

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 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
23 diarrhea or *C. difficile* infections (CDI) including lower diversity compared to communities from
24 healthy patients, which led us to hypothesize that diarrhea may be an indicator of *C. difficile* risk.
25 We explored how osmotic laxatives disrupt the microbiota's colonization resistance to *C. difficile* by
26 administering a laxative to mice either before or after *C. difficile* challenge. Our findings suggest
27 that osmotic laxatives disrupt colonization resistance to *C. difficile*, and prevent clearance among
28 mice already colonized with *C. difficile*. Considering that most hospitals recommend not performing

- 29 *C. difficile* testing on patients taking laxatives and laxatives are used when administering fecal
30 microbiota transplants via colonoscopy to patients with recurrent CDIs, further studies are needed
31 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 are separated into two groups based on stool consistency, there are similar
44 microbiota features between samples from CDI patients and *C. difficile* negative patients with
45 diarrhea compared to samples that were *C. difficile* negative with non-diarrheal stool consistency
46 (12). The similar community features between CDI patients and patients with diarrhea included low
47 alpha diversity and 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 profiled community diversity and composition. We examined the alpha diversity dynamics by

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

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

146 **5-day laxative treatment did not promote more severe CDIs despite altering the mucosal**
147 **microbiota.** Given the findings from a previous study that demonstrated PEG treatment disrupts
148 the mucus layer and alters the immune response in mice (16), we decided to examine the impact of
149 PEG treatment on the mucosal microbiota and CDI severity. To evaluate the mucosal microbiota,
150 we sequenced communities associated with tissues collected from the cecum, proximal colon, and
151 distal colon. Similar to what was observed with the stool samples, the alpha diversity was lower
152 in the PEG-treated mice compared to clindamycin treated mice (Fig. 3A, Data Set S1, sheet 6).
153 The alpha diversity of the tissue-associated community increased in PEG-treated mice collected
154 at 20 and 30 days post-challenge (Fig. 3A). Group ($R^2 = 0.33$), time point ($R^2 = 0.11$), and their
155 interactions with other variables (cage, experiment number, and sample type) explained the majority
156 of the variation observed in mucosal communities (PERMANOVA combined $R^2 = 0.83$, $P < 0.05$,
157 Fig. 3B, Data Set S1, sheet 7). We saw the greatest difference in the relative abundance of the
158 mucosal microbiota between treatment groups (clindamycin, 5-day PEG, and 5-day PEG plus
159 clindamycin) at 6 days post-challenge with 10 genera that were significantly different ($P < 0.05$) in
160 all three of the tissue types we collected (cecum, proximal colon, and distal colon; Fig. S2A, Data
161 Set S1, sheet 8, 9, and 10). Interestingly, *Peptostreptococcaceae* (the family with a sequence that
162 matches *C. difficile*) was one of the genera that had a significant difference in relative abundance
163 between treatment groups at 6 days post-challenge. This population was primarily only present
164 in the 5-day PEG treatment group of mice and decreased in the proximal and distal colon tissues
165 over time (Fig. S2B). By 30 days post-challenge, only the relative abundances of *Bacteroides*,
166 *Clostridiales*, *Firmicutes*, and *Ruminococcaceae* were different between treatment groups and
167 only in the cecum tissues (Fig. 3C, Fig. 2E, Data Set S1, sheet 8). Thus, PEG treatment had a
168 significant impact on the mucosal microbiota and we detected *C. difficile* sequences in the cecum,
169 proximal colon, and distal colon tissue communities.

170 Because there were differences in the mucosal microbiota including detectable *C. difficile* sequences
171 in tissues from PEG-treated mice relative to mice treated with clindamycin, we next examined the
172 severity of *C. difficile* challenge by evaluating cecum and colon histopathology (27). However, we
173 found there was no difference in cecum and colon scores between clindamycin and PEG-treated
174 mice that were challenged with *C. difficile* at 4 days post-challenge (Fig. 3D), the time point

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

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

190 **1-day laxative treatment resulted in transient *C. difficile* colonization and minor microbiota**
191 **disruption.** Next, we examined how a shorter osmotic laxative perturbation would impact the
192 microbiome and susceptibility to *C. difficile*. We administered either a 1-day PEG treatment, a
193 1-day PEG treatment with a 1-day recovery period, or clindamycin to mice before challenging them
194 with *C. difficile* (Fig. 3A). In contrast to the 5-day PEG treated mice, the 1-day PEG groups were
195 only transiently colonized and cleared *C. difficile* by 7 days post-challenge (Fig. 3B). The stool
196 communities of the 1-day PEG treatment groups were also only transiently disrupted, with Shannon
197 diversity recovering by 7 days post-challenge (Fig. 3C-D, Data Set S1, sheets 11 and 12). We found
198 the relative abundances of 14 genera were impacted by treatment, but recovered close to baseline
199 levels by 7 days post-challenge including *Enterobacteriaceae*, *Clostridiales*, *Porphyromonadaceae*,
200 and *Ruminococcaceae* (Fig. 3E, Data Set S1, sheet 13 and 14). These findings suggest the
201 duration of the PEG treatment was relevant, with shorter treatments resulting in a transient loss of
202 *C. difficile* colonization resistance.

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

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

230 **Five-day post-challenge community data can predict which mice will have prolonged**
231 ***C. difficile* colonization.** After identifying bacteria associated with the 5-day, 1-day and

232 post-challenge 1-day PEG treatments, we examined the bacteria that influenced prolonged *C.*
233 *difficile* colonization. We trained 3 machine learning models (random forest, logistic regression,
234 and support vector machine) with bacterial community data from 5 days post-challenge to predict
235 whether the mice were still colonized with *C. difficile* 10 days post-challenge. We chose to predict
236 the status based on communities 5 days post-challenge because that was the earliest time point
237 where we saw a treatment effect in the post-challenge 1-day PEG experiments. The random
238 forest model had the highest performance (median AUROC = 0.90, Data Set S1, sheet 19) and
239 indicated that the 5-day post challenge microbiota was an excellent predictor of prolonged *C.*
240 *difficile* colonization. Next, we performed a permutation importance test to identify the bacteria
241 that were the top contributors to the random forest model for predicting prolonged *C. difficile*
242 colonization. We selected 10 genera that contributed the most to our model's performance (Fig.
243 7A) and examined their relative abundance at 5 days post-challenge, the time point used to predict
244 *C. difficile* colonization status on day 10 (Fig. 7B). Next, we focused on the 5 genera that had a
245 greater than 1% relative abundance in either the cleared or colonized mice and examined how the
246 bacteria changed over time. We found *Enterobacteriaceae* and *Bacteroides* tended to consistently
247 have a higher relative abundance, the relative abundance of *Akkermansia* was initially low and then
248 increased, and *Porphyromonadaceae* and *Lachnospiraceae* had a lower relative abundance in the
249 mice with prolonged colonization compared to the mice that cleared *C. difficile* (Fig. 7C). Together
250 these results suggest a combination of low and high abundance bacterial genera influence the
251 prolonged colonization 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 mice treated with an osmotic laxative over time, we found that a 5-day PEG treatment
256 before challenge resulted in prolonged *C. difficile* colonization, while a 1-day PEG treatment resulted
257 in transient colonization without the use of antibiotics. The differences in *C. difficile* colonization
258 dynamics between the 5- and 1-day PEG treated mice were associated with differences in how
259 much the treatments disrupted the microbiota. Additionally, the intestinal communities of 5-day

260 PEG treated mice did not regain colonization resistance after a 10-day recovery period. In contrast
261 to the other 5-day PEG treatment groups, *C. difficile* was not immediately detectable in the stools of
262 most of the mice in the 10-day recovery group. We also examined the impact of PEG treatment
263 after *C. difficile* challenge. In opposition to the hypothesis suggested by the literature, we found that
264 PEG treatment prolonged colonization relative to mice that only received clindamycin treatment.
265 We identified patterns in the relative abundances of *Bacteroides*, *Enterobacteriaceae*, *Akkermansia*,
266 *Porphyromonadaceae*, and *Lachnospiraceae* that were associated with prolonged *C. difficile*
267 colonization (Fig. 8). Overall, our results demonstrated that osmotic laxative treatment alone
268 rendered mice susceptible to *C. difficile* colonization and the duration of colonization depended on
269 the length of PEG treatment and 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 (30), and separate studies demonstrated valerate is
276 depleted after clindamycin treatment and inhibited *C. difficile* growth *in vitro* and in C57BL/6 mice
277 (31, 32). Similarly, *Acetatifactor* can produce acetate and butyrate (33), SCFAs that are decreased
278 in mice with prolonged *C. difficile* infection after antibiotic treatment (34). Thus protective bacteria
279 and their metabolites could be depleted by osmotive laxative treatment depending on the timing
280 and duration of treatment.

