

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 (dpc). PEG treatment alone was sufficient to render
9 mice susceptible and 5-day PEG-treated mice remain colonized for up to 30 dpc. In contrast, 1-day
10 PEG treated mice were transiently colonized, clearing *C. difficile* within 7 dpc. To examine how
11 PEG treatment impacts clearance, we administered a 1-day PEG treatment to clindamycin-treated,
12 *C. difficile*-challenged mice. Administering PEG to mice after *C. difficile* challenge prolonged
13 colonization up to 30 dpc. When we trained a random forest model with community data from 5
14 dpc, we were able to predict which mice would exhibit prolonged colonization (AUROC = 0.90).
15 Examining the dynamics of these bacterial during the post-challenge period revealed patterns in the
16 relative abundances of *Bacteroides*, *Enterobacteriaceae*, *Porphyromonadaceae*, *Lachnospiraceae*,
17 and *Akkermansia* that were associated with prolonged *C. difficile* colonization in PEG-treated mice.

18 Importance

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

²⁹ impact microbiota colonization resistance in humans.

30 **Introduction**

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

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

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

56 Beyond susceptibility, PEG is also relevant in the context of treating recurrent CDIs via fecal
57 microbiota transplant (FMT) where a healthy microbiota is administered to the patient to restore

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

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

72 **Results**

73 **5-day laxative treatment led to prolonged *C. difficile* colonization in mice.** Building off of
74 previous work that showed treating mice with the osmotic laxative, PEG 3350, rendered mice
75 susceptible to *C. difficile* colonization (15, 17), we decided to test how long *C. difficile* colonization
76 is sustained and how long PEG-treated mice remain susceptible to *C. difficile*. We compared three
77 groups of mice treated with PEG 3350 to one group of mice treated with our standard 10 mg/kg
78 clindamycin treatment, which temporarily renders the mice susceptible to *C. difficile* colonization,
79 with mice typically clearing *C. difficile* within 10 days post-challenge (9, 26). All three groups of
80 PEG-treated mice were administered a 15% PEG solution in the drinking water for 5-days. The
81 first group received no additional treatment. The second group was also treated with clindamycin.
82 A third group was allowed to recover for 10 days prior to challenge (Fig. 1A). The PEG treatment
83 resulted in weight loss for the 3 groups of mice, with the greatest change in weight observed on
84 the fifth day of the PEG treatment. The mice recovered most of the lost weight by five days after
85 treatment (Fig. 1B). After either the PEG, clindamycin, or PEG and clindamycin treatment all mice

86 were challenged with 10^5 *C. difficile* 630 spores (Fig. 1A). All treatments rendered mice susceptible
87 to *C. difficile* colonization. In contrast to the mice that only received clindamycin, PEG-treated
88 mice remained colonized with *C. difficile* at a high level through thirty days post-challenge (Fig.
89 1C). The clindamycin-treated mice cleared *C. difficile* within ten days post-challenge (Fig. 1C).
90 It was noteworthy that PEG-treated mice were still susceptible to *C. difficile* colonization after a
91 10-day recovery period, although *C. difficile* was not detectable in most of the group in the initial
92 five days post-challenge (Fig. 1C, S1A). One mouse was found dead on the 6th day post-challenge,
93 presumably due to *C. difficile* as the bacterium became detectable in stool samples from that mouse
94 on the 4th day post-challenge (Fig. S1A, mouse 10). From 8 days post-challenge onward, the
95 density of *C. difficile* stabilized in the 10-day recovery group and remained high through 20-30
96 days post-challenge (Fig. 1C). Thus, osmotic laxative treatment alone was sufficient to render
97 mice susceptible to prolonged *C. difficile* colonization and PEG-treated mice remained susceptible
98 through ten days post-treatment.

