

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

Sarah Tomkovich¹, Ana Taylor¹, Jacob King¹, Joanna Colovas¹, Lucas Bishop¹, Kathryn McBride¹, Sonya Royzenblat¹, Nicholas A. Lesniak¹, Ingrid L. Bergin², Patrick D. Schloss^{1†}

† To whom correspondence should be addressed: pschloss@umich.edu

1. Department of Microbiology and Immunology, University of Michigan, Ann Arbor, MI, USA
2. The Unit for Laboratory Animal Medicine, University of Michigan, Ann Arbor, MI, USA

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 CDI
5 susceptibility and clearance is unclear. To examine how PEG impacts susceptibility, we treated
6 C57Bl/6 mice with 5-day and 1-day doses of 15% PEG in the drinking water and then challenged the
7 mice with *C. difficile* 630. We used clindamycin-treated mice as a control because they consistently
8 clear *C. difficile* within 10 days post-challenge. PEG treatment alone was sufficient to render mice
9 susceptible and 5-day PEG-treated mice remained colonized for up to 30 days post-challenge.
10 In contrast, 1-day PEG treated mice were transiently colonized, clearing *C. difficile* within 7 days
11 post-challenge. 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
13 *C. difficile* challenge prolonged colonization up to 30 days post-challenge. When we trained a
14 random forest model with community data from 5 days post-challenge, we were able to predict
15 which mice would exhibit prolonged colonization (AUROC = 0.90). Examining the dynamics of these
16 bacterial populations during the post-challenge period revealed patterns in the relative abundances
17 of *Bacteroides*, *Enterobacteriaceae*, *Porphyromonadaceae*, *Lachnospiraceae*, and *Akkermansia*
18 that were associated with prolonged *C. difficile* colonization in PEG-treated mice. Thus, the osmotic
19 laxative, PEG, rendered mice susceptible to *C. difficile* colonization and hindered clearance.

20 Importance

21 Diarrheal samples from patients taking laxatives are typically rejected for *Clostridioides difficile*
22 testing. However, there are similarities between the bacterial communities from people with diarrhea
23 or *C. difficile* infections (CDI) including lower diversity compared to communities from healthy
24 patients. This observation led us to hypothesize that diarrhea may be an indicator of *C. difficile*
25 susceptibility. We explored how osmotic laxatives disrupt the microbiota's colonization resistance to
26 *C. difficile* by administering a laxative to mice either before or after *C. difficile* challenge. Our findings
27 suggest that osmotic laxatives disrupt colonization resistance to *C. difficile*, and prevent clearance
28 among mice already colonized with *C. difficile*. Considering that most hospitals recommend not

29 performing *C. difficile* testing on patients taking laxatives and laxatives are used when administering
30 fecal microbiota transplants via colonoscopy to patients with recurrent CDIs, further studies are
31 needed to evaluate if laxatives impact microbiota 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 samples from patients were separated into two groups based on stool consistency, there were
44 similar microbiota features between samples from CDI patients and *C. difficile* negative patients
45 with diarrhea compared to non-diarrheal samples that were *C. difficile* negative (12). The similar
46 community features between CDI patients and patients with diarrhea included low alpha diversity
47 and there were also only 6 bacterial taxa had higher relative abundances in communities from CDI
48 patients. These results led to the hypothesis that bacterial communities from patients experiencing
49 diarrhea are susceptible to developing CDIs, regardless of how they developed diarrhea.

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

59 Beyond susceptibility, PEG is also relevant in the context of treating recurrent CDIs via fecal

60 microbiota transplant (FMT) where a healthy microbiota is administered to the patient to restore
61 colonization resistance. For FMTs that are delivered via colonoscopy, patients typically undergo
62 bowel preparation by taking an osmotic laxative prior to the procedure. Many of the FMT studies to
63 date rationalize the use of laxatives prior to the FMT (20–22) based on a 1996 case study with 2
64 pediatric patients where the authors suggested in the discussion that the laxative may help flush *C.*
65 *difficile* spores and toxins from the intestine (23).

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

75 **Results**

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

88 treatment (Fig. 1B). After either the PEG, clindamycin, or PEG and clindamycin treatment all mice
89 were challenged with 10^5 *C. difficile* 630 spores (Fig. 1A). All treatments rendered mice susceptible
90 to *C. difficile* colonization. In contrast to the mice that only received clindamycin, PEG-treated
91 mice remained colonized with *C. difficile* at a high level through thirty days post-challenge (Fig.
92 1C). The clindamycin-treated mice cleared *C. difficile* within ten days post-challenge (Fig. 1C).
93 It was noteworthy that 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 bacterium 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 ($R^2 = 0.29$) and treatment group ($R^2 = 0.21$) explained half of the observed
109 variation between fecal communities with most of the remaining variation explained by interactions
110 between treatment group and other experimental variables including time, cage, and sequencing
111 preparation plate (PERMANOVA combined $R^2 = 0.95$, $P < 0.001$, Fig. 2A, Data Set S1, sheet 1).
112 None of the treatment groups recovered to their baseline community structure either 10 or 30 days
113 post-challenge suggesting other community features besides recovery to baseline were responsible
114 for the prolonged *C. difficile* colonization in PEG-treated mice (Fig. 2B).

115 Because time and treatment group influenced most of the variation between communities, we
116 next explored whether there were differences in community diversity and composition between

117 treatment groups. We examined the alpha diversity dynamics by calculating the communities'
118 Shannon diversity. Although both clindamycin and PEG treatments decreased diversity, the
119 Shannon diversity was lower in the groups of mice that received PEG treatment compared to those
120 that received clindamycin alone through thirty days post-challenge (Fig. 2C; Data Set S1, sheet 2).
121 We next identified the bacterial genera whose relative abundances shifted after PEG treatment by
122 comparing the baseline samples of mice treated with only PEG to samples from the same mice one
123 day post-PEG-treatment. We found 18 genera whose relative abundances were altered by PEG
124 treatment (Data Set S1, sheet 3). The majority of the bacterial relative abundances decreased after
125 the PEG treatment, but the relative abundance among members of the *Enterobacteriaceae* and
126 *Bacteroides* increased. The increase in *Bacteroides* relative abundance was unique to PEG treated
127 mice, as the *Bacteroides* relative abundance actually decreased in clindamycin treated mice (Fig.
128 2D). Finally, we identified the genera whose relative abundance differed across treatment groups
129 over multiple time points. Of the 33 genera that were different between treatment groups, 24 genera
130 were different over multiple time points (Fig. 2E, Data Set S1, sheet 4). Thus, PEG had a significant
131 impact on the fecal microbiota that was maintained over time and was distinct from clindamycin
132 treatment.

133 Because *C. difficile* was not immediately detectable in the stools of the PEG-treated mice that
134 were allowed to recover for 10 days prior to challenge, we decided to examine if there were
135 genera that changed during the post-challenge period. We compared the communities from when
136 *C. difficile* shifted from undetectable at 1 day post-challenge to detectable in the stool samples
137 with the density stabilizing around 8 days post-challenge (Fig. S1A). We found no genera with
138 relative abundances that were significantly different over the two time points (Data Set S1, sheet 5).
139 However, there was also wide variation between individual mice regarding when *C. difficile* became
140 detectable (Fig. S1A) as well as the relative abundances of bacterial genera in the communities
141 (Fig. S1B). For example, two mice had a high relative abundance of *Enterobacteriaceae* throughout
142 the post-challenge period. One mouse died on the sixth day post-challenge and in the other *C.*
143 *difficile* was present at a high density from the 4th day post-challenge onward (Fig. S1B). While
144 we did not identify a clear signal to explain the delayed appearance of *C. difficile* in the 5-day PEG
145 mice that were allowed to recover for 10 days prior to challenge, the delay was striking and could

146 reflect changes in microbial activity or metabolites that were not examined in this study.

147 **5-day laxative treatment did not promote more severe CDIs despite altering the mucosal**
148 **microbiota.** Given the findings from a previous study that demonstrated PEG treatment disrupts
149 the mucus layer and alters the immune response in mice (16), we decided to examine the impact of
150 PEG treatment on the mucosal microbiota and CDI severity. To evaluate the mucosal microbiota,
151 we sequenced communities associated with tissues collected from the cecum, proximal colon, and
152 distal colon. Similar to what was observed with the stool samples, the alpha diversity was lower
153 in the PEG-treated mice compared to clindamycin treated mice (Fig. 3A, Data Set S1, sheet 6).
154 The alpha diversity of the tissue-associated community increased in PEG-treated mice collected
155 at 20 and 30 days post-challenge (Fig. 3A). Group ($R^2 = 0.33$), time point ($R^2 = 0.11$), and their
156 interactions with other variables (cage, experiment number, and sample type) explained the majority
157 of the variation observed in mucosal communities (PERMANOVA combined $R^2 = 0.83$, $P < 0.05$,
158 Fig. 3B, Data Set S1, sheet 7). We saw the greatest difference in the relative abundance of the
159 mucosal microbiota between treatment groups (clindamycin, 5-day PEG, and 5-day PEG plus
160 clindamycin) at 6 days post-challenge with 10 genera that were significantly different ($P < 0.05$) in
161 all three of the tissue types we collected (cecum, proximal colon, and distal colon; Fig. S2A, Data
162 Set S1, sheet 8, 9, and 10). Interestingly, *Peptostreptococcaceae* (the family with a sequence that
163 matches *C. difficile*) was one of the genera that had a significant difference in relative abundance
164 between treatment groups at 6 days post-challenge. This population was primarily only present
165 in the 5-day PEG treatment group of mice and decreased in the proximal and distal colon tissues
166 over time (Fig. S2B). By 30 days post-challenge, only the relative abundances of *Bacteroides*,
167 *Clostridiales*, *Firmicutes*, and *Ruminococcaceae* were different between treatment groups and
168 only in the cecum tissues (Fig. 3C, Fig. 2E, Data Set S1, sheet 8). Thus, PEG treatment had a
169 significant impact on the mucosal microbiota and we detected *C. difficile* sequences in the cecum,
170 proximal colon, and distal colon tissue communities.

171 Because there were differences in the mucosal microbiota including detectable *C. difficile* sequences
172 in tissues from PEG-treated mice relative to mice treated with clindamycin, we next examined the
173 severity of *C. difficile* challenge by evaluating cecum and colon histopathology (27). However, we
174 found there was no difference in cecum and colon scores between clindamycin and PEG-treated

175 mice that were challenged with *C. difficile* at 4 days post-challenge (Fig. 3D), the time point
176 typically examined in *C. difficile* 630 challenged mice (28). We also looked at 6 days post-challenge
177 because that was when there was a large difference in *C. difficile* density between PEG- and
178 clindamycin-treated mice (Fig. 1C). Although there was a slight difference in the histopathology
179 score of the colon between PEG and clindamycin-treated mice, there was not a significant difference
180 in the cecum and the overall score was relatively low (1.5 to 2.5 out of 12, Fig. 3E). Therefore,
181 although PEG treatment had a disruptive effect on the mucosal microbiota, the impact of *C. difficile*
182 challenge on the cecum and colon was similar between PEG and clindamycin treated mice.