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

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

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

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

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

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

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

347 in human patients receiving FMTs.

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

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366 Materials and Methods

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

374 at a density of 2-3 mice per cage, with the majority of cages limited to two mice.

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

382 **C. difficile challenge model.** Mice were challenged with 25 microliters of *C. difficile* 630 spores at
383 10^5 concentration, except for 1 experiment where the concentration was 10^3 (Fig. 5A). All mock
384 challenged mice received 25 ul vehicle solution (Ultrapure water). A Dymax stepper pipette was
385 used to administer the same challenge dose to mice via oral gavage. Mice were weighed daily
386 throughout the experiment and stool was collected for quantifying *C. difficile* CFU and 16S rRNA
387 gene sequencing. There were two groups of mice that received either a PBS or fecal microbiota
388 transplant (FMT) gavage post-PEG treatment. The fecal microbiota transplant was prepared with
389 stool samples collected from the mice in the experiment prior to the start of any treatments. The
390 stool samples were transferred to an anaerobic chamber and diluted 1:10 in reduced PBS and
391 glycerol was added to make a 15% glycerol solution. The solution was then aliquoted into tubes and
392 stored at -80 °C until the day of the gavage. An aliquot of both the FMT and PBS solutions were also
393 set aside in the -80 °C for 16S rRNA gene sequencing. The day of the gavage, aliquots were thawed
394 and centrifuged at 7500 RPM for 1 minute. The supernatant was then transferred to a separate
395 tube to prevent the gavage needle from clogging with debris during gavage. The PBS solution that
396 was administered to the other group was also 15% glycerol. Each mouse was administered 100
397 microliters of either the FMT or PBS solution via gavage. When we refer to mice that cleared *C.*
398 *difficile*, we mean that no *C. difficile* was detected in the first serial dilution (limit of detection: 100
399 CFU). In some experiments, we collected tissues for 16SrRNA gene sequencing, histopathology,
400 or both. For 16S rRNA gene sequencing, we collected small snips of cecum, proximal colon,
401 and distal colon tissues in microcentrifuge tubes, snap froze in liquid nitrogen, and stored at
402 -80 °C. For histopathology, cecum and colon tissues were placed into separate cassettes, fixed, and

403 then submitted to McClinchey Histology Labs (Stockbridge, MI) for processing, embedding, and
404 hematoxylin and eosin (H&E) staining.

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

410 **16S rRNA gene sequencing.** Stool samples were stored at -80 °C and were placed into 96-well
411 plates for DNA extractions and library preparation. DNA was extracted using the DNeasy Powersoil
412 HTP 96 kit (Qiagen) and an EpMotion 5075 automated pipetting system (Eppendorf). For library
413 preparation, each plate had a mock community control (ZymoBIOMICS microbial community DNA
414 standards) and a negative control (water). The V4 region of the 16S rRNA gene was amplified with
415 the AccuPrime Pfx DNA polymerase (Thermo Fisher Scientific) using custom barcoded primers, as
416 previously described (71). The PCR amplicons were normalized (SequalPrep normalizatin plate kit
417 from Thermo Fisher Scientific), pooled and quantified (KAPA library quantification kit from KAPA
418 Biosystems), and sequenced with the MiSeq system (Illumina).

419 **16S rRNA gene sequence analysis.** All sequences were processed with mothur (v. 1.43) using
420 a previously published protocol (71, 72). Paired sequencing reads were combined and aligned
421 with the SILVA (v. 132) reference database (73) and taxonomy was assigned with a modified
422 version of the Ribosomal Database Project reference sequences (v. 16) (74). The error rate for
423 sequencing data was 0.0559% based on the 17 mock communities we ran with the samples.
424 Samples were rarefied to 1,000 sequences, 1,000 times for alpha and beta diversity analyses
425 in order to account for uneven sequencing across samples. All but 3 of the 17 water controls
426 had fewer than 1000 sequences. PCoAs were generated based on Bray-Curtis Index distance
427 matrices. Permutational multivariate analysis of variance (PERMANOVA) tests were performed on
428 mothur-generated Bray-Curtis distance matrices with the adonis function from the vegan R package
429 (75).

430 **Histopathology.** H&E stained sections of cecum and colon tissues collected at either 0, 4, or 6

431 days post-challenge were coded to be scored in a blinded manner by a board-certified veterinary
432 pathologist (ILB). Slides were evaluated using a scoring system developed for mouse models of *C.*
433 *difficile* infection (51). Each slide was evaluated for edema, cellular infiltration, and inflammation
434 and given a score ranging from 0-4. The summary score was calculated by combining the scores
435 from the 3 categories and ranged from 0-12.