99 **5-day laxative treatment differentially disrupted the fecal microbiota compared to
100 clindamycin treatment.** Since osmotic laxatives and clindamycin have previously been
101 shown to disrupt the murine microbiota (14–17), we hypothesized the different *C. difficile*
102 colonization dynamics between mice treated with the osmotic laxative or clindamycin were due to
103 the two drugs having differential effects on the microbiota. We profiled the stool microbiota over
104 time by sequencing the V4 region of the 16S rRNA gene to compare changes across treatment
105 groups. We found time ($R^2 = 0.29$) and treatment group ($R^2 = 0.21$) explained half of the observed
106 variation between fecal communities with most of the remaining variation explained by interactions
107 between treatment group and other experimental variables including time, cage, and sequencing
108 preparation plate (PERMANOVA combined $R^2 = 0.95$, $P < 0.001$, Fig. 2A, Data Set S1, sheet 1).
109 None of the treatment groups recovered to their baseline community structure either 10 or 30 days
110 post-challenge suggesting other community features besides recovery to baseline were responsible
111 for the prolonged *C. difficile* colonization in PEG-treated mice (Fig. 2B).

112 Since none of the communities completely recovered in the follow-up period after treatments, we
113 next profiled community diversity and composition. We examined the alpha diversity dynamics by
114 calculating the communities' Shannon diversity. Although both clindamycin and PEG treatments

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

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

142 **5-day laxative treatment did not promote more severe CDIs despite altering the mucosal
143 microbiota.** Given the findings from a previous study that demonstrated PEG treatment disrupts

144 the mucus layer and alters the immune response in mice (16), we decided to examine the impact of
145 PEG treatment on the mucosal microbiota and CDI severity. To evaluate the mucosal microbiota,
146 we sequenced communities associated with tissues collected from the cecum, proximal colon, and
147 distal colon. Similar to what was observed with the stool samples, the alpha diversity was lower
148 in the PEG-treated mice compared to clindamycin treated mice (Fig. 3A, Data Set S1, sheet 6).
149 The alpha diversity of the tissue-associated community increased in PEG-treated mice collected
150 at 20 and 30 days post-challenge (Fig. 3A). Group ($R^2 = 0.33$), time point ($R^2 = 0.11$), and their
151 interactions with other variables (cage, experiment number, and sample type) explained the majority
152 of the variation observed in mucosal communities (PERMANOVA combined $R^2 = 0.83$, $P < 0.05$,
153 Fig. 3B, Data Set S1, sheet 7). We saw the greatest difference in the relative abundance of the
154 mucosal microbiota between treatment groups (clindamycin, 5-day PEG, and 5-day PEG plus
155 clindamycin) at 6 days post-challenge with 10 genera that were significantly different ($P < 0.05$) in
156 all three of the tissue types we collected (cecum, proximal colon, and distal colon; Fig. S2A, Data
157 Set S1, sheet 8, 9, and 10). Interestingly, *Peptostreptococcaceae* (the family with a sequence that
158 matches *C. difficile*) was one of the genera that had a significant difference in relative abundance
159 between treatment groups at 6 days post-challenge. This population was primarily only present
160 in the 5-day PEG treatment group of mice and decreased in the proximal and distal colon tissues
161 over time (Fig. S2B). By 30 days post-challenge, only the relative abundances of *Bacteroides*,
162 *Clostridiales*, *Firmicutes*, and *Ruminococcaceae* were different between treatment groups and
163 only in the cecum tissues (Fig. 3C, Fig. 2E, Data Set S1, sheet 8). Thus, PEG treatment had a
164 significant impact on the mucosal microbiota and we detected *C. difficile* sequences in the cecum,
165 proximal colon, and distal colon tissue communities.

166 Because there were differences in the mucosal microbiota including detectable *C. difficile* sequences
167 in tissues from PEG-treated mice relative to mice treated with clindamycin, we next examined the
168 severity of *C. difficile* challenge by evaluating cecum and colon histopathology (27). However, we
169 found there was no difference in cecum and colon scores between clindamycin and PEG-treated
170 mice that were challenged with *C. difficile* at 4 days post-challenge (Fig. 3D), the time point
171 typically examined in *C. difficile* 630 challenged mice (28). We also looked at 6 days post-challenge
172 because that was when there was a large difference in *C. difficile* density between PEG- and

173 clindamycin-treated mice (Fig. 1C). Although there was a slight difference in the histopathology
174 score of the colon between PEG and clindamycin-treated mice, there was not a significant difference
175 in the cecum and the overall score was relatively low (1.5 to 2.5 out of 12, Fig. 3E). Therefore,
176 although PEG treatment had a disruptive effect on the mucosal microbiota, the impact of *C. difficile*
177 630 challenge on the cecum and colon was similar between PEG and clindamycin treated mice.