183 ***C. difficile* challenge did not have a synergistic disruptive effect on the microbiota of**
184 **PEG-treated mice.** Because *C. difficile* itself can have an impact on the microbiota (29), we
185 also sequenced the tissue and stools of mock-challenged mice treated with clindamycin or PEG.
186 Examining the stools of the mock-challenged mice revealed similar bacterial disruptions as the *C.*
187 *difficile* challenged mice (Fig. S3A-C). Similarly, there was no difference between the communities
188 found in the tissues of mock and *C. difficile* challenged mice (Fig. S3D-F). Thus, most of the
189 microbiota alterations we observed in the PEG-treated mice were a result of the laxative and not an
190 interaction between the laxative and *C. difficile*.

191 **1-day laxative treatment resulted in transient *C. difficile* colonization and minor microbiota**
192 **disruption.** Next, we examined how a shorter osmotic laxative perturbation would impact the
193 microbiome and susceptibility to *C. difficile*. We administered either a 1-day PEG treatment, a
194 1-day PEG treatment with a 1-day recovery period, or clindamycin to mice before challenging them
195 with *C. difficile* (Fig. 3A). In contrast to the 5-day PEG treated mice, the 1-day PEG groups were
196 only transiently colonized and cleared *C. difficile* by 7 days post-challenge (Fig. 3B). The stool
197 communities of the 1-day PEG treatment groups were also only transiently disrupted, with Shannon
198 diversity recovering by 7 days post-challenge (Fig. 3C-D, Data Set S1, sheets 11 and 12). We found
199 the relative abundances of 14 genera were impacted by treatment, but recovered close to baseline
200 levels by 7 days post-challenge including *Enterobacteriaceae*, *Clostridiales*, *Porphyromonadaceae*,
201 and *Ruminococcaceae* (Fig. 3E, Data Set S1, sheet 13 and 14). These findings suggest the
202 duration of the PEG treatment was relevant, with shorter treatments resulting in a transient loss of
203 *C. difficile* colonization resistance.

204 **Post-challenge laxative treatment disrupted clearance in clindamycin-treated mice**
205 **regardless of whether an FMT was also administered.** Since a 1-day PEG treatment resulted
206 in a more mild perturbation of the microbiota, we decided to use the 1-day treatment to examine the
207 hypothesis that PEG helps to flush *C. difficile* spores from the intestine. This hypothesis is proposed
208 in the discussion section of FMT studies where bowel prep is part of the preparation undergone by
209 patients receiving FMTs via colonoscopy (20–23). To examine the impact of PEG treatment on
210 *C. difficile* clearance, we treated 4 groups of mice with clindamycin and then challenged all mice
211 with *C. difficile* before administering the following treatments: no additional treatment, 1-day PEG
212 immediately after challenge, and 1-day PEG treatment 3 days after challenge followed by either
213 administration of an FMT or PBS solution by oral gavage (Fig. 5A). Contrary to the hypothesis, all
214 groups of mice that received PEG exhibited prolonged *C. difficile* colonization (Fig. 5B).

215 We were also interested in exploring whether PEG might help with engraftment in the context of
216 FMTs. An FMT was prepared under anaerobic conditions using stool collected from the same
217 group of mice pre-treatment representing the baseline community. The FMT appeared to partially
218 restore Shannon diversity but not richness (Fig. 5C-D, Data Set S1, sheets 15 and 16). Similarly,
219 we saw some overlap between the communities of mice that received FMT and the mice treated
220 with only clindamycin after 5 days post-challenge (Fig. 6A, Data Set S1, sheet 17). The increase
221 in Shannon diversity suggests that the FMT did have an impact on the microbiota, despite seeing
222 prolonged *C. difficile* colonization in the FMT treated mice. However, only the relative abundances
223 of *Bacteroidales* and *Porphyromonadaceae* consistently differed between the mice that received
224 either an FMT or PBS gavage (Fig. 6B). Overall, we found the relative abundances of 24 genera
225 were different between treatment groups over multiple time points (Data Set S1, sheet 18). For
226 example, the relative abundance of *Akkermansia* was increased and the relative abundances of
227 *Ruminococcaceae*, *Clostridiales*, *Lachnospiraceae*, and *Oscillibacter* were decreased in mice that
228 received PEG after *C. difficile* challenge relative to clindamycin treated mice (Fig. 6C). In sum,
229 administering PEG actually prolonged *C. difficile* colonization, including in mice that received an
230 FMT, which only restored 2 bacterial genera.

231 **Five-day post-challenge community data can predict which mice 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 examined the bacteria that influenced prolonged *C.*
234 *difficile* colonization. We trained 3 machine learning models (random forest, logistic regression,
235 and support vector machine) with bacterial community data from 5 days post-challenge to predict
236 whether the mice were still colonized with *C. difficile* 10 days post-challenge. We chose to predict
237 the status based on communities 5 days post-challenge because that was the earliest time point
238 where we saw 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 19) and
240 indicated that the 5-day post challenge microbiota was an excellent predictor of prolonged *C.*
241 *difficile* colonization. Next, we performed a permutation importance test to identify the bacteria
242 that were the top contributors to the random forest model for predicting prolonged *C. difficile*
243 colonization. We selected 10 genera that contributed the most to our model's performance (Fig.
244 7A) and examined their relative abundance at 5 days post-challenge, the time point used to predict
245 *C. difficile* colonization status on day 10 (Fig. 7B). Next, we focused on the 5 genera that had a
246 greater than 1% relative abundance in either the cleared or colonized mice and examined how the
247 bacteria changed over time. We found *Enterobacteriaceae* and *Bacteroides* tended to consistently
248 have a higher relative abundance, the relative abundance of *Akkermansia* was initially low and then
249 increased, and *Porphyromonadaceae* and *Lachnospiraceae* had a lower relative abundance in the
250 mice with prolonged colonization compared to the mice that cleared *C. difficile* (Fig. 7C). Together
251 these results suggest a combination of low and high abundance bacterial genera influence the
252 prolonged colonization observed in 5-day PEG and post-challenge 1-day PEG treated mice.

253 Discussion

254 While the disruptive effect of antibiotics on *C. difficile* colonization resistance is well established,
255 the extent to which other drugs such as laxatives disrupt colonization resistance was unclear. By
256 following mice treated with an osmotic laxative over time, we found that a 5-day PEG treatment
257 before challenge resulted in prolonged *C. difficile* colonization, while a 1-day PEG treatment resulted
258 in transient colonization without the use of antibiotics. The differences in *C. difficile* colonization
259 dynamics between the 5- and 1-day PEG treated mice were associated with differences in how
260 much the treatments disrupted the microbiota. Additionally, the intestinal communities of 5-day

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

271 In addition to altering composition, laxative treatment may alter microbiota-produced metabolites. A
272 previous study demonstrated that a 5-day treatment of 10% PEG depleted acetate and butyrate
273 and increased succinate compared to untreated mice (15). While we did not perform metabolomic
274 analysis, we did see bacteria known to produce beneficial metabolites were depleted in mice
275 that cleared *C. difficile* compared to mice with prolonged colonization. For example, *Oscillibacter*
276 *valericigenes* can produce the SCFA valerate (30), and separate studies demonstrated valerate is
277 depleted after clindamycin treatment and inhibited *C. difficile* growth *in vitro* and in C57BL/6 mice
278 (31, 32). Similarly, *Acetatifactor* can produce acetate and butyrate (33), SCFAs that are decreased
279 in mice with prolonged *C. difficile* infection after antibiotic treatment (34). Thus protective bacteria
280 and their metabolites could be depleted by osmotive laxative treatment depending on the timing
281 and duration of treatment.

282 One possible explanation for the prolonged *C. difficile* colonization in 5-day PEG treated mice, might
283 be due to the bacteria's persistence in the mucosal compartment. In fact, it has been hypothesized
284 that *C. difficile* biofilms may serve as reservoirs for recurrent infections (35) and *C. difficile* biofilms
285 in the mucus layer were recently identified in patients as aggregates with *Fusobacterium nucleatum*
286 (36). There was an interesting pattern of increased *Enterobacteriaceae*, *Bacteroides*, and *C.*
287 *difficile* in both the stool and mucosal communities of PEG-treated mice suggesting a potential
288 synergy. *Bacteroides* has the potential to degrade mucus and the osmotic laxative may have allowed
289 *Bacteroides* to colonize the mucosal niche by degrading mucin glycans with glycosyl hydrolases

290 that are absent in *C. difficile* (37). *Bacteroides* persistence in the mucosal tissue might also have
291 helped *Enterobacteriaceae* to colonize the region, as a synergy between mucus-degrading *B.*
292 *fragilis* and *E. coli* has previously been described (38). A separate study demonstrated *C. difficile*
293 was present in the outer mucus layer and associated with *Enterobacteriaceae* and *Bacteroidaceae*
294 using fluorescent in situ hybridization (FISH) staining (39). However, protective roles for *Bacteroides*
295 have also been demonstrated. For example, *B. fragilis* prevented CDI morbidity in a mouse model
296 and inhibited *C. difficile* adherence *in vitro* (40). In coculture experiments *B. longum* decreased
297 *C. difficile* biofilm formation while *B. thetaiotamicron* enhanced biofilm formation (41) and *B. dorei*
298 reduced *C. difficile* growth in a 9-species community *in vitro* (42). Therefore, whether *Bacteroides*
299 is detrimental or beneficial in the context of *C. difficile* infection or colonization is still unclear, but
300 the niche and interactions with other bacteria may contribute.

301 *Akkermansia* is also a mucin degrader with potentially beneficial or detrimental roles depending on
302 context in other diseases (43, 44). In our study the relative abundance of *Akkermansia* shifted over
303 time between groups of mice that either cleared *C. difficile* or had prolonged colonization. In the stool
304 it was initially increased in mice that cleared *C. difficile*, but shifted after 5-days post-challenge so
305 that it was increased in mice that had prolonged colonization. In the context of CDIs, some studies
306 suggest a protective role (45, 46), while others suggest a detrimental role because *Akkermansia*
307 was positively correlated with *C. difficile* (47–50). Because the relative abundance of *Akkermansia*
308 was dynamic in our study, it is unclear whether *Akkermansia* helps with clearance of *C. difficile* or
309 allows it to persist. A better understanding how *C. difficile* interacts with the mucosal microbiota
310 may lead to insights into CDIs, asymptomatic *C. difficile* carriage, and colonization resistance.

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

319 the host mucosal barrier in mice (52, 53). Further research is needed to tease out the interplay
320 between medications that influence the mucus layer and different strains of *C. difficile* in the context
321 of CDIs.

322 It is more difficult to interpret what are findings mean in the context of *C. difficile* colonization
323 resistance in human patients. The main difficulty being that most hospitals recommend not
324 performing *C. difficile* testing if the patient is currently taking a laxative. This recommendation
325 is in accordance with the Infectious Diseases Society of America and Society for Healthcare
326 Epidemiology of America guidelines (54). The rationale behind the recommendation is that patients
327 taking laxatives may be asymptotically colonized with *C. difficile*, resulting in unnecessary
328 antibiotic treatment (55–57). Furthermore, some studies identified laxatives as a risk factor for
329 developing CDIs or recurrent CDIs (58–60) and a recent study found the proportion of severe CDIs
330 was similar between patients taking and not taking laxatives (61). However, there have also been
331 some studies that suggest laxatives are not a risk factor for developing CDIs (62, 63). Although, it
332 is unclear whether laxatives impact CDI susceptibility in human paitents, it is clear that laxatives
333 also have a transient impact on the human microbiota (13, 64–67). Additional studies to examine
334 the relationship between laxatives, *C. difficile* colonization, and CDIs are warranted.

