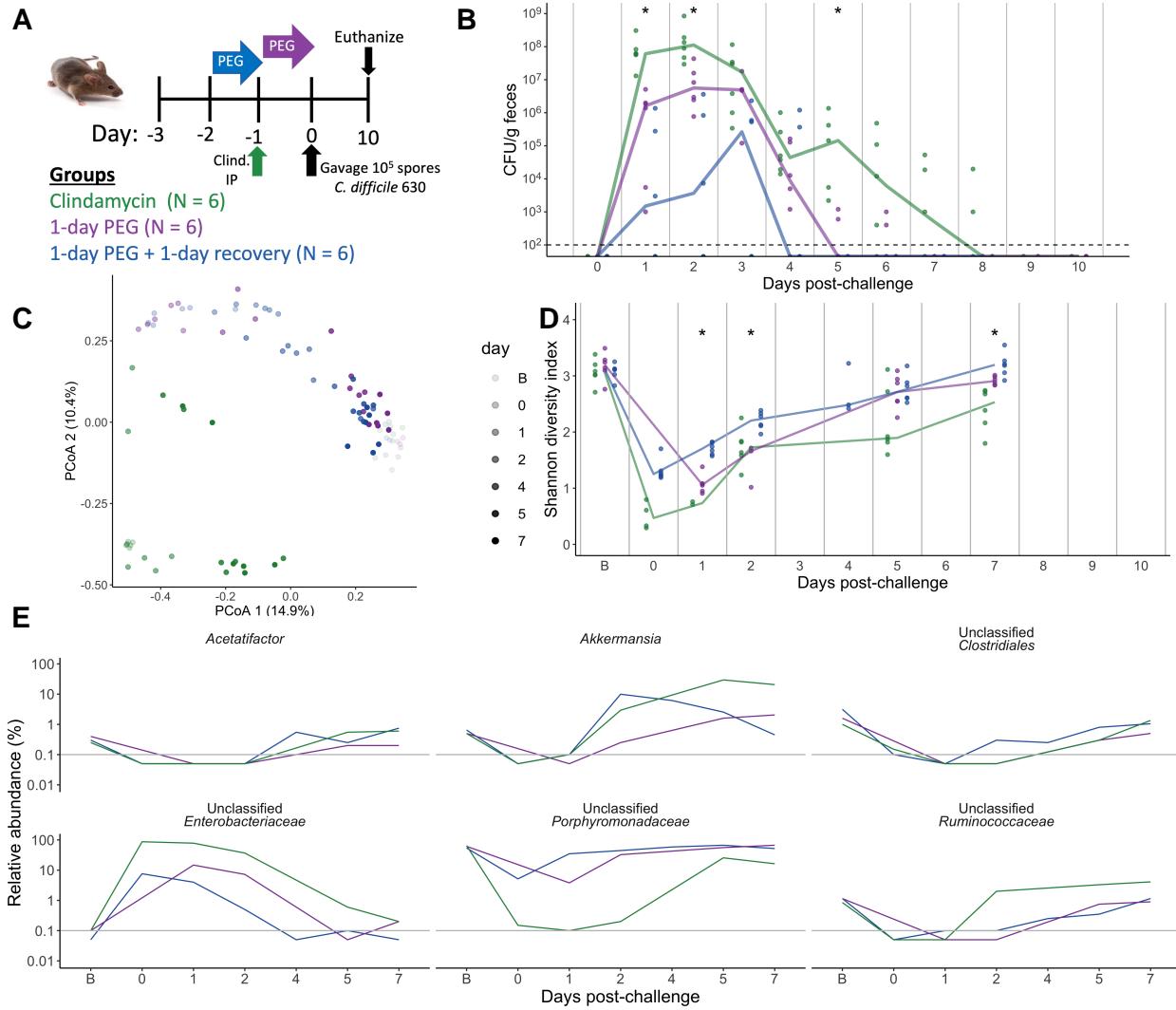
677 **compared to clindamycin-treated mice.** A. Principal Coordinate analysis (PCoA) of Bray-Curtis  
678 distances from stool samples collected throughout the experiment. Each circle represents  
679 a sample from an individual mouse and the transparency of the symbol corresponds to the  
680 day post-challenge. B. Bray-Curtis distances of stool samples collected on either day 10 or  
681 30 post-challenge relative to the baseline sample collected for each mouse (before any drug  
682 treatments were administered). C. Shannon diversity in stool communities over time. The line  
683 indicates the median value for each treatment group. For B-C, the symbols represent samples  
684 from individual mice and the line indicates the median value for each treatment group. D. 14 of  
685 the 33 genera affected by PEG treatment (Data Set S1, sheet X). The symbols represent the  
686 median relative abundance for a treatment group at either baseline (open circle) or 1-day post  
687 treatment (closed circle). Relative abundance data from paired baseline and 1-day post treatment  
688 stool samples from the 5-day PEG and 5-day PEG plus 10-day recovery groups were analyzed  
689 by paired Wilcoxon signed-rank test with Benjamini-Hochberg correction for testing all identified  
690 genera. The clindamycin and 5-day PEG plus clindamycin treatment groups are shown on the  
691 plot for comparison. E. 6 of the 24 genera that were significantly different between the treatment  
692 groups over multiple time points, the 5-day PEG plus clindamycin treatment group was only  
693 followed through 6-days post-challenge. Differences between treatment groups were identified by  
694 Kruskal-Wallis test with Benjamini-Hochberg correction for testing all identified genera. The gray  
695 vertical line (D) and horizontal vertical lines (E) indicate the limit of detection.