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

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

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

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

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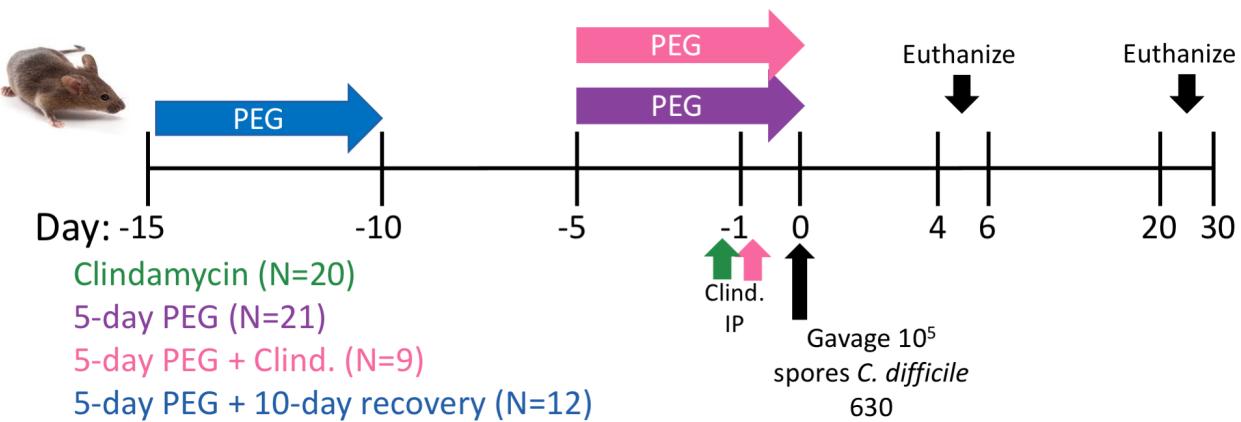
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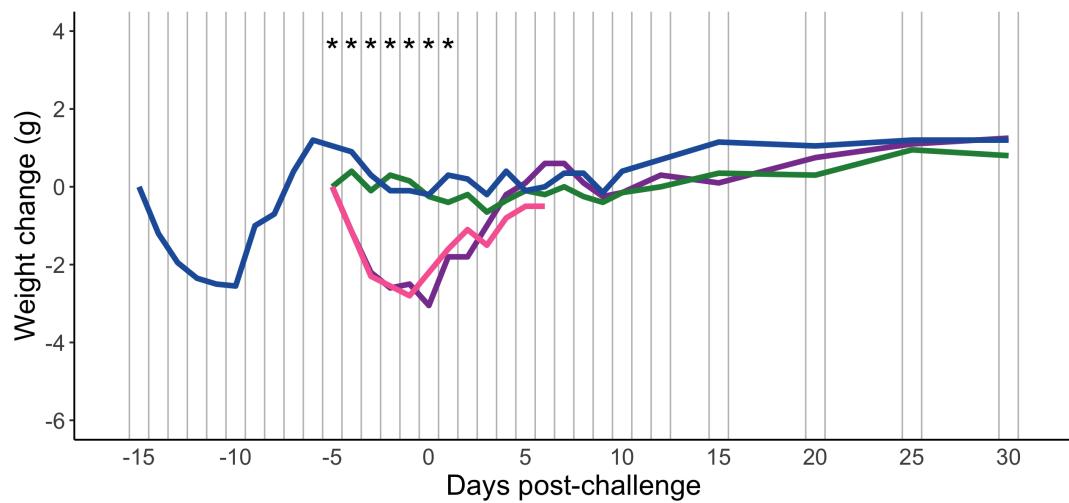
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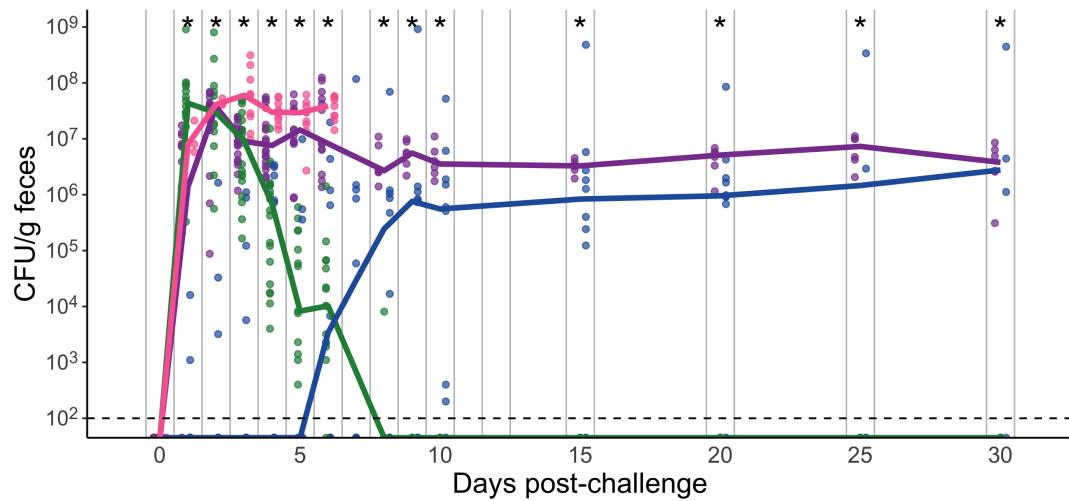
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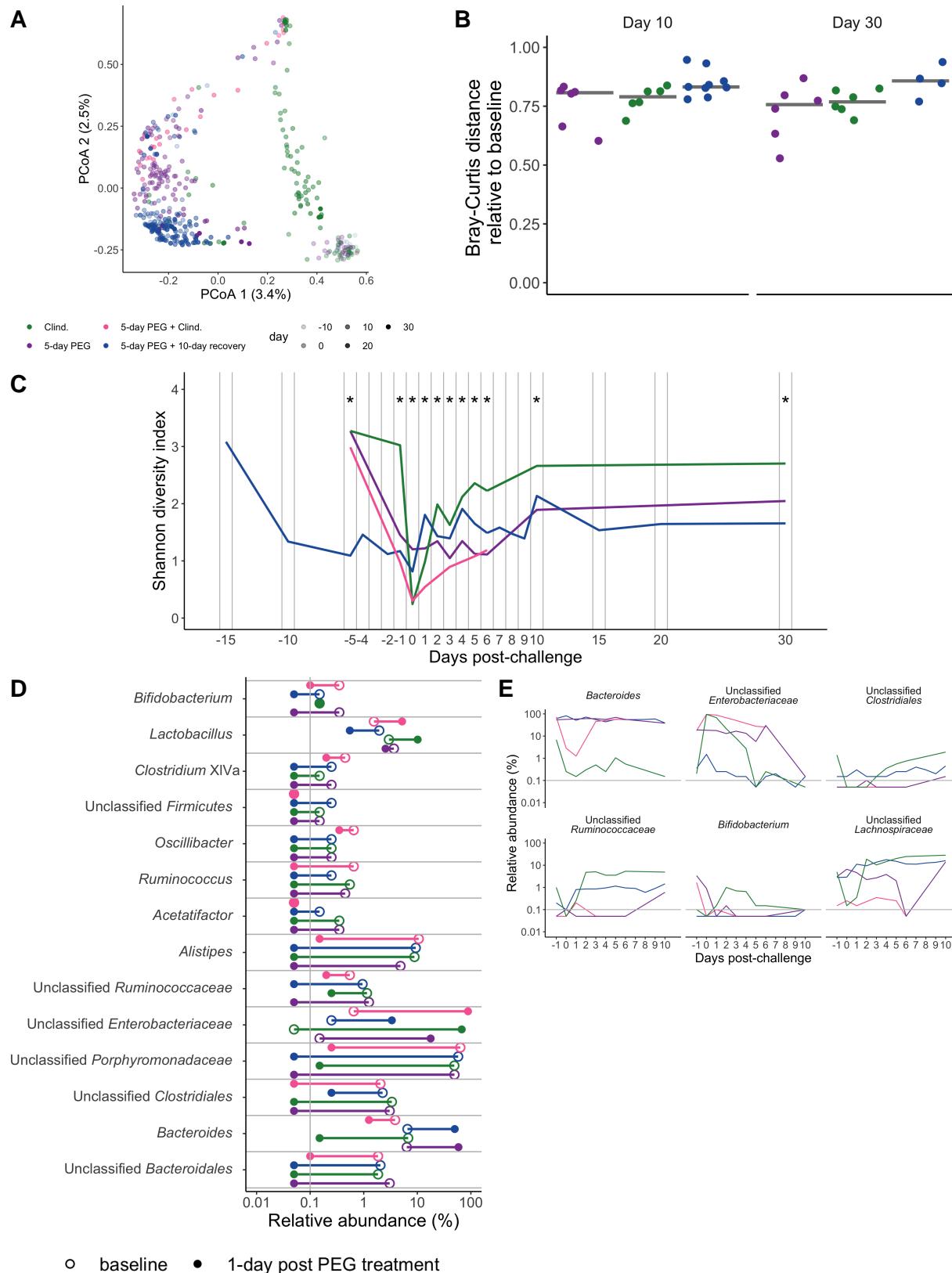
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701