335 Considering laxatives are also used to prepare patients when administering fecal microbiota
336 transplants via colonoscopy to treat recurrent CDIs, it will be important to determine whether
337 osmotic laxatives impact *C. difficile* clearance in the human intestinal tract. It is still unclear what
338 the best administration route is because there have been no studies designed to evaluate the best
339 administration route for FMTs (68). Nevertheless, results from the FMT National Registry where
340 85% of FMTs were delivered by colonoscopy demonstrate FMTs are highly effective treatment for
341 recurrent CDIs with 90% achieving resolution in the 1 month follow-up window (69). A surprising
342 number of studies continue to hypothesize that PEG or bowel preparation can clear *C. difficile*
343 spores and toxins despite the paucity of supporting evidence (20–23). There was even a clinical
344 trial (NCT01630096) designed to examine whether administering PEG 3350 (NuLYTELY) prior to
345 antibiotic treatment reduced disease severity that started recruitment in 2012 (70), but no results
346 have been posted to date. Here we sought to evaluate the impact of treating *C. difficile* colonized
347 mice with PEG (with or without FMT) and found clearance was delayed. Further studies are needed

348 to understand the impact of osmotic laxatives on *C. difficile* colonization resistance and clearance
349 in human patients receiving FMTs.

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

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368 Materials and Methods

369 **Animals.** All experiments were approved by the University of Michigan Animal Care and Use
370 Committee IACUC (protocol numbers PRO00006983 and PRO00008975). All mice were C57Bl/6
371 and part of the Schloss lab colony which was established in 2010 with mice donated from Vincent
372 Young's lab colony (established with mice purchased from The Jackson Laboratory in 2002). We
373 used 7-19 week old female mice for all experiments. This allowed us to break up littermates and
374 distribute them as evenly as possible across treatment groups in order to minimize microbiota

375 differences prior to starting treatments with medications. During the experiment, mice were housed
376 at a density of 2-3 mice per cage, with the majority of cages limited to two mice.

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

384 **C. difficile challenge model.** Mice were challenged with 25 microliters containing 10^5 *C. difficile*
385 630 spores, except for 1 experiment where the concentration was 10^3 (Fig. 5A). All mock challenged
386 mice received 25 ul vehicle solution (Ultrapure water). A Dymax stepper pipette was used to
387 administer the same challenge dose to mice via oral gavage. Mice were weighed daily throughout
388 the experiment and stool was collected for quantifying *C. difficile* CFU and 16S rRNA gene
389 sequencing. There were two groups of mice that received either a PBS or fecal microbiota
390 transplant (FMT) gavage post-PEG treatment. The fecal microbiota transplant was prepared with
391 stool samples collected from the mice in the experiment prior to the start of any treatments. The
392 stool samples were transferred to an anaerobic chamber and diluted 1:10 in reduced PBS and
393 glycerol was added to make a 15% glycerol solution. The solution was then aliquoted into tubes and
394 stored at -80 °C until the day of the gavage. An aliquot of both the FMT and PBS solutions were also
395 set aside in the -80 °C for 16S rRNA gene sequencing. The day of the gavage, aliquots were thawed
396 and centrifuged at 7500 RPM for 1 minute. The supernatant was then transferred to a separate
397 tube to prevent the gavage needle from clogging with debris during gavage. The PBS solution that
398 was administered to the other group was also 15% glycerol. Each mouse was administered 100
399 microliters of either the FMT or PBS solution via gavage. When we refer to mice that cleared *C.*
400 *difficile*, we mean that no *C. difficile* was detected in the first serial dilution (limit of detection: 100
401 CFU). In some experiments, we collected tissues for 16SrRNA gene sequencing, histopathology,
402 or both. For 16S rRNA gene sequencing, we collected small snips of cecum, proximal colon, and
403 distal colon tissues in microcentrifuge tubes, snap froze in liquid nitrogen, and stored at -80 °C.

404 For histopathology, cecum and colon tissues were placed into separate cassettes, fixed, and
405 then submitted to McClinchey Histology Labs (Stockbridge, MI) for processing, embedding, and
406 hematoxylin and eosin (H&E) staining.

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

412 **16S rRNA gene sequencing.** Stool samples were stored at -80 °C and were placed into 96-well
413 plates for DNA extractions and library preparation. DNA was extracted using the DNeasy Powersoil
414 HTP 96 kit (Qiagen) and an EpMotion 5075 automated pipetting system (Eppendorf). For library
415 preparation, each plate had a mock community control (ZymoBIOMICS microbial community DNA
416 standards) and a negative control (water). The V4 region of the 16S rRNA gene was amplified with
417 the AccuPrime Pfx DNA polymerase (Thermo Fisher Scientific) using custom barcoded primers, as
418 previously described (71). The PCR amplicons were normalized (SequalPrep normalizatin plate kit
419 from Thermo Fisher Scientific), pooled and quantified (KAPA library quantification kit from KAPA
420 Biosystems), and sequenced with the MiSeq system (Illumina).

421 **16S rRNA gene sequence analysis.** All sequences were processed with mothur (v. 1.43) using
422 a previously published protocol (71, 72). Paired sequencing reads were combined and aligned
423 with the SILVA (v. 132) reference database (73) and taxonomy was assigned with a modified
424 version of the Ribosomal Database Project reference sequences (v. 16) (74). The error rate for
425 sequencing data was 0.0559% based on the 17 mock communities we ran with the samples.
426 Samples were rarefied to 1,000 sequences, 1,000 times for alpha and beta diversity analyses
427 in order to account for uneven sequencing across samples. All but 3 of the 17 water controls
428 had fewer than 1000 sequences. PCoAs were generated based on Bray-Curtis Index distance
429 matrices. Permutational multivariate analysis of variance (PERMANOVA) tests were performed on
430 mothur-generated Bray-Curtis distance matrices with the adonis function from the vegan R package
431 (75).

432 **Histopathology.** H&E stained sections of cecum and colon tissues collected at either 0, 4, or 6
433 days post-challenge were coded to be scored in a blinded manner by a board-certified veterinary
434 pathologist (ILB). Slides were evaluated using a scoring system developed for mouse models of *C.*
435 *difficile* infection (51). Each slide was evaluated for edema, cellular infiltration, and inflammation
436 and given a score ranging from 0-4. The summary score was calculated by combining the scores
437 from the 3 categories and ranged from 0-12.

438 **Classification model training and evaluation.** We used the mikropml package to train and
439 evaluate models to predict *C. difficile* colonization status 10 days post-challenge where mice were
440 categorized as either cleared or colonized (76, 77). We removed the *C. difficile* genus relative
441 abundance data prior to training the model. Input community relative abundance data at the
442 genus level from 5 days post-challenge was used to generate random forest, logistic regression,
443 and support vector machine classification models to predict *C. difficile* colonization status 10 days
444 post-challenge. To accommodate the small number of samples in our data set we used 50% training
445 and 50% testing splits with repeated 2-fold cross-validation of the training data for hyperparameter
446 tuning. Permutation importance was performed as described previously (78) using mikropml (76,
447 77) with the random forest model because it had the highest AUROC value.

448 **Statistical analysis.** R (v. 4.0.2) and the tidyverse package (v. 1.3.0) were used for statistical
449 analysis (79, 80). Kruskal-Wallis tests with Benjamini-Hochberg correction for testing multiple time
450 points were used to analyze differences in *C. difficile* CFU, mouse weight change, and alpha
451 diversity between treatment groups. Paired Wilcoxon rank signed rank tests were used to identify
452 genera impacted by treatments on matched pairs of samples from 2 time points. Bacterial relative
453 abundances that varied between treatment groups at the genus level were identified with the
454 Kruskal-Wallis test with Benjamini-Hochberg correction for testing all identified OTUs, followed by
455 pairwise Wilcoxon comparisons with Benjamini-Hochberg correction.

456 **Code availability.** Code for data analysis and generating this paper with accompanying figures is
457 available at https://github.com/SchlossLab/Tomkovich_PEG3350_mSphere_2021.