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

186 **1-day laxative treatment resulted in transient *C. difficile* colonization and minor microbiota**
187 **disruption.** Next, we examined how a shorter osmotic laxative perturbation would impact the
188 microbiome and susceptibility to *C. difficile*. We administered either a 1-day PEG treatment, a
189 1-day PEG treatment with a 1-day recovery period, or clindamycin to mice before challenging them
190 with *C. difficile* (Fig. 3A). In contrast to the 5-day PEG treated mice, the 1-day PEG groups were
191 only transiently colonized and cleared *C. difficile* by 7 days post-challenge (Fig. 3B). The stool
192 communities of the 1-day PEG treatment groups were also only transiently disrupted, with Shannon
193 diversity recovering by 7 days post-challenge (Fig. 3C-D, Data Set S1, sheets 11 and 12). We found
194 the relative abundances of 14 genera were impacted by treatment, but recovered close to baseline
195 levels by 7 days post-challenge including *Enterobacteriaceae*, *Clostridiales*, *Porphyromonadaceae*,
196 and *Ruminococcaceae* (Fig. 3E, Data Set S1, sheet 13 and 14). These findings suggest the
197 duration of the PEG treatment was relevant, with shorter treatments resulting in a transient loss of
198 *C. difficile* colonization resistance.

199 **Post-challenge laxative treatment disrupted clearance in clindamycin-treated mice**
200 **regardless of whether an FMT was also administered.** Since a 1-day PEG treatment resulted

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

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

226 **Five-day post-challenge community data can predict which mice will have prolonged**
227 ***C. difficile* colonization.** After identifying bacteria associated with the 5-day, 1-day and
228 post-challenge 1-day PEG treatments, we examined the bacteria that influenced prolonged *C.*
229 *difficile* colonization. We trained 3 machine learning models (random forest, logistic regression,

230 and support vector machine) with bacterial community data from 5 days post-challenge to predict
231 whether the mice were still colonized with *C. difficile* 10 days post-challenge. We chose 5 days
232 post-challenge because that was the earliest time point where we saw a treatment effect in the
233 post-challenge 1-day PEG experiments. The random forest model had the highest performance
234 (median AUROC = 0.90, Data Set S1, sheet 19), suggesting the 5-day post challenge microbiota
235 is an excellent predictor of prolonged *C. difficile* colonization. Next, we performed permutation
236 importance to examine the bacteria that were the top contributors to the random forest model
237 predicting prolonged *C. difficile* colonization. We selected 10 genera that contributed the most to
238 our model's performance (Fig. 7A) and examined their relative abundance at 5 days post-challenge,
239 the time point used to predict *C. difficile* colonization status on day 10 (Fig. 7B). Next, we focused
240 on the 5 genera that had a greater than 1% relative abundance in either the cleared or colonized
241 mice and examined how the bacteria changed over time. We found *Enterobacteriaceae* and
242 *Bacteroides* tended to have a higher relative abundance, the relative abundance of *Akkermansia*
243 was initially decreased and then increased, and *Porphyromonadaceae* and *Lachnospiraceae* had a
244 lower relative abundance in the mice with prolonged colonization compared to the mice that cleared
245 *C. difficile* (Fig. 7C). Together these results suggest a combination of low and high abundance
246 bacterial genera influence the prolonged colonization observed in 5-day PEG and post-challenge
247 1-day PEG treated mice.