696

697 **Figure 3. 5-day PEG treatment does not result in more severe CDIs, although mucosal**

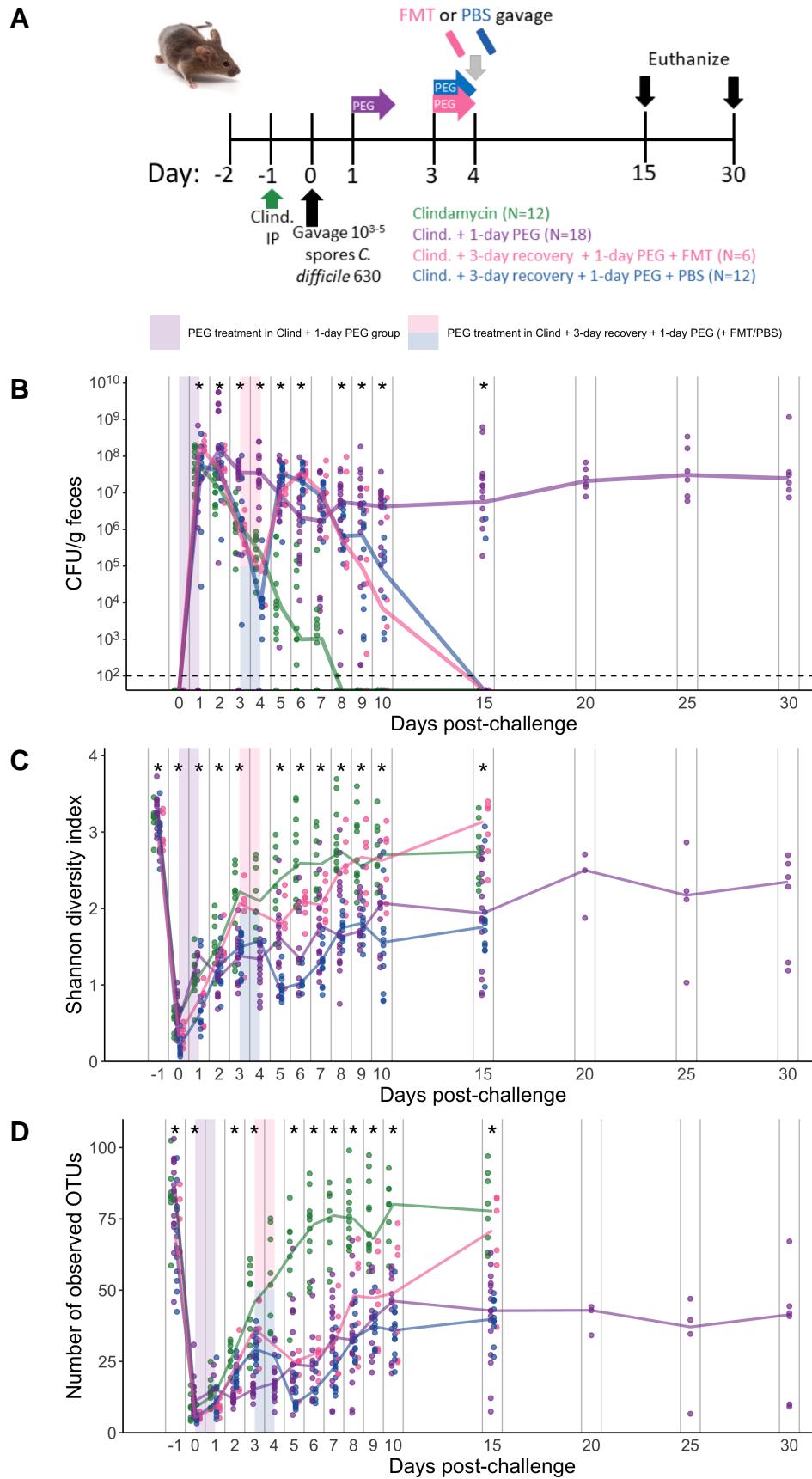
698 **microbiota is altered.** A. Shannon diversity in cecum communities over time. The line indicates  
699 the median value for each treatment group. The colors of the symbols and lines represent  
700 individual and median relative abundance values for four treatment groups. A similar pattern was  
701 observed with the proximal and distal colon communities (Data Set S1, sheet X-X). B. PCoA of  
702 Bray-Curtis distances from mucosal samples collected throughout the experiment. Circles, triangles,  
703 and squares indicate the cecum, proximal colon, and distal colon communities, respectively.  
704 Transparency of the symbol corresponds to the day post-challenge that the sample was collected.  
705 C. The median relative abundance of the 4 genera that were significantly different between the  
706 cecum communities of different treatment groups on day 6 and day 30 post-challenge (Data Set S1,  
707 sheet X). The gray vertical lines indicate the limit of detection. D-E. The histopathology summary  
708 scores from cecum and colon H&E stained tissue sections. The summary score is the total score  
709 based on evaluation of edema, cellular infiltration, and inflammation in either the cecum or colon  
710 tissue. Each category is given a score ranging from 0-4, thus the maximum possible summary  
711 score is 12. The tissue for histology was collected at either 4 (D) or 6 (E) days post-challenge  
712 with the exception that one set of 5-day PEG treated mock-challenged mice were collected on  
713 day 0 post-challenge (first set of open purple circles in D). Histology data were analyzed with the  
714 Kruskal-Wallis test followed by pairwise Wilcoxon comparisons with Benjamini-Hochberg correction.