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

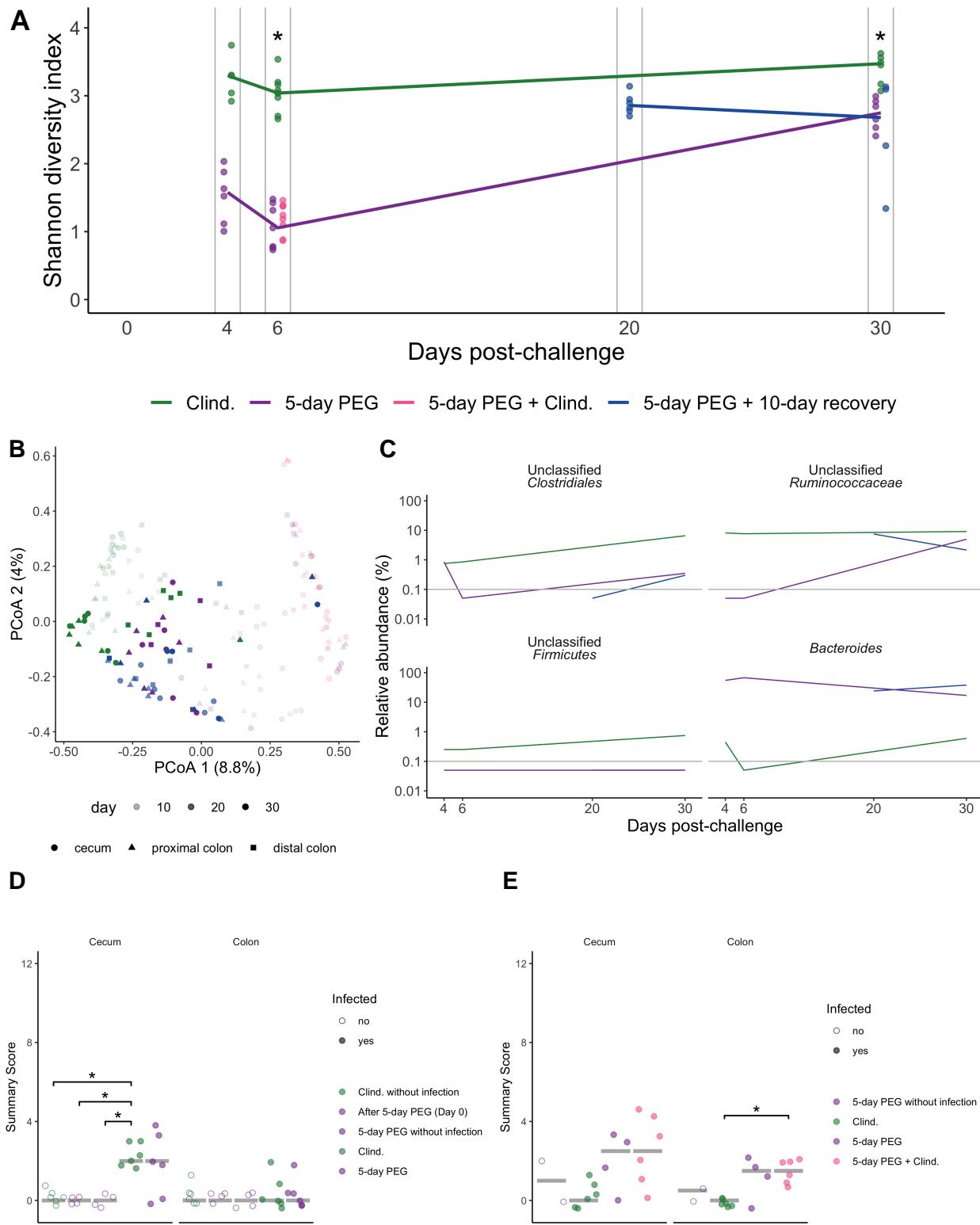
705 intraperitoneal injection. 2. 15% PEG 3350 was administered in the drinking water for five days. 3.
706 5-day PEG plus clindamycin treatment. 4. 5-day PEG plus 10-day recovery treatment. All treatment
707 groups were then challenged with 10^5 *C. difficile* 630 spores. A subset of mice were euthanized
708 on either 4 or 6 days post-challenge and tissues were collected for histopathology analysis, the
709 remaining mice were followed through 20 or 30 days post-challenge. B. Weight change from
710 baseline weight in groups after treatment with PEG and/or clindamycin, followed by *C. difficile*
711 challenge. C. *C. difficile* CFU/gram stool measured over time via serial dilutions(N = 10-59 mice
712 per time point). The black line represents the limit of detection for the first serial dilution. CFU
713 quantification data was not available for each mouse due to stool sampling difficulties (particularly
714 the day the mice came off of the PEG treatment) or early deaths. For B-C, lines represent the
715 median for each treatment group and circles represent samples from individual mice. Asterisks
716 indicate time points where the weight change or CFU/g was significantly different ($P < 0.05$)
717 between groups by the Kruskal-Wallis test with Benjamini-Hochberg correction for testing multiple
718 time points. The data presented are from a total of 5 separate experiments.



719

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

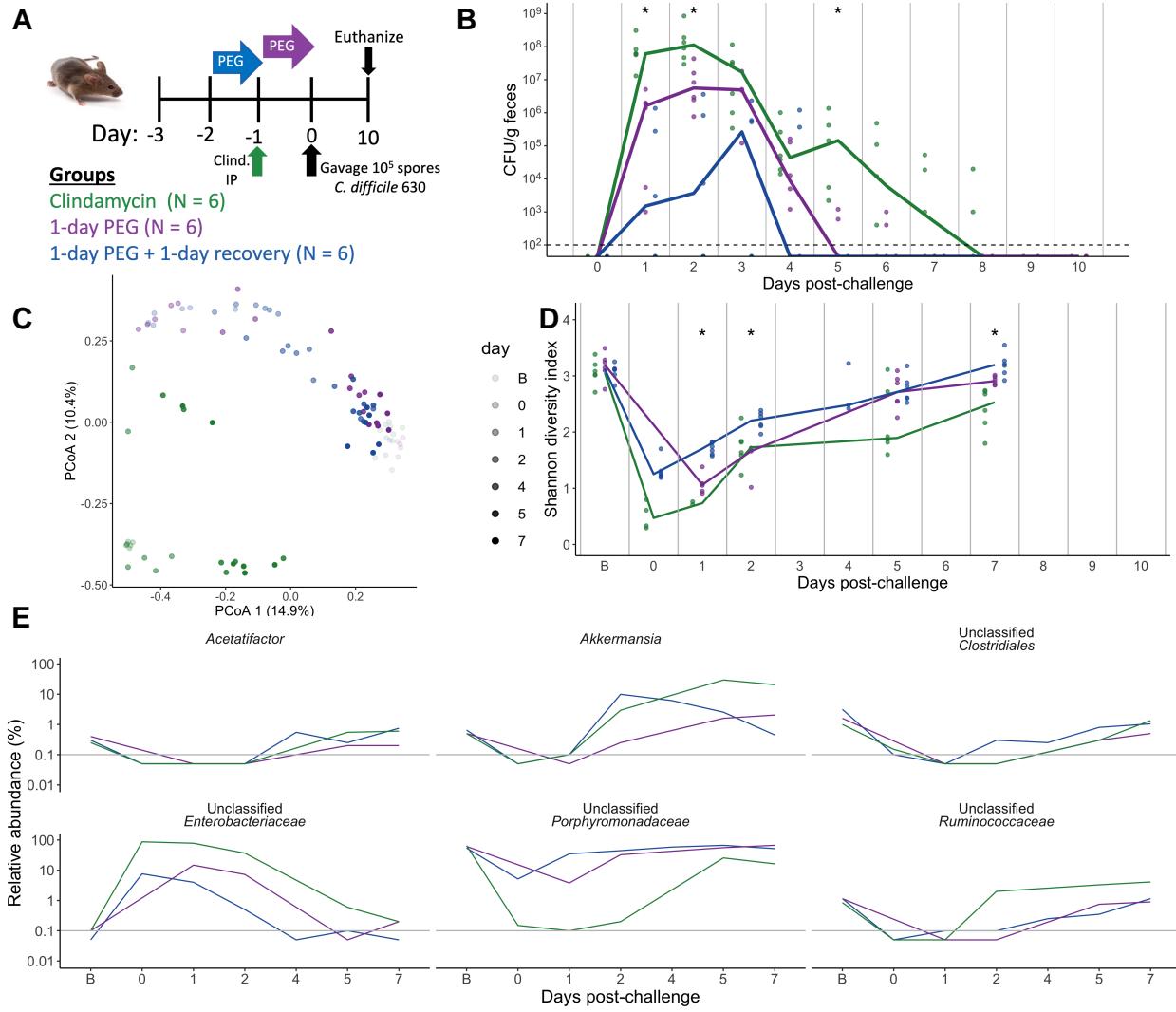
721 **compared to clindamycin-treated mice.** A. Principal Coordinate analysis (PCoA) of Bray-Curtis
722 distances from stool samples collected throughout the experiment. Each circle represents a
723 sample from an individual mouse and the transparency of the symbol corresponds to the day
724 post-challenge.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