248 Discussion

249 While the disruptive effect of antibiotics on *C. difficile* colonization resistance is well established,
250 the extent to which other drugs such as laxatives disrupt colonization resistance was unclear. By
251 following osmotic laxative treated mice over time, we found that a 5-day PEG treatment before
252 challenge resulted in prolonged *C. difficile* colonization, while a 1-day PEG treatment resulted in
253 transient colonization. The differences in *C. difficile* colonization dynamics between the 5- and
254 1-day PEG treated mice were associated with differences in how much the treatments disrupted
255 the microbiota. Additionally, the intestinal communities of 5-day PEG treated mice did not regain
256 colonization resistance after a 10-day recovery period. In contrast to the other 5-day PEG treatment
257 groups, *C. difficile* was not immediately detectable in the stools of most of the mice in the 10-day

recovery group. We also examined the impact of PEG treatment after *C. difficile* challenge. In opposition to the hypothesis suggested by the literature, we found that PEG treatment prolonged colonization relative to mice that only received clindamycin treatment. We identified patterns in the relative abundances of *Bacteroides*, *Enterobacteriaceae*, *Akkermansia*, *Porphyromonadaceae*, and *Lachnospiraceae* that were associated with prolonged *C. difficile* colonization (Fig. 8). Overall, our results demonstrated that osmotic laxative treatment alone rendered mice susceptible to *C. difficile* colonization and the duration of colonization depended on the length of PEG treatment and whether treatment was administered before or after challenge.

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

One possible explanation for the prolonged *C. difficile* colonization in 5-day PEG treated mice, might be due to the bacteria's persistence in the mucosal compartment. In fact, it has been hypothesized that *C. difficile* biofilms may serve as reservoirs for recurrent infections (35) and *C. difficile* biofilms in the mucus layer were recently identified in patients as aggregates with *Fusobacterium nucleatum* (36). There was an interesting pattern of increased *Enterobacteriaceae*, *Bacteroides*, and *C. difficile* in both the stool and mucosal communities of PEG-treated mice suggesting a potential synergy. *Bacteroides* has the potential to degrade mucus and the osmotic laxative may have allowed *Bacteroides* to colonize the mucosal niche by degrading mucin glycans with glycosyl hydrolases that are absent in *C. difficile* (37). *Bacteroides* persistent in the mucosal tissue might also have helped *Enterobacteriaceae* to colonize the region, as a synergy between mucus-degrading *B.*

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

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

306 Despite identifying an altered compositional profile that included higher relative abundance of
307 the *C. difficile* sequence in the mucosal tissues of mice treated with 5-day PEG compared to the
308 clindamycin group, we did not see a difference in histopathology scores between the groups. One
309 reason there was no difference could be the *C. difficile* strain used, *C. difficile* 630 results in mild
310 histopathology summary scores in mice compared to VPI 10463 despite both strains producing
311 toxin in mice (51). Part of our hypothesis for why there could have been increased histopathology
312 scores in PEG-treated mice was because PEG was previously shown to disrupt the mucus layer in
313 mice. However, recent studies demonstrated that broad spectrum antibiotics can also disrupt the
314 host mucosal barrier in mice (52, 53). Future research is needed to tease out the interplay between
315 medications that influence the mucus layer and different strains of *C. difficile* in the context of CDIs.

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

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

344 We have demonstrated that osmotic laxative treatment alone has a substantial impact on the

345 microbiota and rendered mice susceptible to prolonged *C. difficile* colonization in contrast to
346 clindamycin-treated mice. The duration and timing of the laxative treatment impacted the duration of
347 *C. difficile* colonization, with only 5-day PEG and post-challenge 1-day PEG treatments prolonging
348 colonization compared to clindamycin treated mice. Further studies are warranted to ascertain
349 whether laxatives have a similar impact on *C. difficile* colonization resistance on the human
350 microbiota.