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

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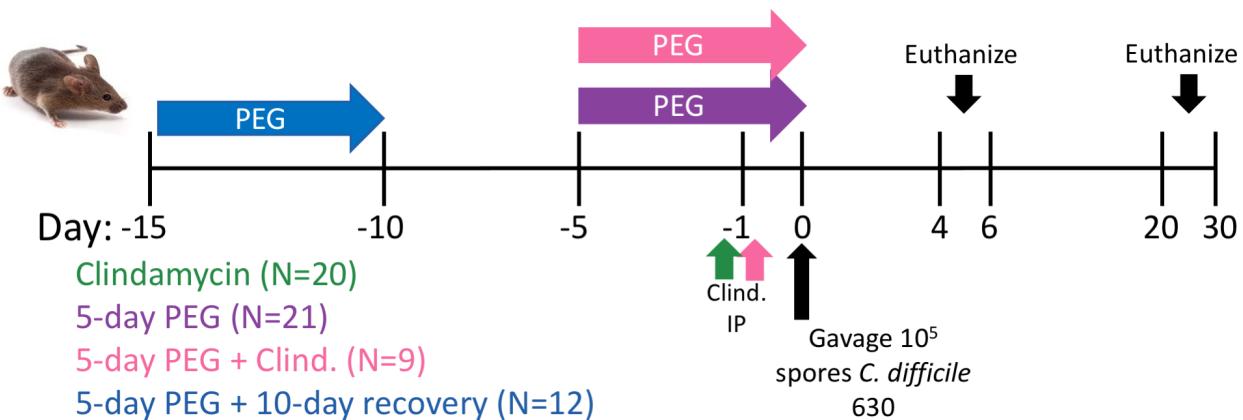
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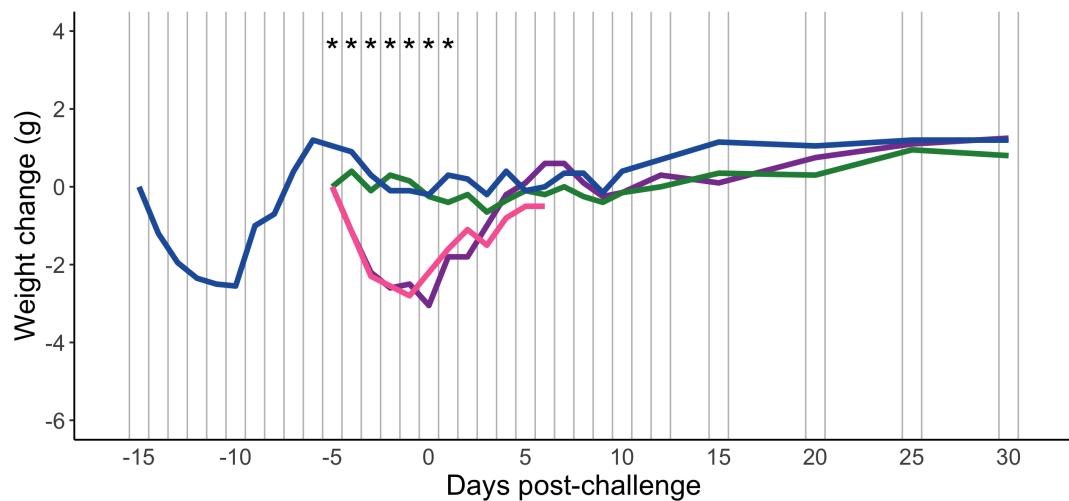
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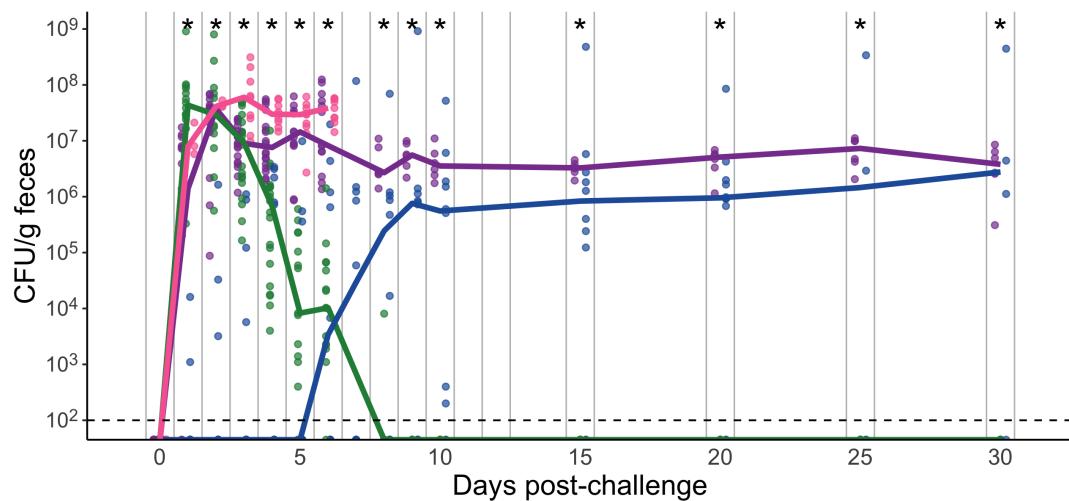
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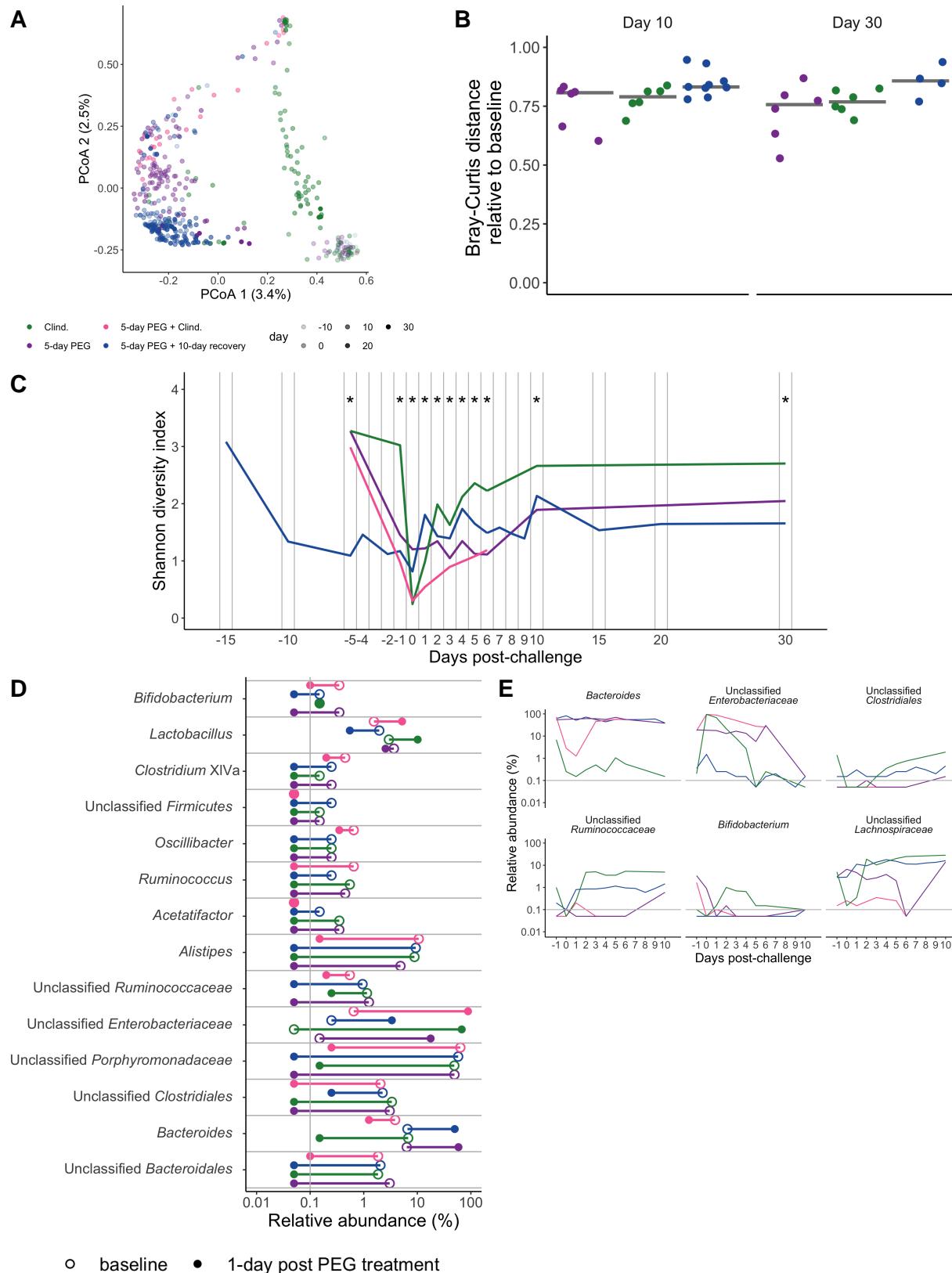
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703

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

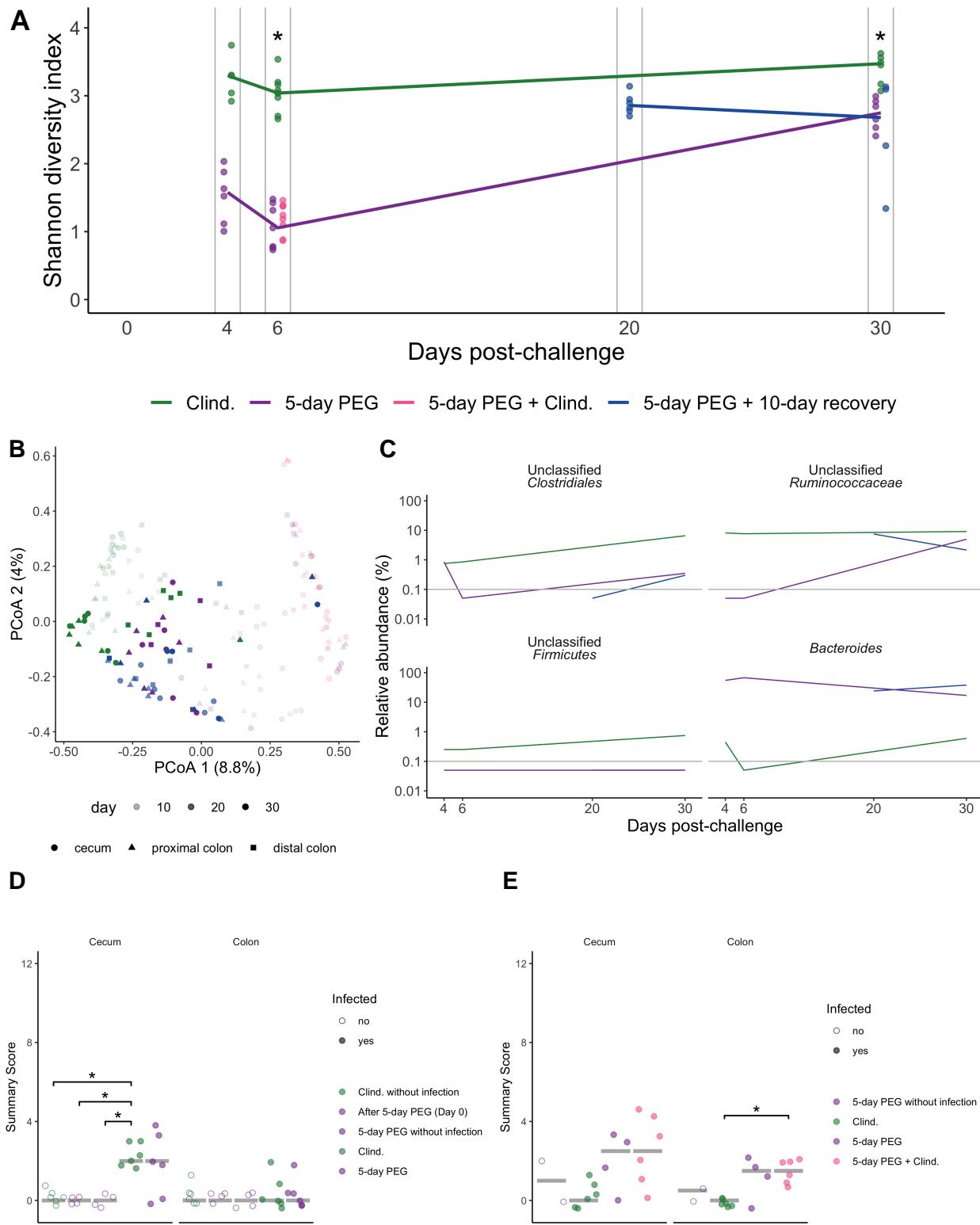
707 intraperitoneal injection. 2. 15% PEG 3350 was administered in the drinking water for five days. 3.
708 5-day PEG plus clindamycin treatment. 4. 5-day PEG plus 10-day recovery treatment. All treatment
709 groups were then challenged with 10^5 *C. difficile* 630 spores. A subset of mice were euthanized
710 on either 4 or 6 days post-challenge and tissues were collected for histopathology analysis, the
711 remaining mice were followed through 20 or 30 days post-challenge. B. Weight change from
712 baseline weight in groups after treatment with PEG and/or clindamycin, followed by *C. difficile*
713 challenge. C. *C. difficile* CFU/gram stool measured over time via serial dilutions(N = 10-59 mice
714 per time point). The black line represents the limit of detection for the first serial dilution. CFU
715 quantification data was not available for each mouse due to stool sampling difficulties (particularly
716 the day the mice came off of the PEG treatment) or early deaths. For B-C, lines represent the
717 median for each treatment group and circles represent samples from individual mice. Asterisks
718 indicate time points where the weight change or CFU/g was significantly different ($P < 0.05$)
719 between groups by the Kruskal-Wallis test with Benjamini-Hochberg correction for testing multiple
720 time points. The data presented are from a total of 5 separate experiments.