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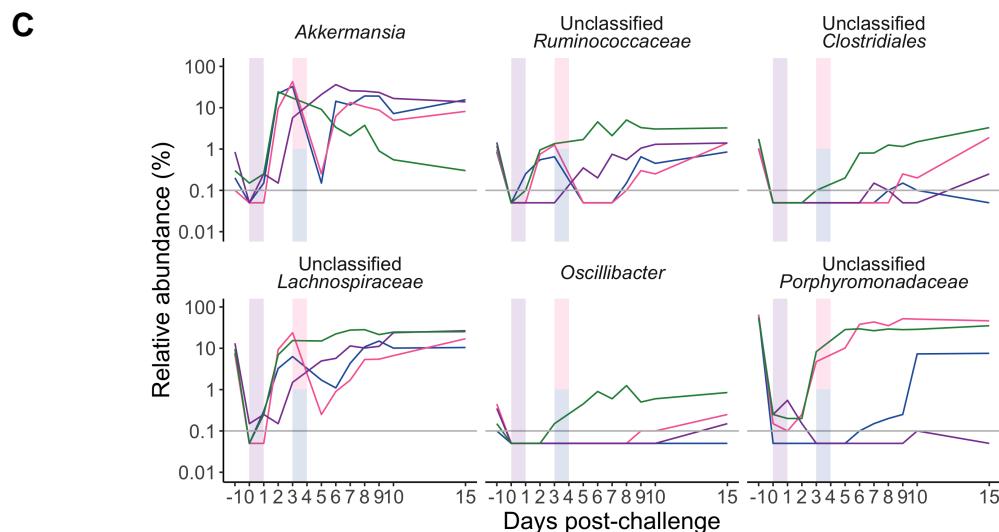
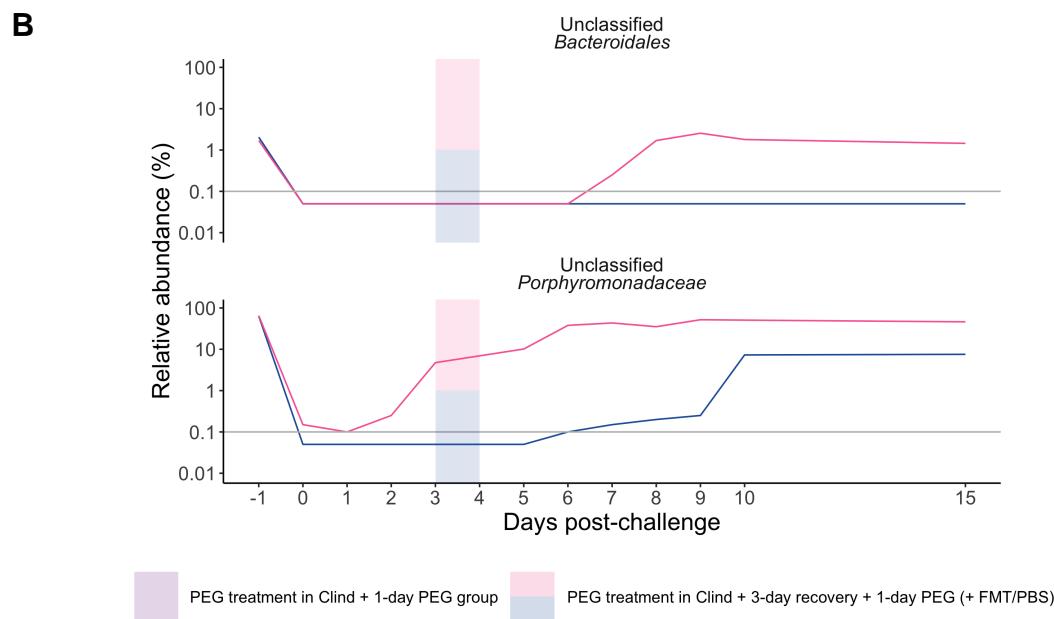
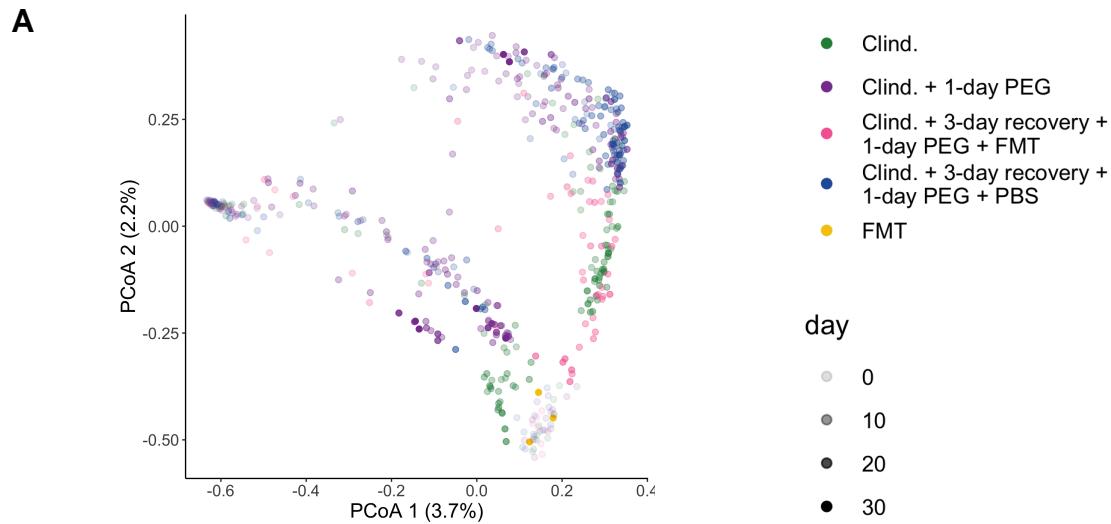
716 **Figure 4. 1-day PEG treatment renders mice susceptible to transient *C. difficile***  
 717 **colonization.** A. Setup of the experimental time line for the 1-day PEG treated mice consisting of 3  
 718 treatment groups. 1. Clindamycin was administered at 10 mg/kg by intraperitoneal injection. 2.  
 719 15% PEG 3350 was administered in the drinking water for 1 day. 3. 1-day PEG plus 1-day recovery.  
 720 The three treatment groups were then challenged with  $10^5$  *C. difficile* 630 spores. B. *C. difficile*  
 721 CFU/gram stool measured over time (N = 12-18 mice per time point) by serial dilutions. The black  
 722 dashed horizontal line represents the limit of detection for the first serial dilution. For B and D,  
 723 asterisks indicate time points where there was a significant difference between treatment groups by  
 724 Kruskall-Wallis test with Benjamini-Hochberg correction for testing multiple time points. For B-D,  
 725 each symbol represents a sample from an individual mouse and lines indicate the median value for