741

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

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



760

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

⁷⁷¹ lines indicate the median value for each treatment group. C. PCoA of Bray-Curtis distances from
⁷⁷² stool communities collected over time (day: $R^2 = 0.43$; group: $R^2 = 0.19$, Data Set S1, sheet 11).
⁷⁷³ Symbol transparency represents the day post-challenge of the experiment. For C-E, the B on the
⁷⁷⁴ day legend or days post-challenge X-axis stands for baseline and represents the sample that was
⁷⁷⁵ collected prior to any drug treatments. D. Shannon diversity in stool communities over time (Data
⁷⁷⁶ Set S1, sheet 12). E. Median relative abundances per treatment group for 6 out of the 14 genera
⁷⁷⁷ that were affected by treatment, but recovered close to baseline levels by 7 days post-challenge
⁷⁷⁸ (Fig. 3E, Data Set S1, sheets 13 and 14). Paired stool sample relative abundance values either
⁷⁷⁹ baseline and day 1 or baseline and day 7 were analyzed by paired Wilcoxon signed-rank test with
⁷⁸⁰ Benjamini-Hochberg correction for testing all identified genera. Only genera that were different
⁷⁸¹ between baseline and 1-day post-challenge, but not baseline and 7-days post-challenge are shown.
⁷⁸² The gray horizontal lines represents the limit of detection.