721

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

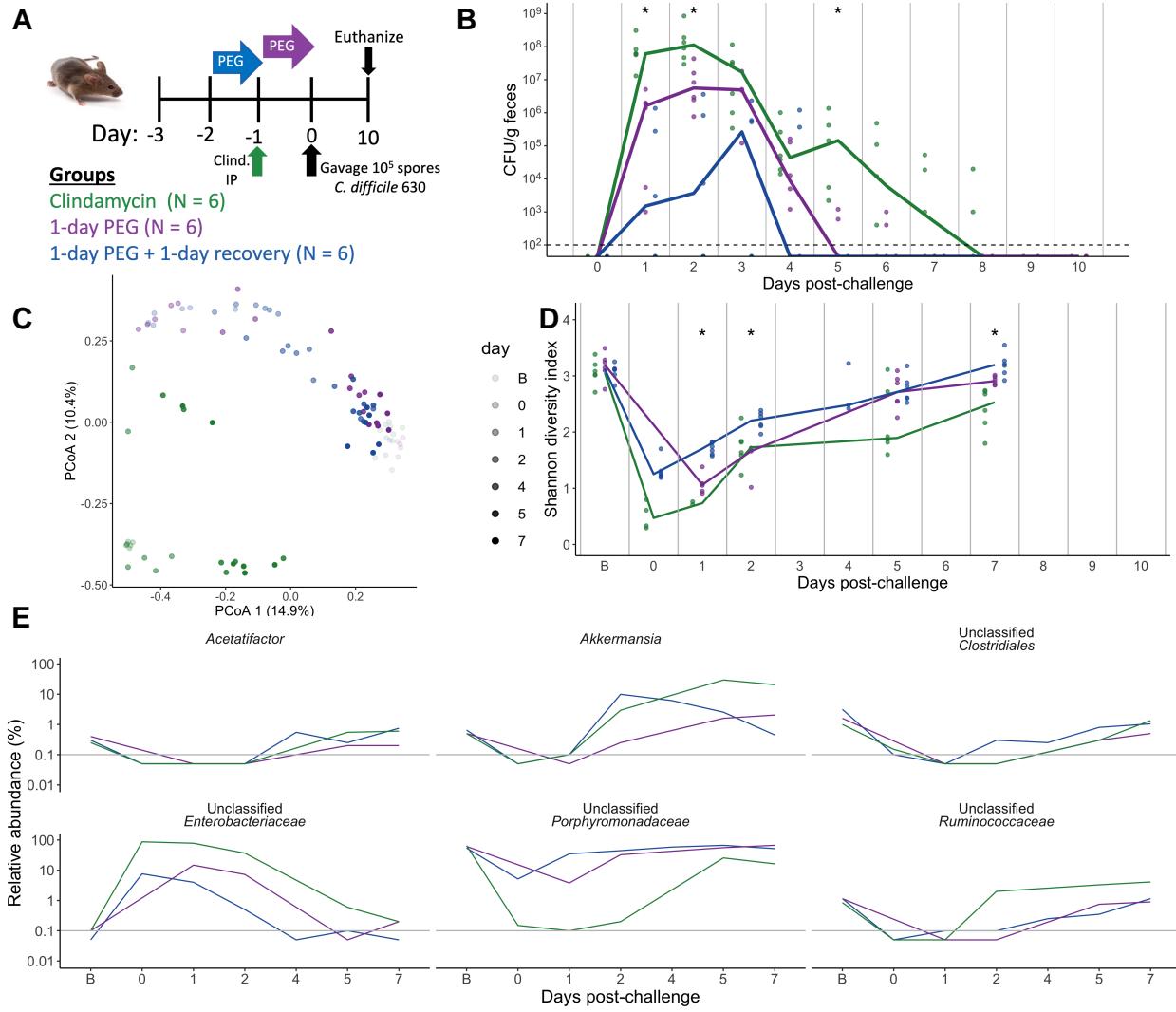
723 **compared to clindamycin-treated mice.** A. Principal Coordinate analysis (PCoA) of Bray-Curtis
724 distances from stool samples collected throughout the experiment. Each circle represents a
725 sample from an individual mouse and the transparency of the symbol corresponds to the day
726 post-challenge. See Data Set S1, sheet 1 for PERMANOVA results. B. Bray-Curtis distances of
727 stool samples collected on either day 10 or 30 post-challenge relative to the baseline sample
728 collected for each mouse (before any drug treatments were administered). The symbols represent
729 samples from individual mice and the line indicates the median value for each treatment group.
730 C. Shannon diversity in stool communities over time. The line indicates the median value for
731 each treatment group (Data Set S1, sheet 2). D. 14 of the 33 genera affected by PEG treatment
732 (Data Set S1, sheet 3). The symbols represent the median relative abundance for a treatment
733 group at either baseline (open circle) or 1-day post treatment (closed circle). Relative abundance
734 data from paired baseline and 1-day post treatment stool samples from the 5-day PEG and
735 5-day PEG plus 10-day recovery groups were analyzed by paired Wilcoxon signed-rank test with
736 Benjamini-Hochberg correction for testing all identified genera. The clindamycin and 5-day PEG
737 plus clindamycin treatment groups are shown on the plot for comparison. E. 6 of the 24 genera
738 that were significantly different between the treatment groups over multiple time points (see Data
739 Set S1, sheet 4 for complete list). The 5-day PEG plus clindamycin treatment group was only
740 followed through 6-days post-challenge. Differences between treatment groups were identified by
741 Kruskal-Wallis test with Benjamini-Hochberg correction for testing all identified genera (*, $P < 0.05$).
742 The gray vertical line (D) and horizontal vertical lines (E) indicate the limit of detection.



743

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

745 **microbiota is altered.** A. Shannon diversity in cecum communities over time. The colors of the
746 symbols and lines represent individual and median relative abundance values for four treatment
747 groups (Data Set S1, sheet 6). B. PCoA of Bray-Curtis distances from mucosal samples collected
748 throughout the experiment. Circles, triangles, and squares indicate the cecum, proximal colon,
749 and distal colon communities, respectively. Transparency of the symbol corresponds to the day
750 post-challenge that the sample was collected. See Data Set S1, sheet 7 for PERMANOVA results.
751 C. The median relative abundance of the 4 genera that were significantly different between the
752 cecum communities of different treatment groups on day 6 and day 30 post-challenge (Data Set S1,
753 sheet 8). The gray vertical lines indicate the limit of detection. D-E. The histopathology summary
754 scores from cecum and colon H&E stained tissue sections. The summary score is the total score
755 based on evaluation of edema, cellular infiltration, and inflammation in either the cecum or colon
756 tissue. Each category is given a score ranging from 0-4, thus the maximum possible summary
757 score is 12. The tissue for histology was collected at either 4 (D) or 6 (E) days post-challenge
758 with the exception that one set of 5-day PEG treated mock-challenged mice were collected on
759 day 0 post-challenge (first set of open purple circles in D). Histology data were analyzed with the
760 Kruskal-Wallis test followed by pairwise Wilcoxon comparisons with Benjamini-Hochberg correction.
761 *, $P < 0.05$.



762

763 **Figure 4. 1-day PEG treatment renders mice susceptible to transient *C. difficile* colonization.** A. Setup of the experimental time line for the 1-day PEG treated mice
 764 consisting of 3 treatment groups. 1. Clindamycin was administered at 10 mg/kg by intraperitoneal
 765 injection. 2. 15% PEG 3350 was administered in the drinking water for 1 day. 3. 1-day PEG
 766 plus 1-day recovery. The three treatment groups were then challenged with 10^5 *C. difficile* 630
 767 spores. B. *C. difficile* CFU/gram stool measured over time (N = 12-18 mice per time point) by serial
 768 dilutions. The black dashed horizontal line represents the limit of detection for the first serial dilution.
 769 For B and D, asterisks indicate time points where there was a significant difference ($P < 0.05$)
 770 between treatment groups by Kruskall-Wallis test with Benjamini-Hochberg correction for testing
 771 multiple time points. For B-D, each symbol represents a sample from an individual mouse and
 772

⁷⁷³ lines indicate the median value for each treatment group. C. PCoA of Bray-Curtis distances from
⁷⁷⁴ stool communities collected over time (day: $R^2 = 0.43$; group: $R^2 = 0.19$, Data Set S1, sheet 11).
⁷⁷⁵ Symbol transparency represents the day post-challenge of the experiment. For C-E, the B on the
⁷⁷⁶ day legend or days post-challenge X-axis stands for baseline and represents the sample that was
⁷⁷⁷ collected prior to any drug treatments. D. Shannon diversity in stool communities over time (Data
⁷⁷⁸ Set S1, sheet 12). E. Median relative abundances per treatment group for 6 out of the 14 genera
⁷⁷⁹ that were affected by treatment, but recovered close to baseline levels by 7 days post-challenge
⁷⁸⁰ (Fig. 3E, Data Set S1, sheets 13 and 14). Paired stool sample relative abundance values either
⁷⁸¹ baseline and day 1 or baseline and day 7 were analyzed by paired Wilcoxon signed-rank test with
⁷⁸² Benjamini-Hochberg correction for testing all identified genera. Only genera that were different
⁷⁸³ between baseline and 1-day post-challenge, but not baseline and 7-days post-challenge are shown.
⁷⁸⁴ The gray horizontal lines represents the limit of detection.