726 each treatment group. C. PCoA of Bray-Curtis distances from stool communities collected over  
727 time (day:  $R^2 = 0.43$ ; group:  $R^2 = 0.19$ ). Symbol transparency represents the day post-challenge of  
728 the experiment. For C-E, the B on the day legend or days post-challenge X axis stands for baseline  
729 and represents the sample that was collected prior to any drug treatments. D. Shannon diversity  
730 in stool communities over time. E. Median relative abundances per treatment group for 6 out of  
731 the 14 genera that were affected by treatment, but recovered close to baseline levels by 7 days  
732 post-challenge (Fig. 3E, Data Set S1, Sheet X). Paired stool sample relative abundance values  
733 either baseline and day 1 or baseline and day 7 were analyzed by paired Wilcoxon signed-rank test  
734 with Benjamini-Hochberg correction for testing all identified genera. Only genera that were different  
735 between baseline and 1-day post-challenge, but not baseline and 7-days post-challenge. The gray  
736 horizontal lines represents the limit of detection.

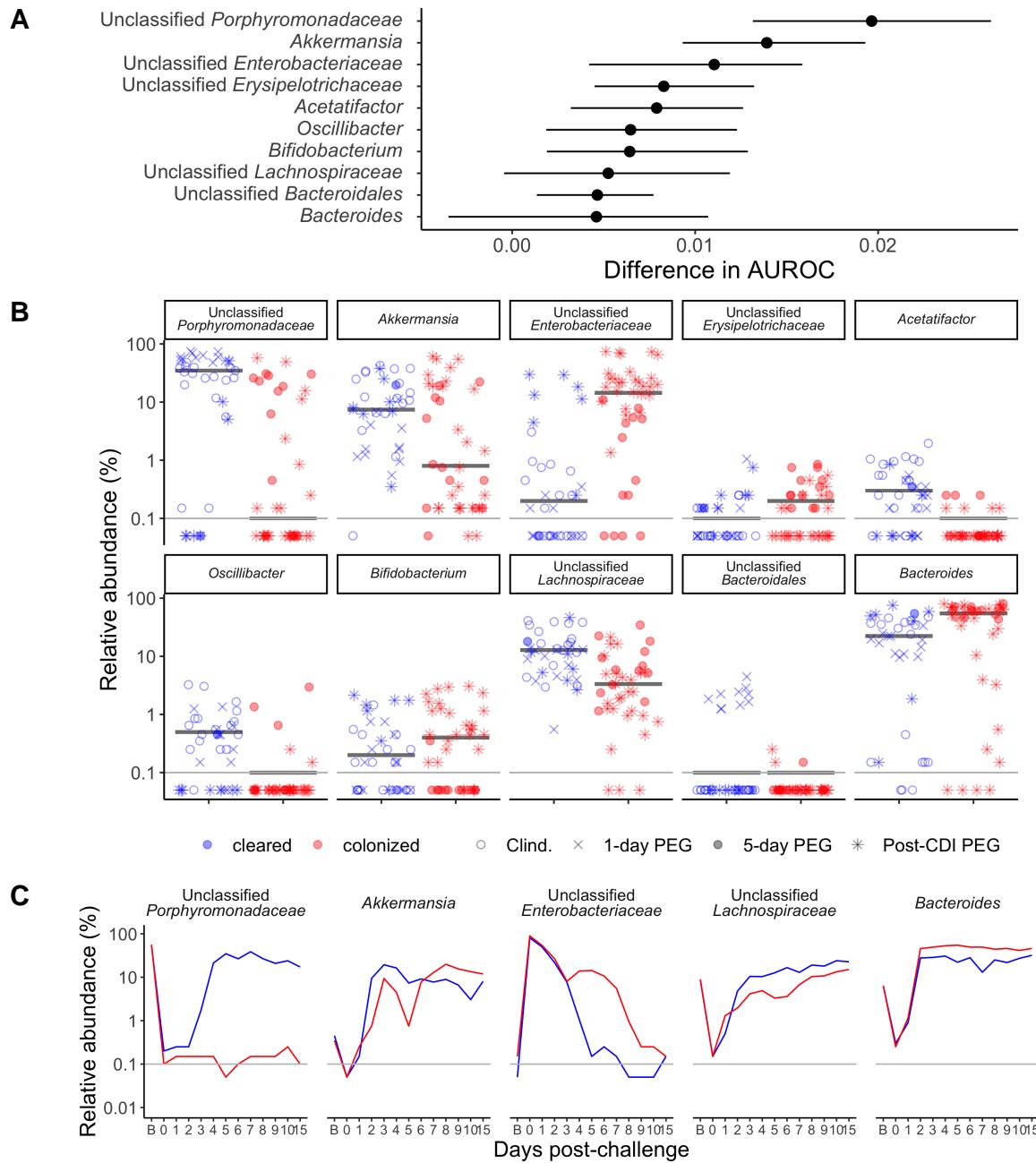


**Figure 5.**

738 **1-day PEG treatment post *C. difficile* challenge prolongs colonization regardless of**  
739 **whether an FMT is also administered.** A. Setup of the experimental time line for experiments  
740 with post-challenge PEG treated mice. There were a total of 4 different treatment groups. All mice  
741 were administered 10 mg/kg clindamycin intraperitoneally (IP) 1 day before challenge with  $10^{3-5}$   
742 *C. difficile* 630 spores. 1. Received no additional treatment (Clindamycin). 2. Immediately after  
743 *C. difficile* challenge, mice received 15% PEG 3350 in the drinking water for 1 day. 3-4. 3-days  
744 after challenge, mice received 1-day PEG treatment and then received either 100 microliters a  
745 fecal microbiota transplant (3) or PBS (4) solution by oral gavage. Mice were followed through  
746 15-30 days post-challenge (only the post-CDI 1-day PEG group was followed through 30 days  