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362 Materials and Methods

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

371 **Drug treatments.** For PEG treatment groups, fifteen percent PEG 3350 (Miralax) was administered

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

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

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

406 **16S rRNA gene sequencing.** Stool samples that were stored in the -80°C were placed into 96-well
407 plates for DNA extractions and library preparation. DNA was extracted using the DNeasy Powersoil
408 HTP 96 kit (Qiagen) and an EpMotion 5075 automated pipetting system (Eppendorf). For library
409 preparation, each plate had a mock community control (ZymoBIOMICS microbial community DNA
410 standards) and a negative control (water). The V4 region of the 16S rRNA gene was amplified with
411 the AccuPrime Pfx DNA polymerase (Thermo Fisher Scientific) using custom barcoded primers, as
412 previously described (71). The PCR amplicons were normalized (SequalPrep normalizatin plate kit
413 from Thermo Fisher Scientific), pooled and quantified (KAPA library quantification kit from KAPA
414 Biosystems), and sequenced with the MiSeq system (Illumina).

415 **16S rRNA gene sequence analysis.** All sequences were processed with mothur (v. 1.43)
416 using a previously published protocol (71, 72). Paired sequencing reads were combined and
417 aligned with the SILVA (v. 132) reference database (73) and taxonomy was assigned with a
418 modified version (v. 16) of the Ribosomal Database Project (v. 11.5) (74). The error rate for
419 sequencing data was 0.0559% based on the 17 mock communities we ran with the samples.
420 Samples were rarefied to 1,000 sequences, 1,000 times for alpha and beta diversity analyses
421 in order to account for uneven sequencing across samples. All but 3 out of 17 water controls
422 had less than 1000 sequences. PCoAs were generated based on Bray-Curtis Index distance
423 matrices. Permutational multivariate analysis of variance (PERMANOVA) tests were performed on
424 mothur-generated Bray-Curtis distance matrices with the adonis function from the vegan R package
425 (75).

426 **Histopathology.** H&E stained sections of cecum and colon tissues collected at either 0, 4, or 6
427 days post-challenge were coded to be scored in a blinded manner by a board-certified veterinary
428 pathologist (ILB). Slides were evaluated using a scoring system developed for mouse models of *C.*

429 *difficile* infection (51). Each slide was evaluated for edema, cellular infiltration, and inflammation
430 and given a score ranging from 0-4. The summary score was calculated by combining the scores
431 from the 3 categories and ranged from 0-12.

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

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

450 **Code availability.** Code for data analysis and generating this paper with accompanying figures is
451 available at https://github.com/SchlossLab/Tomkovich_PEG3350_XXXX_2021.