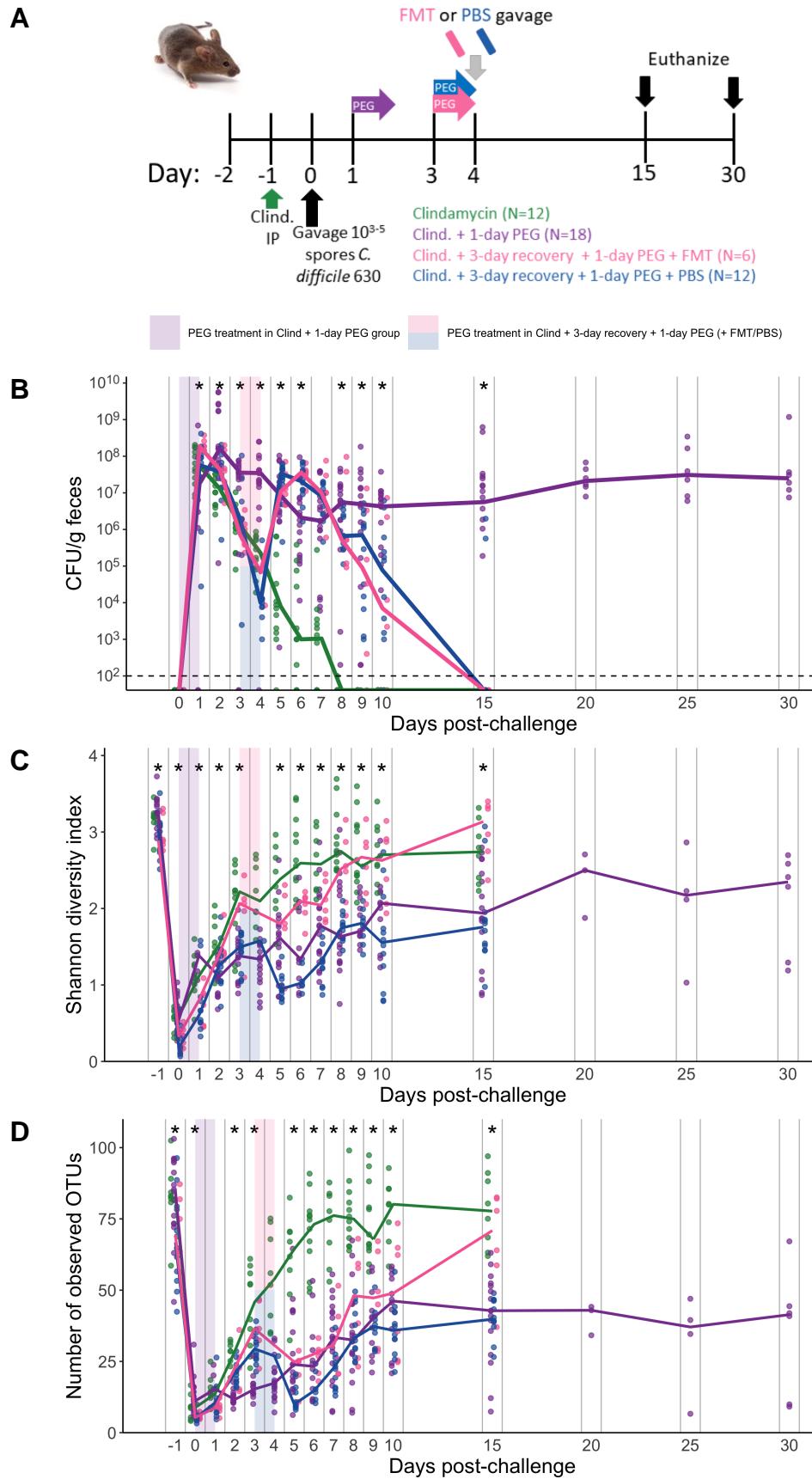
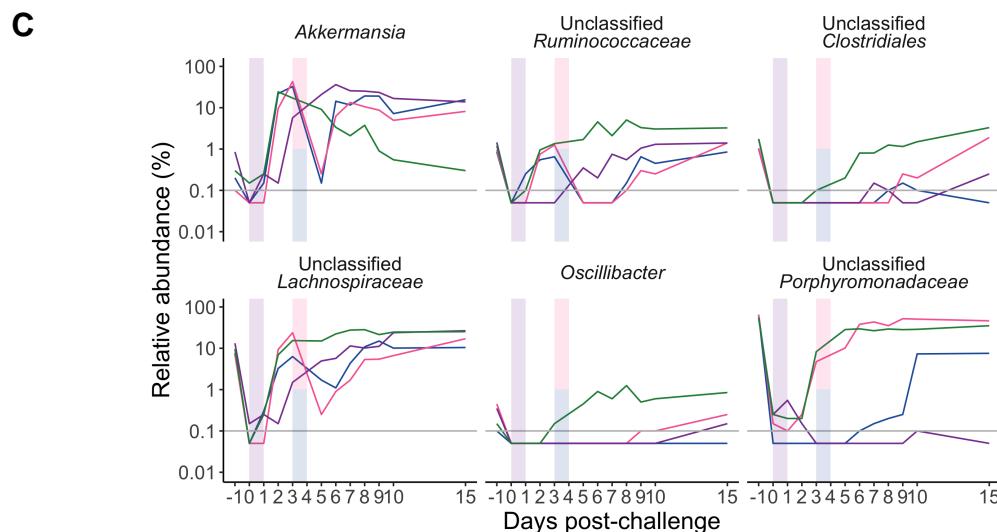
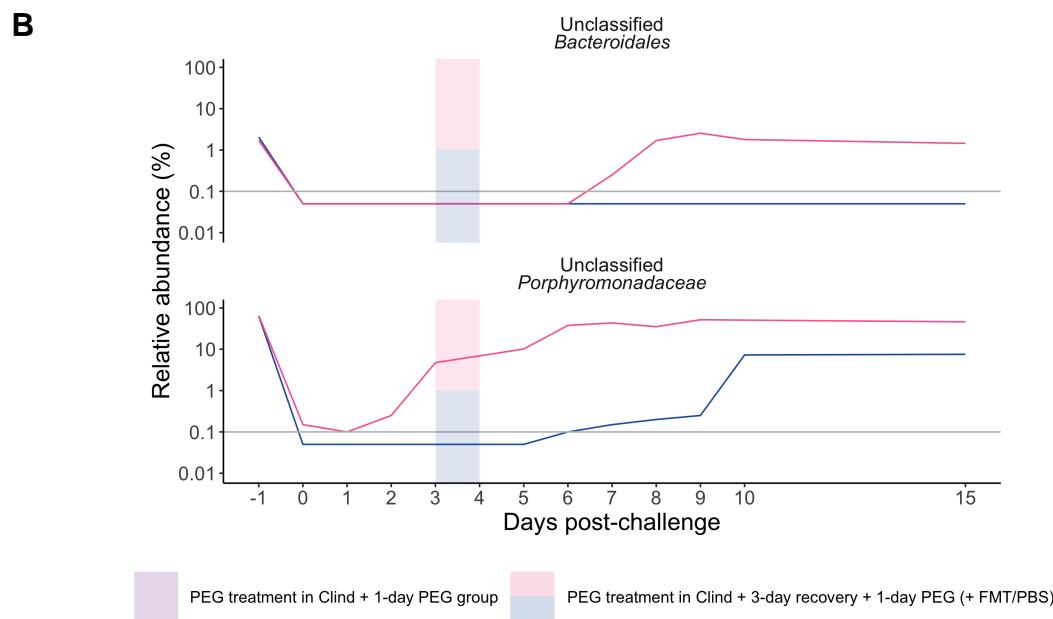
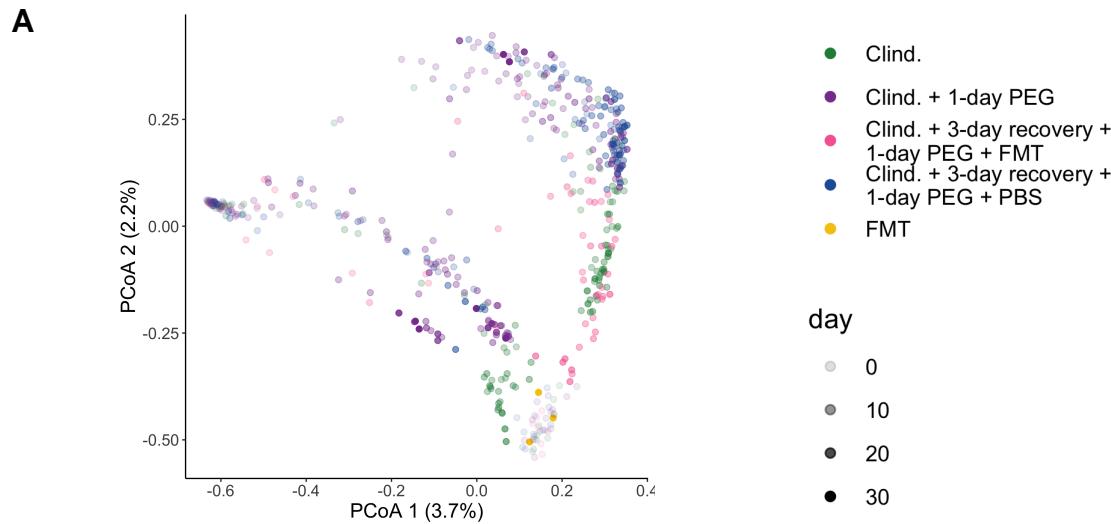
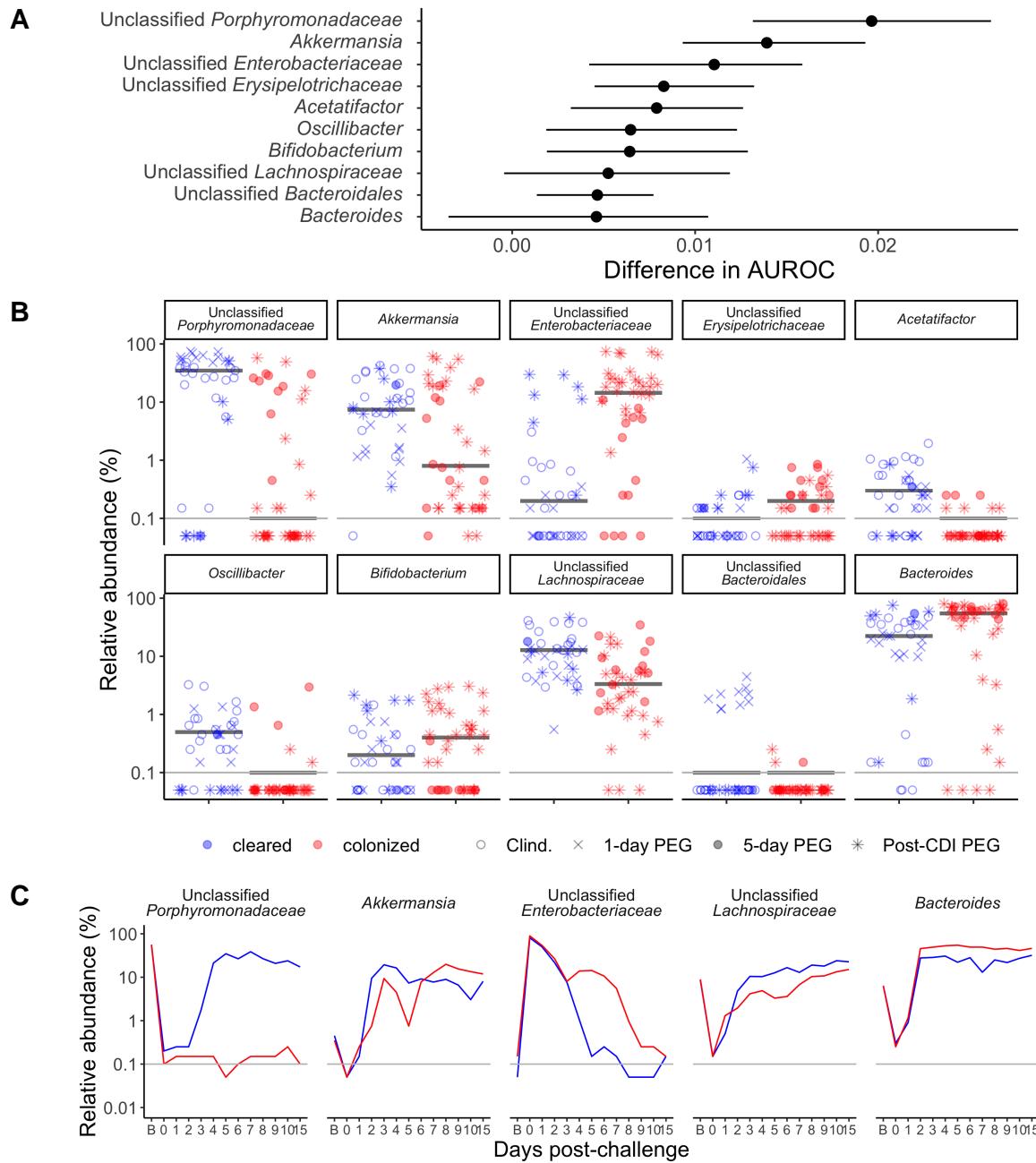


Figure 5.