747 post-challenge). B. CFU/g of *C. difficile* stool measured over time via serial dilutions. The black  
748 line represents the limit of detection for the first serial dilution. C-D. Shannon diversity (C) and  
749 richness (D) in stool communities over time. B-D. Each symbol represents a stool sample from an  
750 individual mouse with the lines representing the median value for each treatment group. Asterisks  
751 indicate time points with significant differences between groups by a Kruskall-Wallis test with a  
752 Benjamini-Hochberg correction for testing multiple times points. Colored rectangles indicates  
753 the 1-day PEG treatment period for applicable groups. The data presented are from a total of 3  
754 separate experiments.



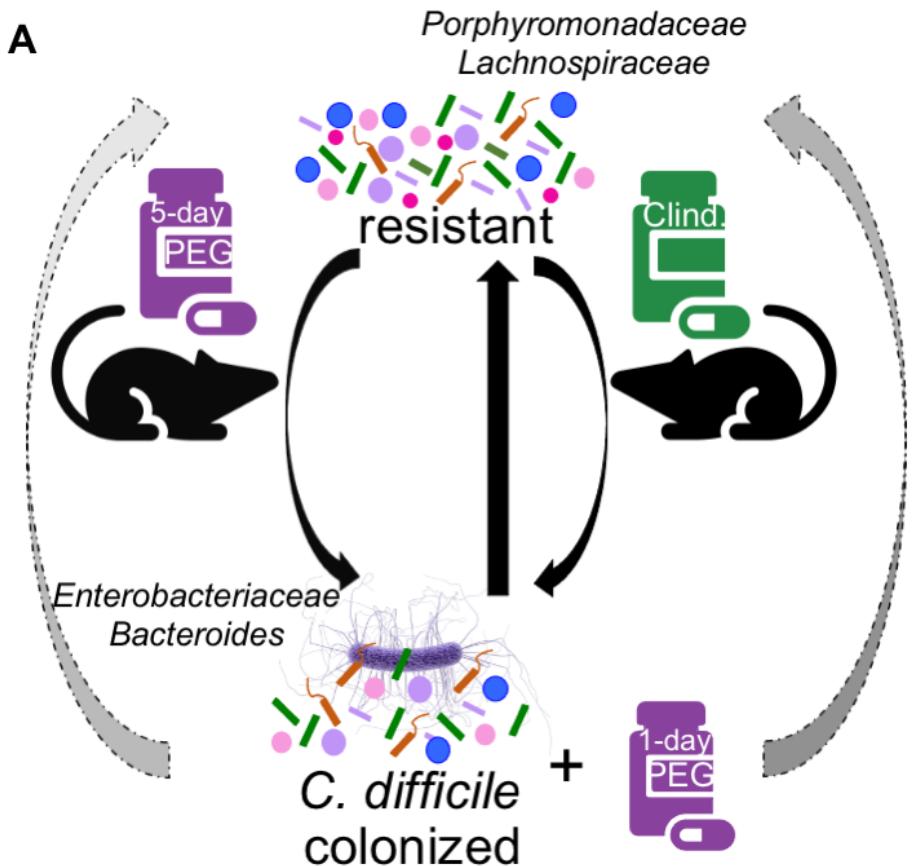
756 **6. For 1-day PEG treatment post *C. difficile* challenge mice that also receive an FMT only**  
757 **some bacterial genera were restored.** A. PCoA of Bray-Curtis distances from stool samples  
758 collected over time as well as the FMT solution that was administered to one of the treatment  
759 groups. Each circle represents an individual sample, the transparency of the circle corresponds  
760 to day post-challenge. B. Median relative abundances of 2 genera that were significantly different  
761 over multiple time points in mice that were administered either FMT or PBS solution via gavage C.  
762 Median relative abundances of the top 6 out of 24 genera that were significant over multiple  
763 timepoints, plotted over time (Data Set S1, Sheet X). For B-C, colored rectangles indicates  
764 the 1-day PEG treatment period for applicable groups. Gray horizontal lines represent the limit  
765 of detection. Differences between treatment groups were identified by Kruskal-Wallis test with  
766 Benjamini-Hochberg correction for testing all identified genera. For pairwise comparisons of the  
767 groups (B), we performed pairwise Wilcoxon comparisons with Benjamini-Hochberg correction for  
768 testing all combinations of group pairs.