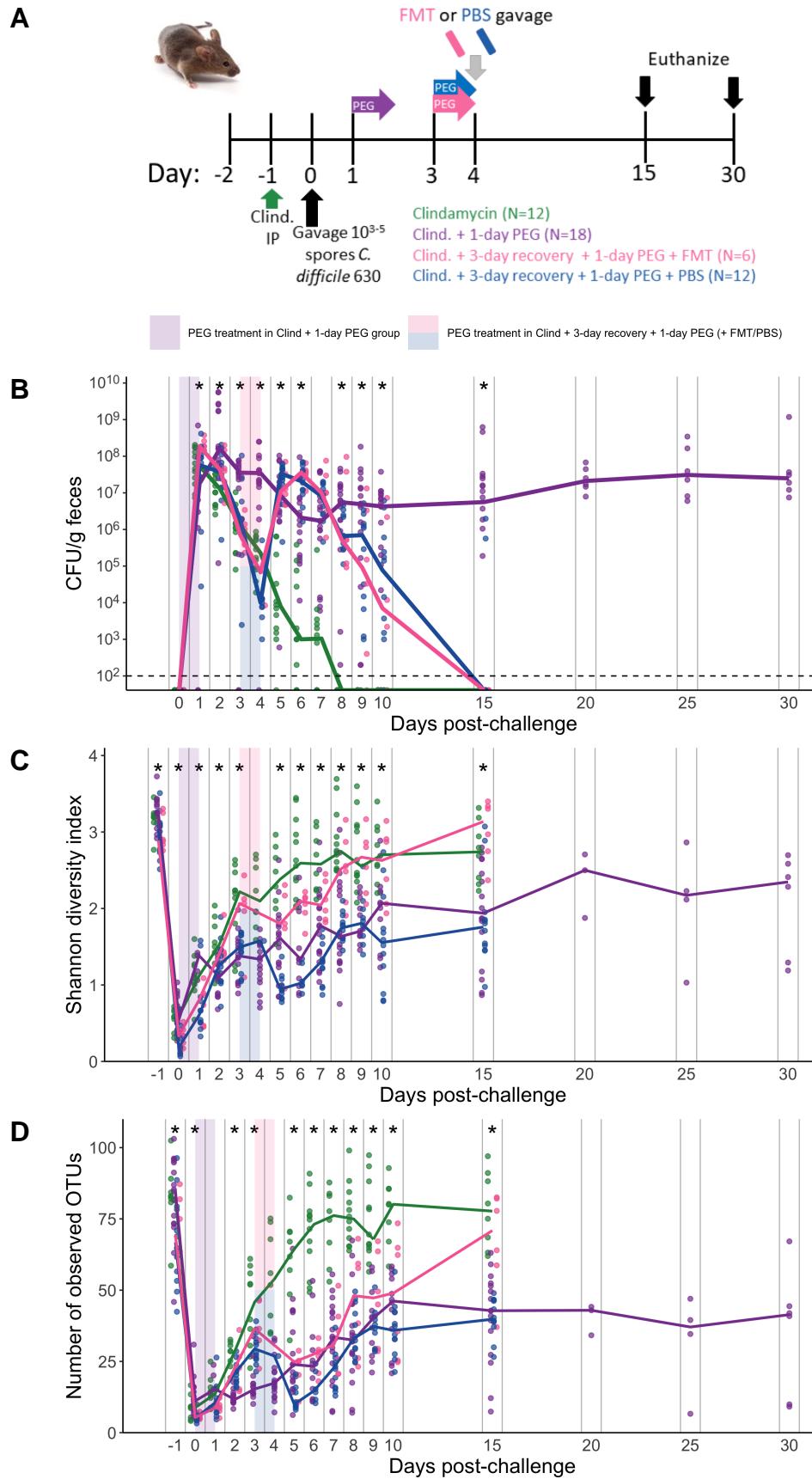
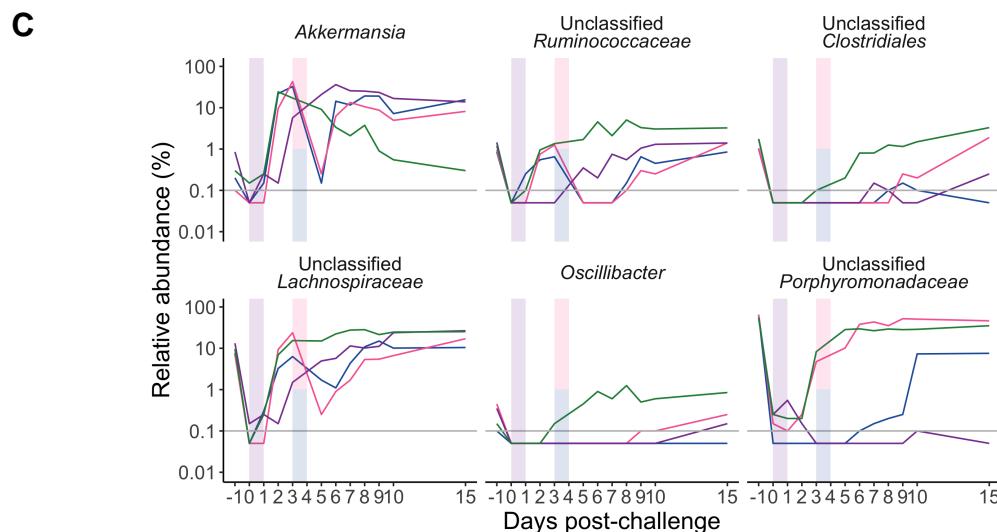
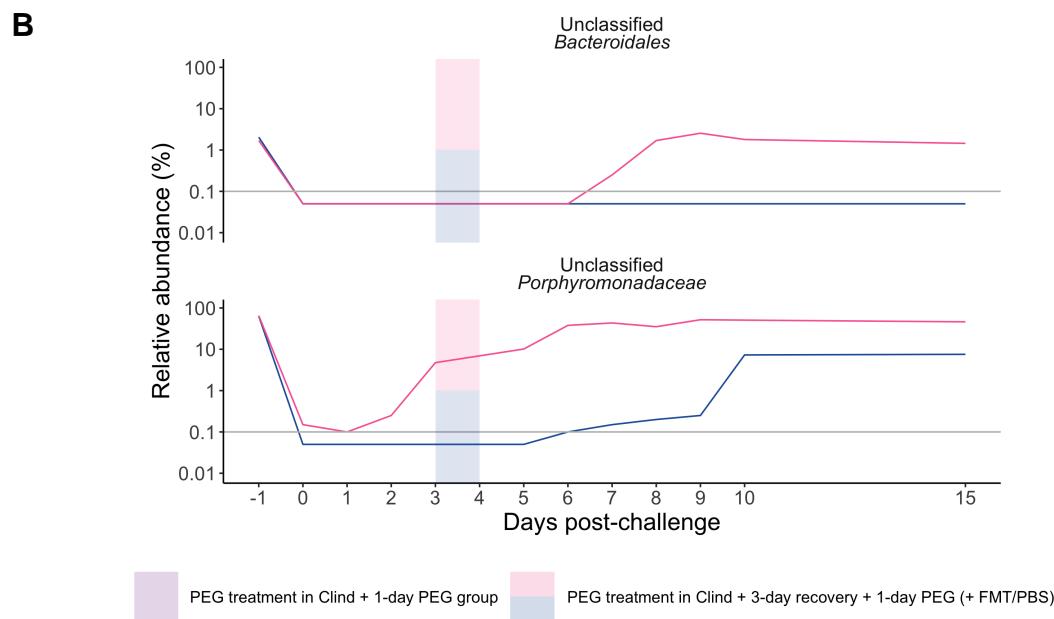
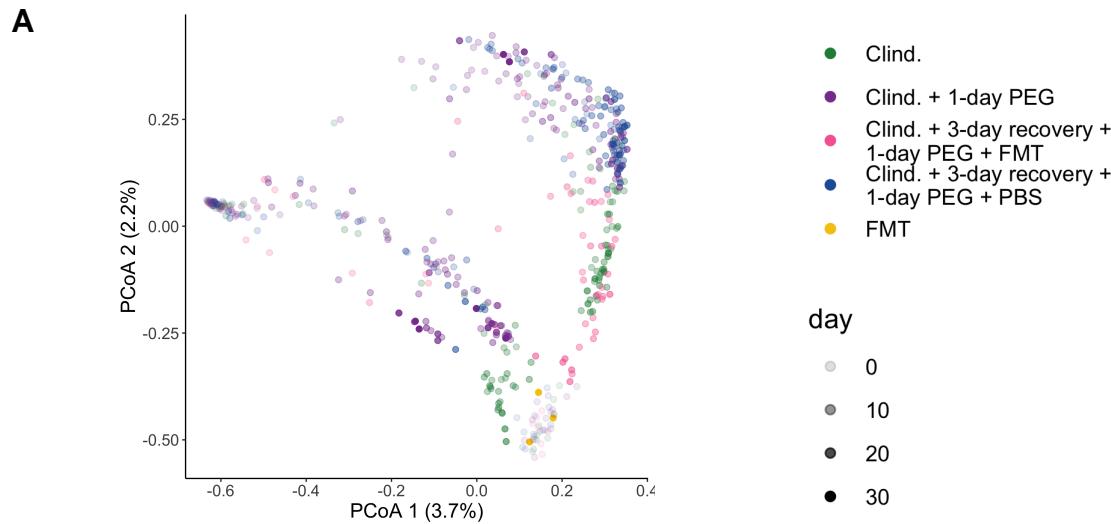
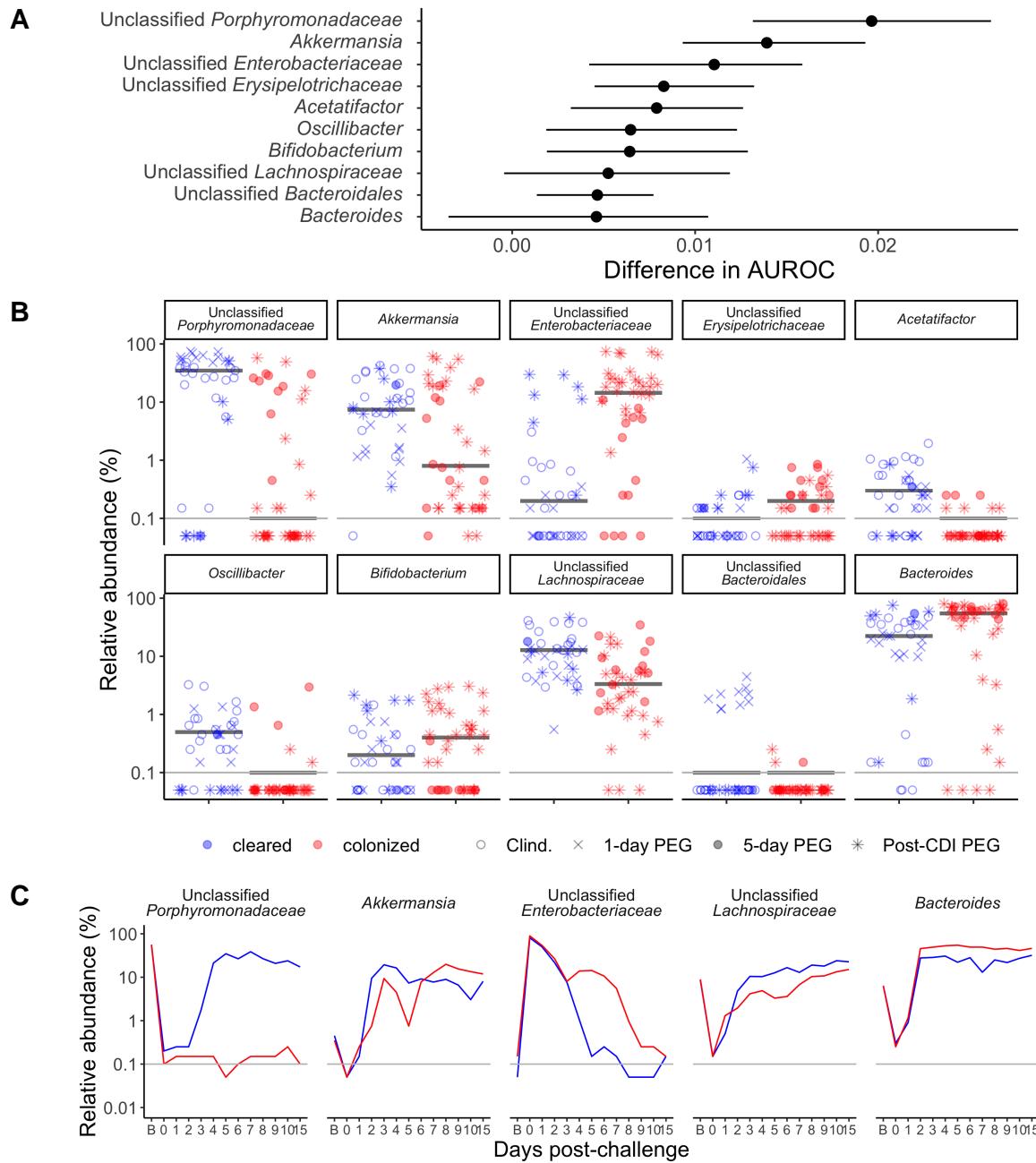


Figure 5.

784 **1-day PEG treatment post *C. difficile* challenge prolongs colonization regardless of**
785 **whether an FMT is also administered.** A. Setup of the experimental time line for experiments
786 with post-challenge PEG treated mice. There were a total of 4 different treatment groups. All mice
787 were administered 10 mg/kg clindamycin intraperitoneally (IP) 1 day before challenge with 10^{3-5}
788 *C. difficile* 630 spores. 1. Received no additional treatment (Clindamycin). 2. Immediately after
789 *C. difficile* challenge, mice received 15% PEG 3350 in the drinking water for 1 day. 3-4. 3-days
790 after challenge, mice received 1-day PEG treatment and then received either 100 microliters a
791 fecal microbiota transplant (3) or PBS (4) solution by oral gavage. Mice were followed through
792 15-30 days post-challenge (only the post-CDI 1-day PEG group was followed through 30 days
793 post-challenge). B. CFU/g of *C. difficile* stool measured over time via serial dilutions. The black line
794 represents the limit of detection for the first serial dilution. C-D. Shannon diversity (C) and richness
795 (D) in stool communities over time (Data Set S1, sheets 15 and 16). B-D. Each symbol represents
796 a stool sample from an individual mouse with the lines representing the median value for each
797 treatment group. Asterisks indicate time points with significant differences ($P < 0.05$) between
798 groups by the Kruskall-Wallis test with a Benjamini-Hochberg correction for testing multiple times
799 points. Colored rectangles indicates the 1-day PEG treatment period for applicable groups. The
800 data presented are from a total of 3 separate experiments.