786 **1-day PEG treatment post *C. difficile* challenge prolongs colonization regardless of**
787 **whether an FMT is also administered.** A. Setup of the experimental time line for experiments
788 with post-challenge PEG treated mice. There were a total of 4 different treatment groups. All mice
789 were administered 10 mg/kg clindamycin intraperitoneally (IP) 1 day before challenge with 10^{3-5}
790 *C. difficile* 630 spores. 1. Received no additional treatment (Clindamycin). 2. Immediately after
791 *C. difficile* challenge, mice received 15% PEG 3350 in the drinking water for 1 day. 3-4. 3-days
792 after challenge, mice received 1-day PEG treatment and then received either 100 microliters a
793 fecal microbiota transplant (3) or PBS (4) solution by oral gavage. Mice were followed through
794 15-30 days post-challenge (only the post-CDI 1-day PEG group was followed through 30 days
795 post-challenge). B. CFU/g of *C. difficile* stool measured over time via serial dilutions. The black line
796 represents the limit of detection for the first serial dilution. C-D. Shannon diversity (C) and richness
797 (D) in stool communities over time (Data Set S1, sheets 15 and 16). B-D. Each symbol represents
798 a stool sample from an individual mouse with the lines representing the median value for each
799 treatment group. Asterisks indicate time points with significant differences ($P < 0.05$) between
800 groups by the Kruskall-Wallis test with a Benjamini-Hochberg correction for testing multiple times
801 points. Colored rectangles indicates the 1-day PEG treatment period for applicable groups. The
802 data presented are from a total of 3 separate experiments.



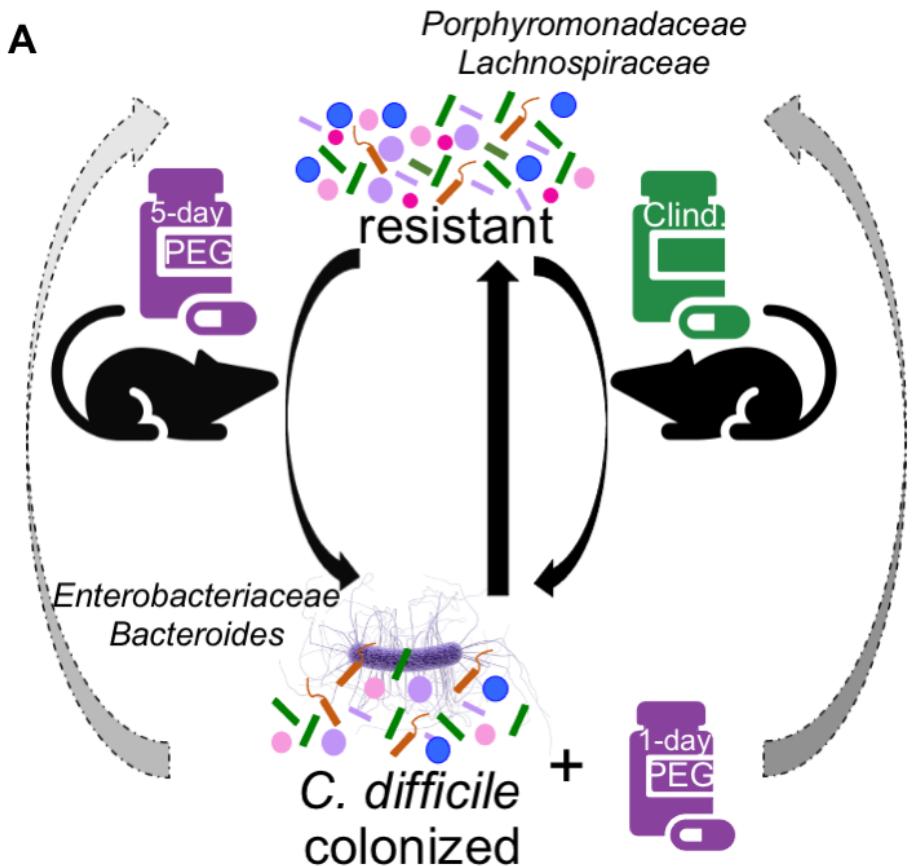
804 **6. For 1-day PEG treatment post *C. difficile* challenge mice that also receive an FMT only**
805 **some bacterial genera were restored.** A. PCoA of Bray-Curtis distances from stool samples
806 collected over time as well as the FMT solution that was administered to one of the treatment
807 groups. Each circle represents an individual sample, the transparency of the circle corresponds
808 to day post-challenge. See Data Set S1, sheet 17 for PERMANOVA results. B. Median relative
809 abundances of 2 genera that were significantly different over multiple time points in mice that were
810 administered either FMT or PBS solution via gavage C. Median relative abundances of the top 6
811 out of 24 genera that were significant over multiple time points, plotted over time (see Data Set S1,
812 sheet 18 for complete list). For B-C, colored rectangles indicates the 1-day PEG treatment period
813 for applicable groups. Gray horizontal lines represent the limit of detection. Differences between
814 treatment groups were identified by Kruskal-Wallis test with Benjamini-Hochberg correction for
815 testing all identified genera. For pairwise comparisons of the groups (B), we performed pairwise
816 Wilcoxon comparisons with Benjamini-Hochberg correction for testing all combinations of group
817 pairs.



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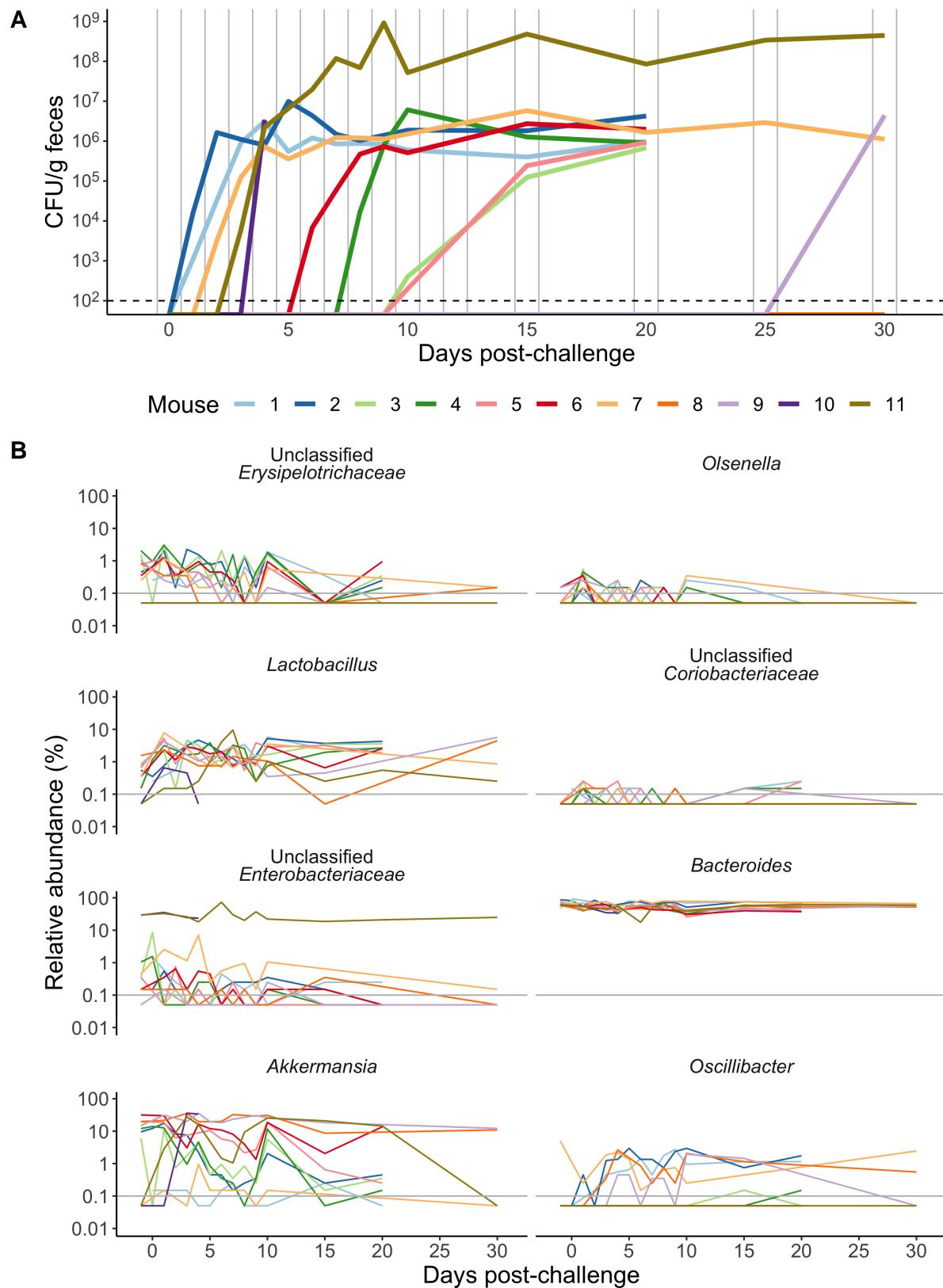
819 **Figure 7. Specific microbiota features associated with prolonged *C. difficile* colonization**
 820 **in PEG treated mice.** A. Top ten bacteria that contributed to the random forest model trained on
 821 5-day post-challenge community relative abundance data, predicting whether mice would still be
 822 colonized with *C. difficile* 10 days post-challenge. The median (point) and interquartile range (lines)
 823 change in AUROC when the bacteria were left out of the model by permutation feature importance
 824 analysis. B. The median relative abundances of the top ten bacteria that contributed to the random
 825 forest classification model at 5 days post-challenge . Red indicates the mice were still colonized

826 with *C. difficile* while blue indicates mice that cleared *C. difficile* 10 days post-challenge and the
827 black horizontal line represents the median relative abundance for the two categories. Each symbol
828 represents a stool sample from an individual mouse and the shape of the symbol indicates whether
829 the PEG-treated mice received a 5-day (Fig. 1-3), 1-day (Fig. 4) or post-challenge PEG (Fig. 5-6)
830 treatment. C. The median relative abundances of the 5 genera with greater than 1% median
831 relative abundance in the stool community over time. For B-C, the gray horizontal lines represents
832 the limit of detection.