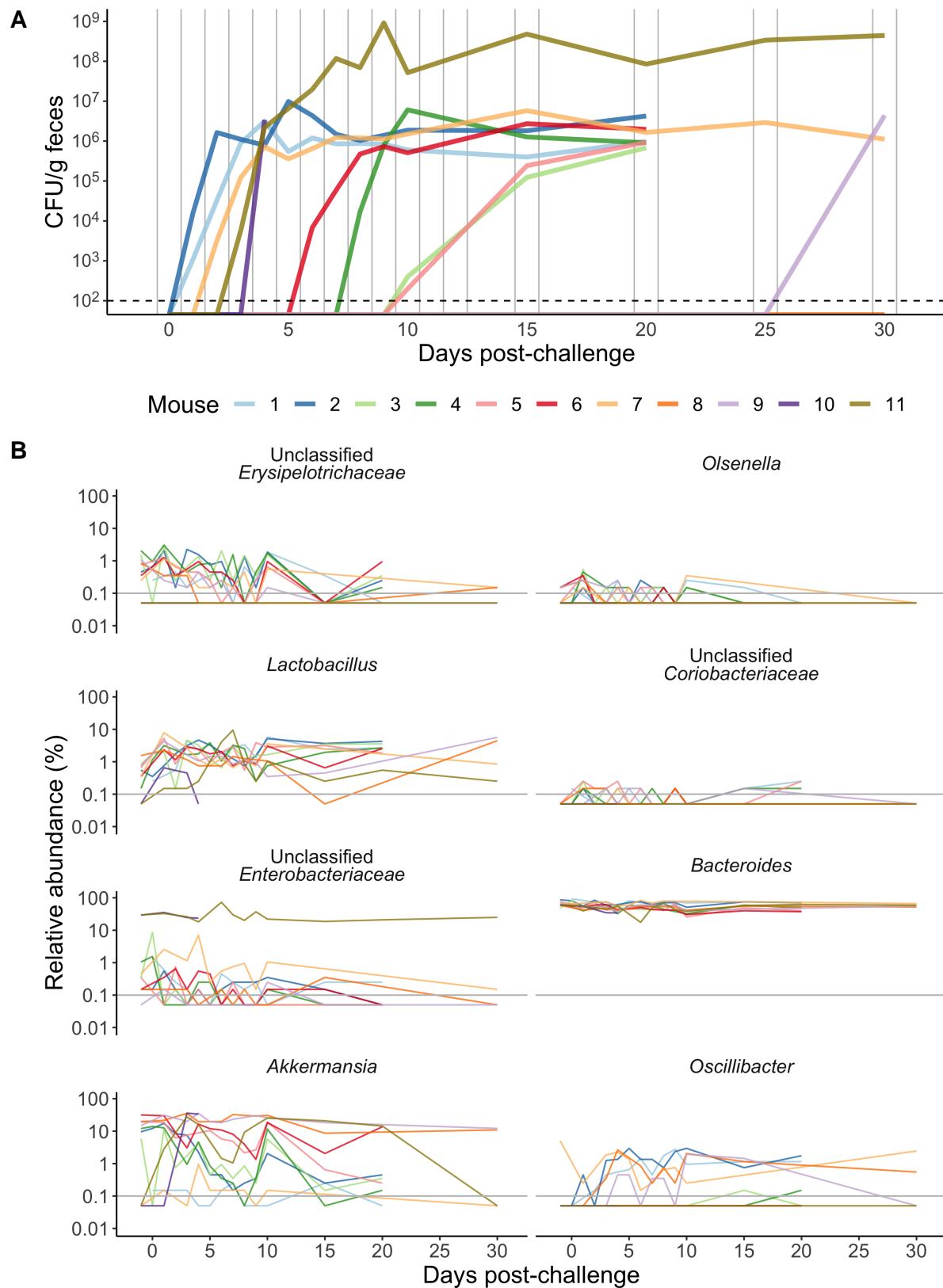
769

770 **Figure 7. Specific microbiota features associated with prolonged *C. difficile* colonization**  
 771 **in PEG treated mice.** A. Top ten bacteria that contributed to the random forest model trained  
 772 on five day post-challenge community relative abundance data to predict whether mice would  
 773 still be colonized with *C. difficile* 10 days post-challenge. The median (point) and interquartile  
 774 range (lines) change in AUROC when the bacteria is left out of the model by permutation feature  
 775 importance analysis. B. The median relative abundances of the top ten bacteria that contributed to  
 776 the random forest classification model at 5 days post-challenge . Color indicates whether the mice

777 were still colonized with *C. difficile* 10 days post-challenge and the black horizontal line represents  
778 the median relative abundance for the two categories. Each symbol represents a stool sample  
779 from an individual mouse and the shape of the symbol indicates whether the PEG-treated mice  
780 received a 5-day (Fig. 1-3), 1-day (Fig. 4) or post-challenge PEG (Fig. 5-6) treatment. C. The  
781 median relative abundances of the 5 genera with greater than 1% median relative abundance in the  
782 stool community over time. For B-C, the gray horizontal lines represents the limit of detection.