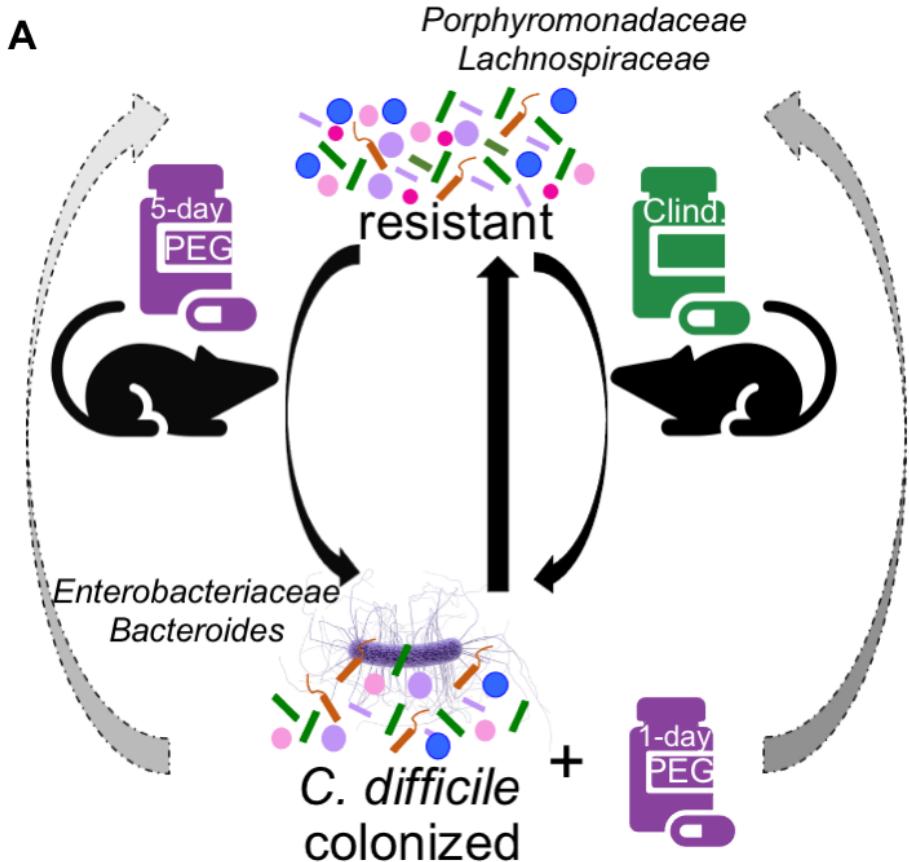
802 **6. For 1-day PEG treatment post *C. difficile* challenge mice that also receive an FMT only**
803 **some bacterial genera were restored.** A. PCoA of Bray-Curtis distances from stool samples
804 collected over time as well as the FMT solution that was administered to one of the treatment
805 groups.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



816

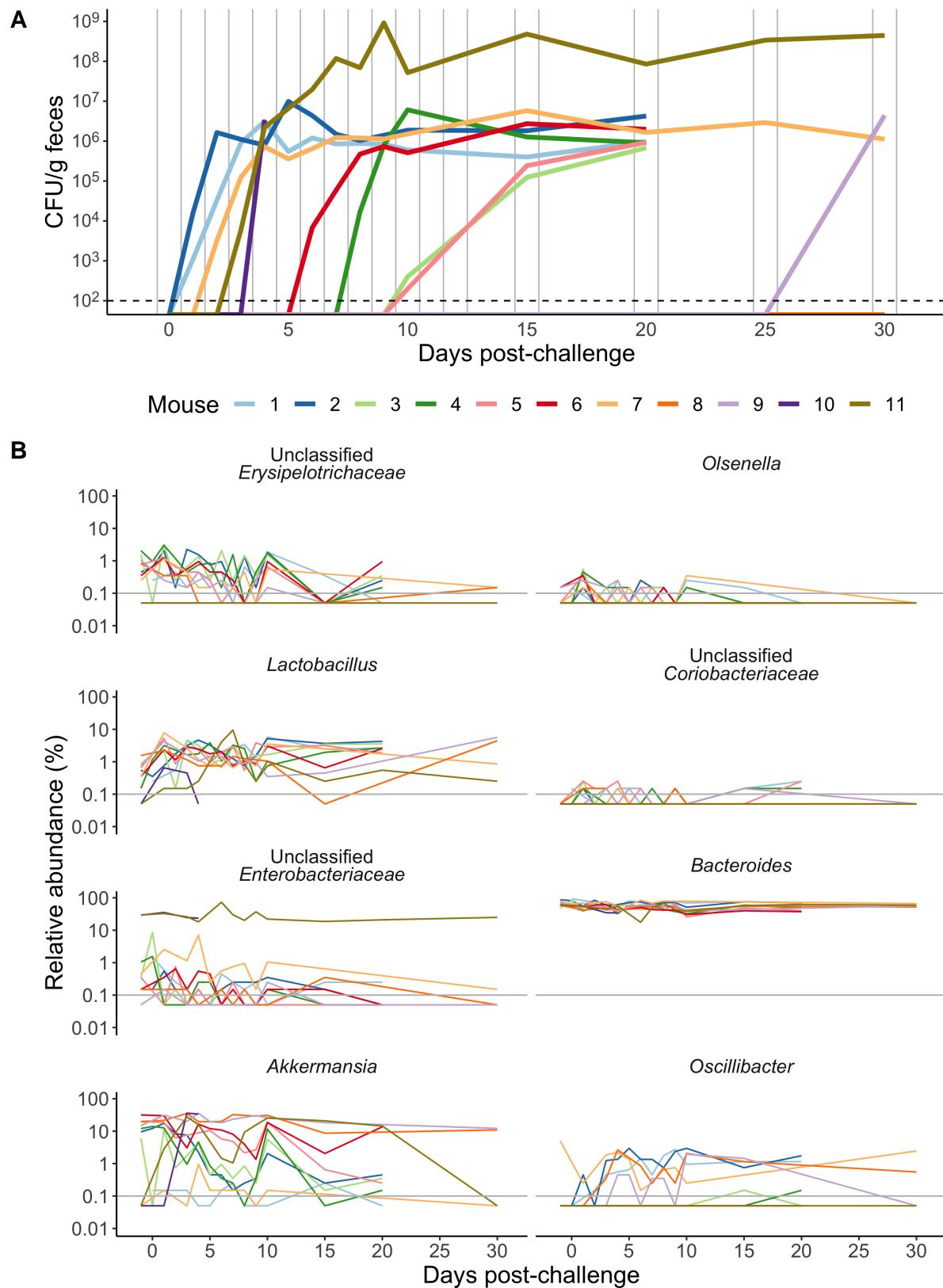
817 **Figure 7. Specific microbiota features associated with prolonged *C. difficile* colonization**
818 **in PEG treated mice.** A. Top ten bacteria that contributed to the random forest model trained on
819 5-day post-challenge community relative abundance data, predicting whether mice would still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