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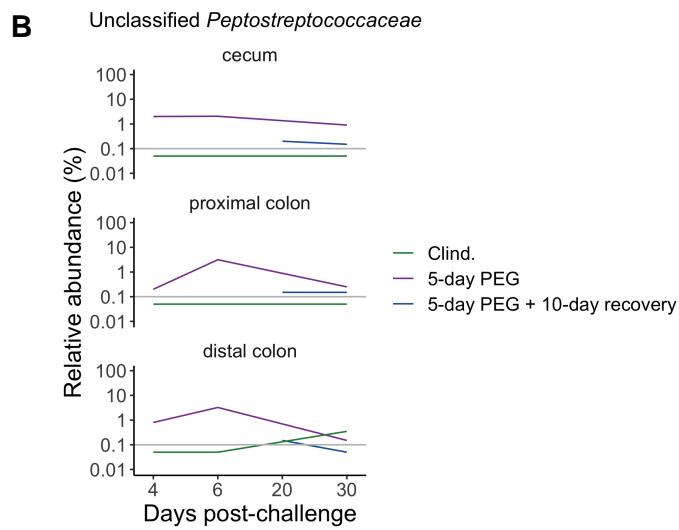
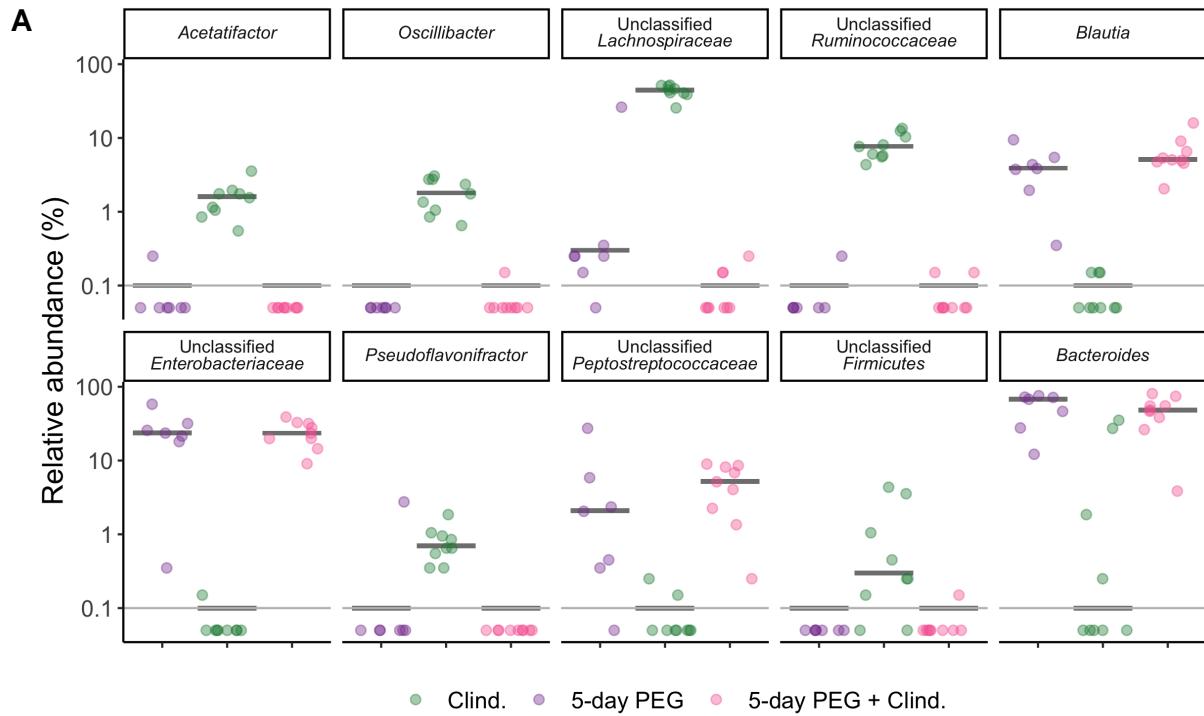
834 **Figure 8. Schematic summarizing findings.** The gut microbiota of our C57Bl/6 mice is resistant
 835 to *C. difficile* but treatment with either the antibiotic, clindamycin, or the osmotic laxative, PEG
 836 3350, renders the mice susceptible to *C. difficile* colonization. Recovery of colonization resistance
 837 in clindamycin-treated mice is relatively straightforward and the mice clear *C. difficile* within 10
 838 days post-challenge. However, for mice that received either a 5-day PEG treatment prior to *C.*
 839 *difficile* challenge or a 1-day PEG treatment post-challenge recovery of colonization resistance was
 840 delayed because most mice were still colonized with *C. difficile* 10 days post-challenge. We found
 841 increased relative abundances of *Porphyromonadaceae* and *Lachnospiraceae* were associated
 842 with recovery of colonization resistance, while increased relative abundances of *Enterobacteriaceae*
 843 and *Bacteroides* were associated with prolonged *C. difficile* colonization.



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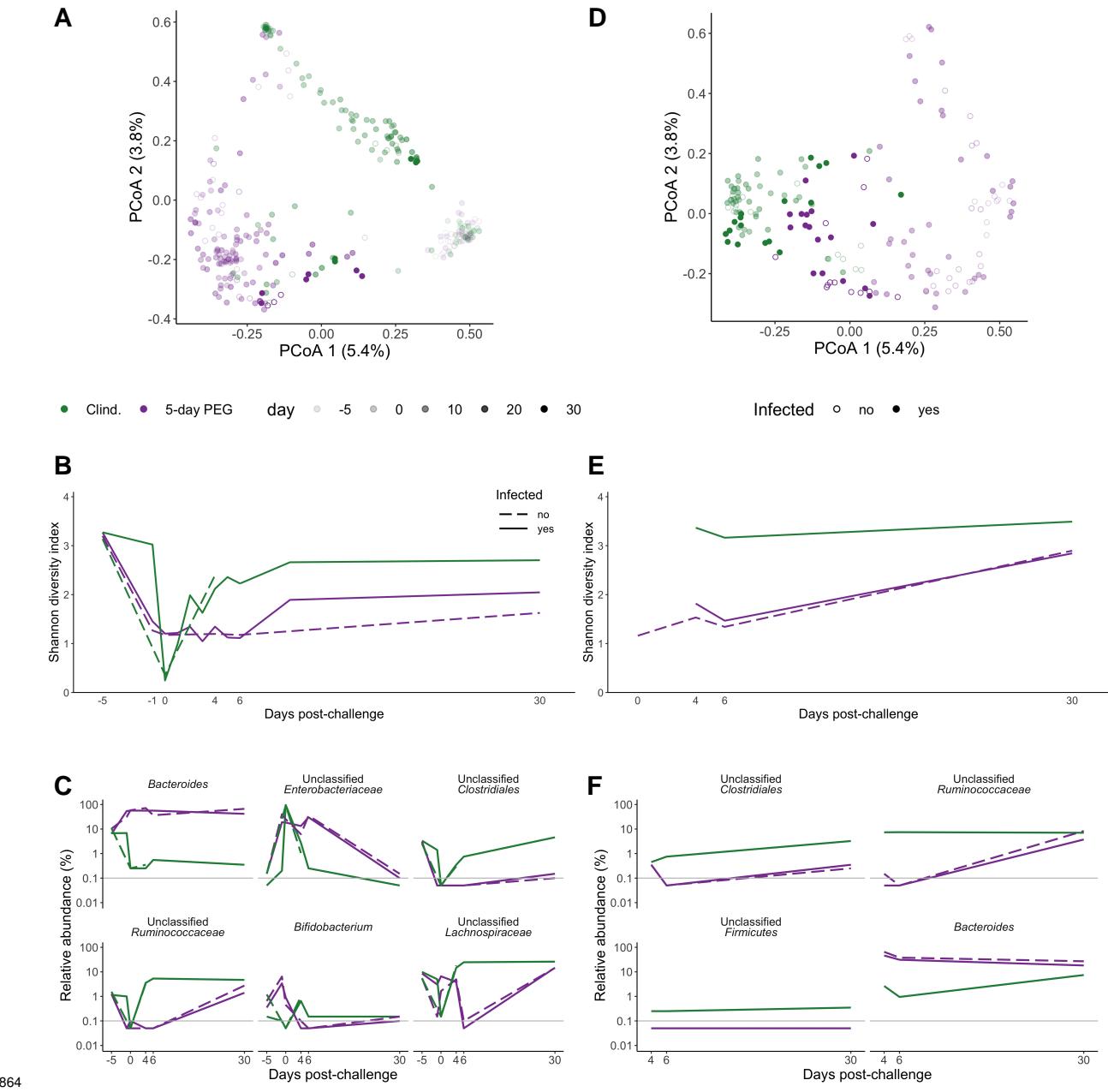
845 **Figure S1.** Microbiota dynamics post-challenge in the 5-day PEG treatment plus 10-day

846 **recovery mice.** A. *C. difficile* CFU/g over time in the stool samples collected from 5-day PEG
847 treated mice that were allowed to recover for 10 days prior to challenge. Same data presented in
848 Fig. 1C, but the data for the other 3 treatment groups have been removed and each line represents
849 the CFU over time for an individual mouse. Mouse 10 was found dead 6 days post-challenge.
850 B. Relative abundances of eight bacterial genera from day 0 post-challenge onward in each of
851 the 10-day recovery mice. We analyzed samples from day 0 and day 8 post-challenge, which
852 represented the time points where mice were challenged with *C. difficile* and when the median
853 relative *C. difficile* CFU stabilized for the group using the paired Wilcoxon signed-rank test, but no
854 genera were significantly different after Benjamini-Hochberg correction (Data Set S1, sheet 5).



855 **Figure S2. PEG treatment still has a large impact on the mucosal microbiota 6 days**
 856 **post-challenge A.** The relative abundances of the 10 bacterial genera that were significantly
 857 different between treatment groups at 6 days post-infection in the cecum tissue (the relative
 858 abundances of the 10 genera were also significantly different in the proximal and distal colon
 859 tissues, Data Set S1, sheets 8, 9, and 10). Each symbol represents a tissue sample from an
 860 individual mouse, the black horizontal lines represents the median relative abundances for each
 861 treatment group. B. The relative abundance of *Peptostreptococcaceae* in the three types of tissue

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



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

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

876 **Data Set S1**

877 **Data Set S1, Sheets 1-19. Excel workbook with 19 sheets.**

878 **Data Set S1, Sheet 1. PERMANOVA results for the stool communities from mice in the 5-day PEG subset.**

880 **Data Set S1, Sheet 2. Shannon diversity analysis for the stool communities from mice in the 5-day PEG subset.**

882 **Data Set S1, Sheet 3. Genera with relative abundances impacted by PEG treatment based on stool communities of 5-day PEG treated mice.**

884 **Data Set S1, Sheet 4. Genera with relative abundances that vary between treatment groups in the stool communities from mice in the 5-day PEG subset.**

886 **Data Set S1, Sheet 5. Statistical analysis results for genera with relative abundances that varied in stool communities in the 5-day PEG plus 10-day recovery mice between the day 1 and day 8 time points.**

889 **Data Set S1, Sheet 6. Shannon diversity analysis for the cecum communities from mice in the 5-day PEG experiments.**

891 **Data Set S1, Sheet 7. PERMANOVA results for the tissue communities from mice in the 5-day PEG subset.**

893 **Data Set S1, Sheet 8. Genera with relative abundances that vary between treatment groups in the cecum communities from mice in the 5-day PEG esubset.**

895 **Data Set S1, Sheet 9. Genera with relative abundances that vary between treatment groups in the proximal colon communities from mice in the 5-day PEG subset.**

897 **Data Set S1, Sheet 10. Genera with relative abundances that vary between treatment groups in the distal colon communities from mice in the set of 5-day PEG subset.**

899 **Data Set S1, Sheet 11. PERMANOVA results for the stool communities from mice in the set**

900 **of 1-day PEG subset.**

901 **Data Set S1, Sheet 12. Shannon diversity analysis for the stool communities from mice in**
902 **the 1-day PEG experiments.**

903 **Data Set S1, Sheet 13. Genera with different relative abundances between the baseline and**
904 **day 1 time points in the 1-day PEG subset.**

905 **Data Set S1, Sheet 14. Genera with different relative abundances between the baseline and**
906 **day 7 time points in the 1-day PEG subset..**

907 **Data Set S1, Sheet 15. Shannon diversity analysis for the stool communities from mice in**
908 **the post-challenge PEG experiments.**

909 **Data Set S1, Sheet 16. Richness analysis for the stool communities from mice in the**
910 **post-challenge PEG experiments.**

911 **Data Set S1, Sheet 17. PERMANOVA results for the stool communities from mice in the**
912 **post-challenge PEG subset.**

913 **Data Set S1, Sheet 18. Genera with relative abundances that vary between treatment groups**
914 **in the stool communities from mice in the post-challenge PEG subset.**

915 **Data Set S1, Sheet 19. AUROC results for the 100 different seeds from each of the 3 models**
916 **tested.**