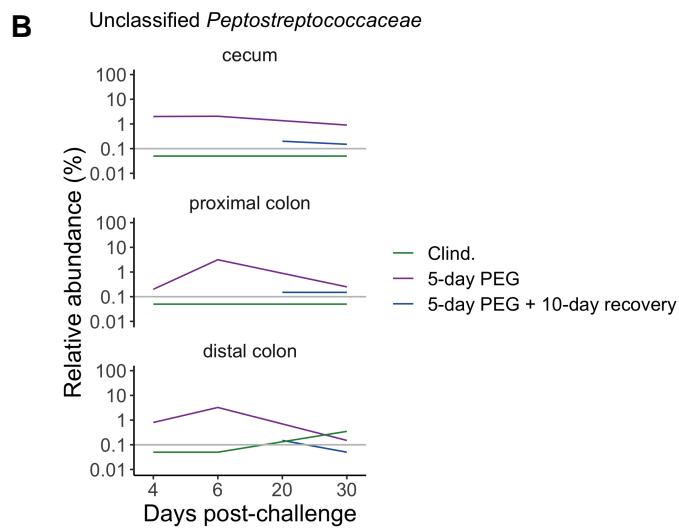
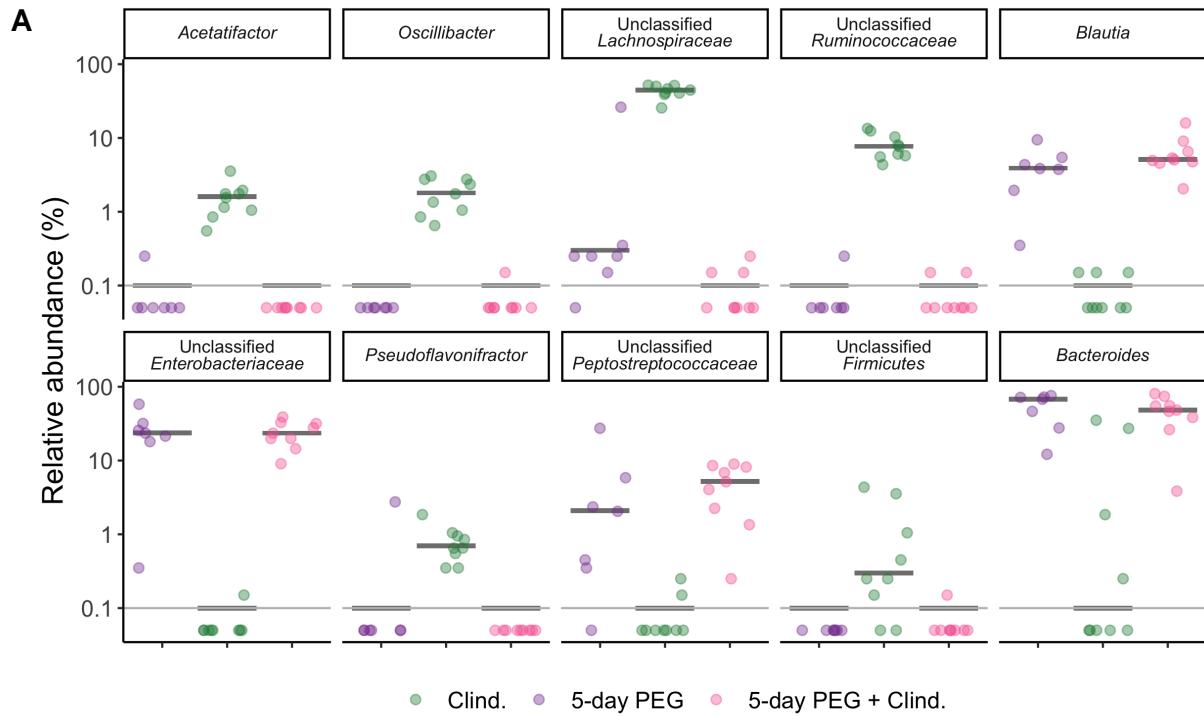
784 **Figure 8. Schematic summarizing findings.** The gut microbiota of our C57Bl/6 mice is resistant  
 785 to *C. difficile* but treatment with either the antibiotic, clindamycin, or the osmotic laxative, PEG  
 786 3350, renders the mice susceptible to *C. difficile* colonization. Recovery of colonization resistance  
 787 in clindamycin-treated mice is relatively straightforward and the mice clear *C. difficile* within 10  
 788 days post-challenge. However, for mice that received either a 5-day PEG treatment pre-*C. difficile*  
 789 challenge or a 1-day PEG treatment post-challenge recovery of colonization resistance was  
 790 delayed because most mice were still colonized with *C. difficile* 10 days post-challenge. We found  
 791 increased relative abundances of *Porphyromonadaceae* and *Lachnospiraceae* were associated  
 792 with recovery of colonization resistance, while increased relative abundances of *Enterobacteriaceae*  
 793 and *Bacteroides* were associated with prolonged *C. difficile* colonization.