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



831

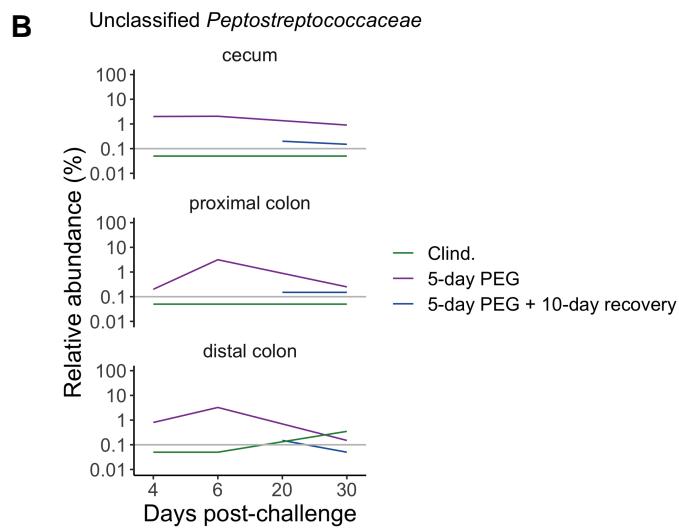
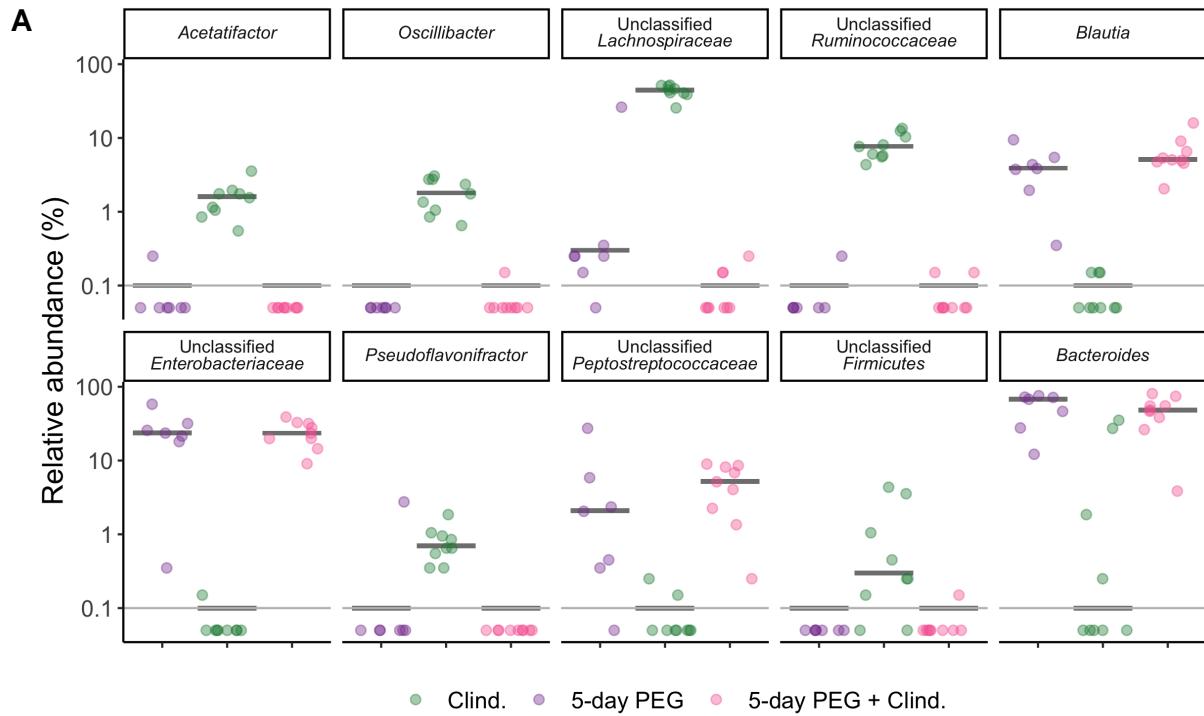
832 **Figure 8. Schematic summarizing findings.** The gut microbiota of our C57Bl/6 mice is resistant
 833 to *C. difficile* but treatment with either the antibiotic, clindamycin, or the osmotic laxative, PEG
 834 3350, renders the mice susceptible to *C. difficile* colonization. Recovery of colonization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



842

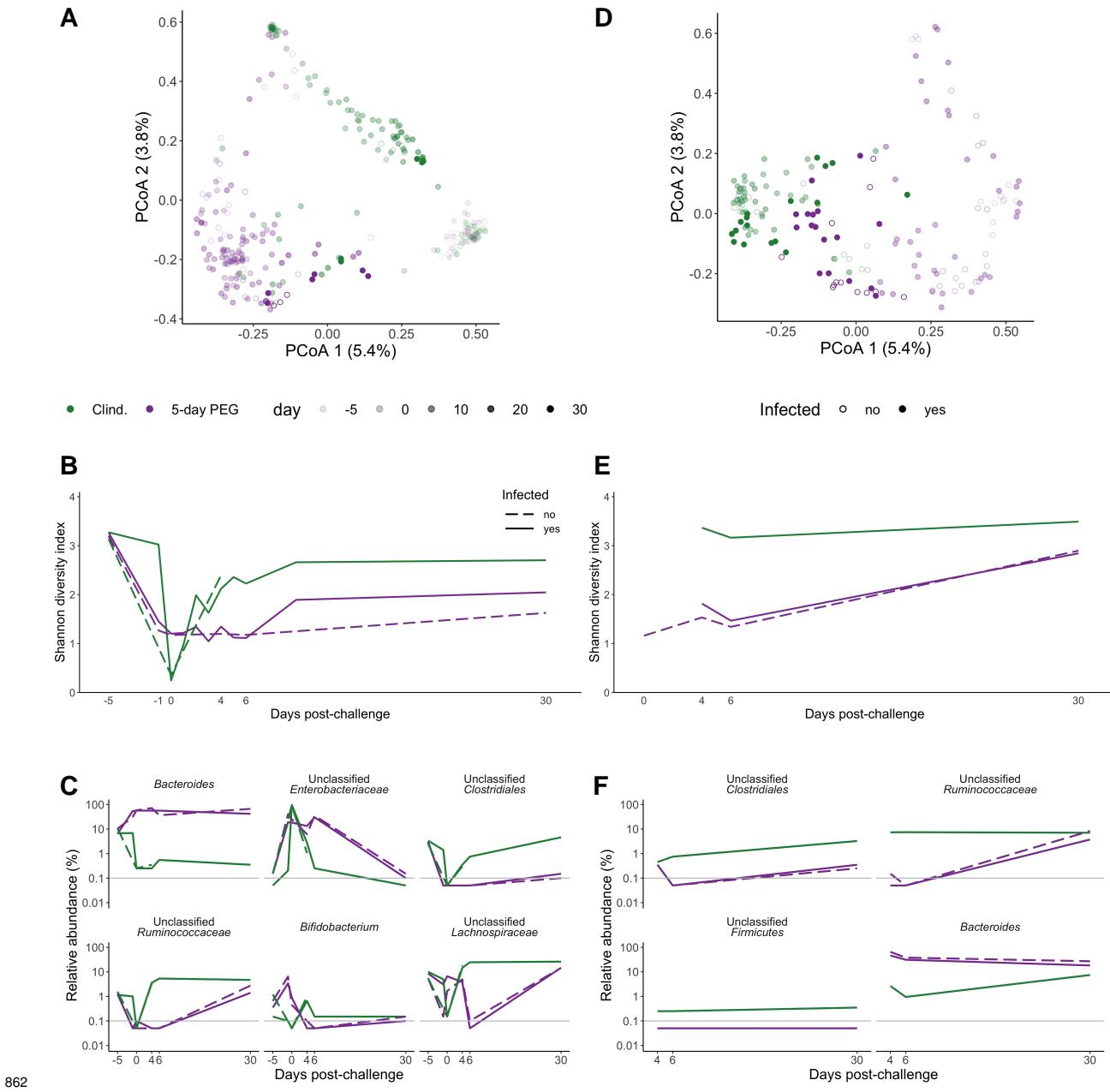
843 **Figure S1.** Microbiota dynamics post-challenge in the 5-day PEG treatment plus 10-day

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



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

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



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

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

874 **Data Set S1**

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

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

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

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

882 **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.**

884 **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.**

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

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

891 **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.**

893 **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.**

895 **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.**

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

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

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

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

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

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

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

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

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

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