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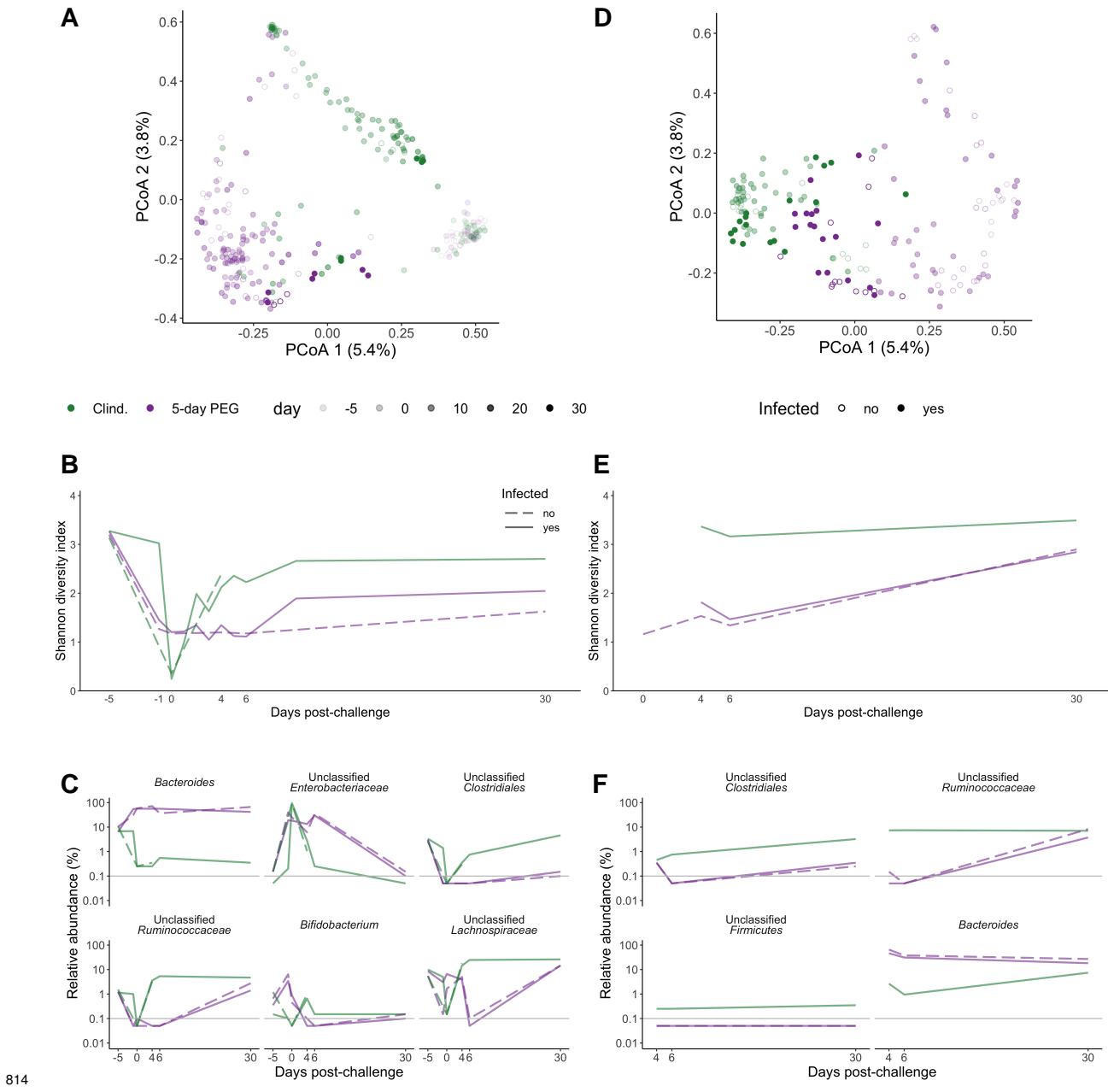
795 **Figure S1. Microbiota dynamics post-challenge in the 5-day PEG treatment plus 10-day**

796 **recovery mice.** A. *C. difficile* CFU/g over time in the stool samples collected from 5-day PEG  
797 treated mice that were allowed to recover for 10 days prior to challenge. Same data presented in  
798 Fig. 1C, but the data for the other 3 treatment groups have been removed and each line represents  
799 the CFU over time for an individual mouse. Mouse 10 was found dead 6 days post-challenge.  
800 B. Relative abundances of eight bacterial genera from day 0 post-challenge onward in each off  
801 the 10-day recovery mice. We analyzed samples from day 0 and day 8 post-challenge, which  
802 represented the the time points where mice were challenged with *C. difficile* and when the median  
803 relative *C. difficile* CFU stabilized for the group using the paired Wilcoxon signed-rank test, but no  
804 genera were significantly different after Benjamini-Hochberg correction.



805  
806 **Figure S2. PEG treatment still has a large impact on the mucosal microbiota 6 days**  
807 **post-challenge A.** The relative abundances of the 10 bacterial genera that were significantly  
808 different between treatment groups at 6 days post-infection in the cecum tissue (the relative  
809 abundances of the 10 genera were also significantly different in the proximal and distal colon  
810 tissues, Data Sheet S1, Sheet X). Each symbol represents a tissue sample from an individual  
811 mouse, the black horizontal lines represents the median relative abundances for each treatment  
812 group. B. The relative abundance of *Peptostreptococcaceae* in the three types of tissue sample

813 communities over time. For A-B, the gray horizontal lines represent the limit of detection.



815 **Figure S3. *C. difficile* challenge does not enhance the disruptive effect of PEG on the**  
 816 **microbiota.** A, D. PCoAs of the Bray-Curtis distances from the stool (A) and tissue (D) communities  
 817 from mock- and *C. difficile*-challenged treatment groups. Each symbol represents a sample from an  
 818 individual mouse with open and closed circles representing mock and *C. difficile*-challenged mice,  
 819 respectively. B, E. Median Shannon diversity in stool (B) and tissue (E) communities collected over  
 820 time. C, F. The median relative abundances of genera that were significantly different between  
 821 the *C. difficile* challenged treatment groups in either the stool (Fig. 2E) or cecum tissue (Fig. 3C)

822 communities in the stool (C) and tissue (F) communities from mock- and *C. difficile*-challenged mice.  
823 For B-F, the dashed and solid lines represent the median value for mock and *C. difficile*-challenged  
824 mice, respectively. For E-F, tissues from mock-challenged clindamycin treated mice were only  
825 collected 4 days post-challenge so there is no dashed line for this group.