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

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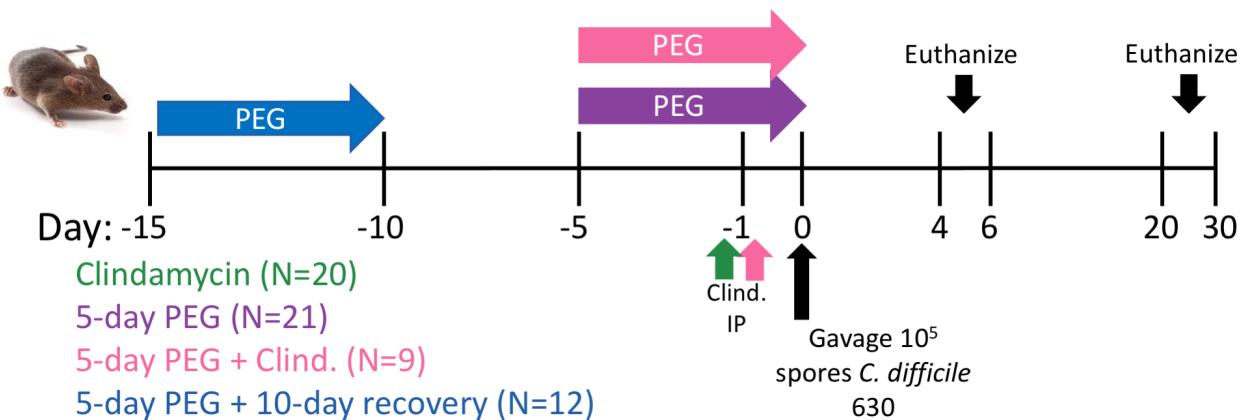
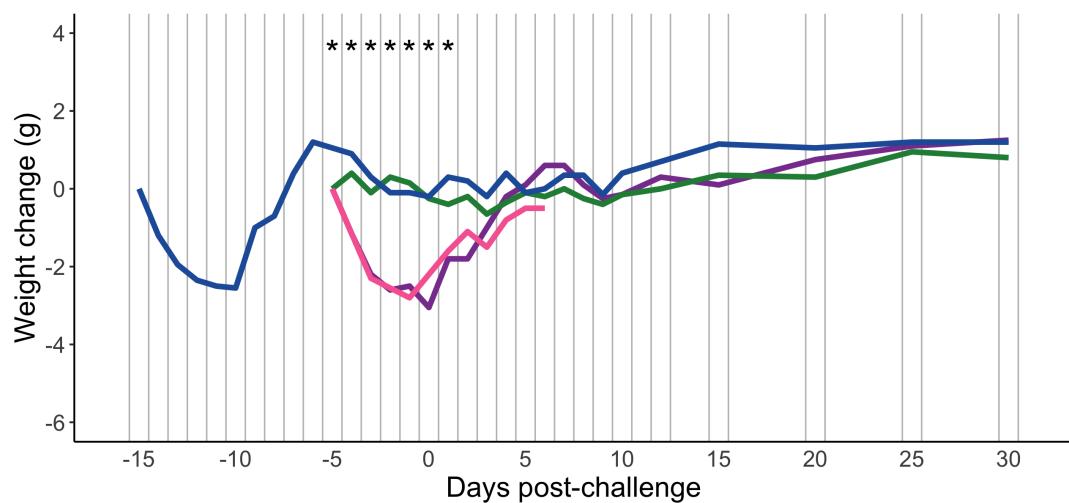
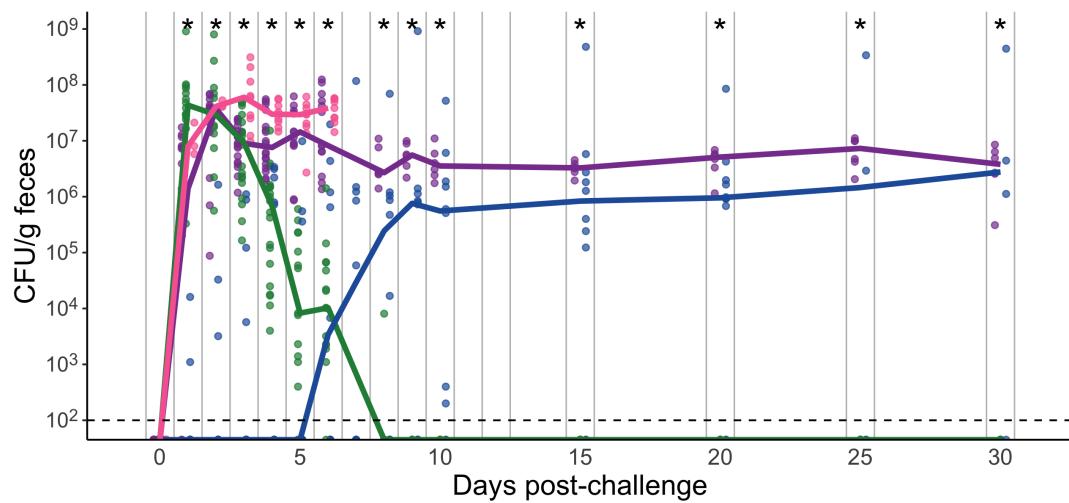
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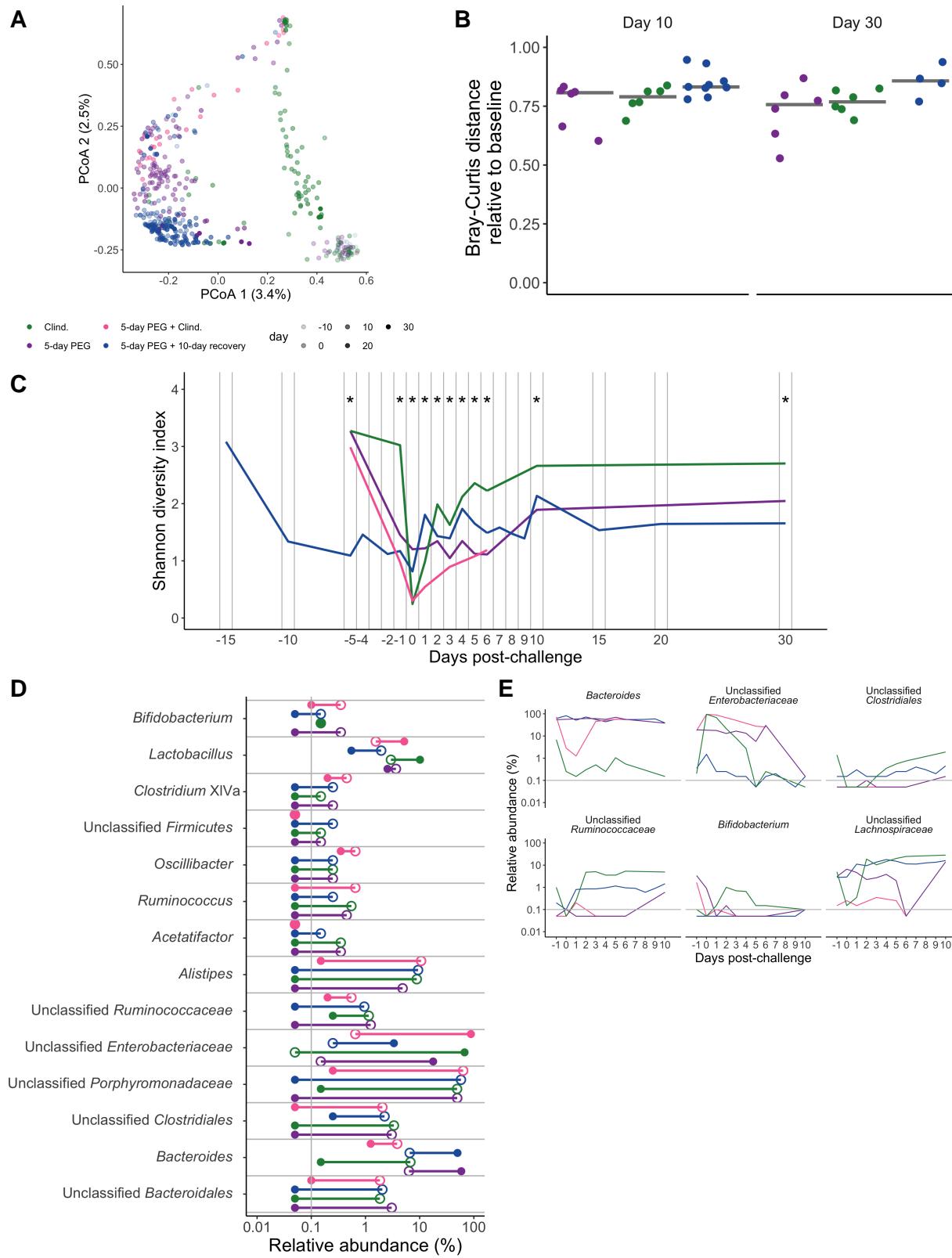
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A**B****C**

726

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

730 intraperitoneal injection. 2. 15% PEG 3350 was administered in the drinking water for five days. 3.
731 5-day PEG plus clindamycin treatment. 4. 5-day PEG plus 10-day recovery treatment. All treatment
732 groups were then challenged with 10^5 *C. difficile* 630 spores. A subset of mice were euthanized
733 on either 4 or 6 days post-challenge and tissues were collected for histopathology analysis, the
734 remaining mice were followed through 20 or 30 days post-challenge. B. Weight change from
735 baseline weight in groups after treatment with PEG and/or clindamycin, followed by *C. difficile*
736 challenge. C. *C. difficile* CFU/gram stool measured over time via serial dilutions(N = 10-59 mice
737 per time point). The black line represents the limit of detection for the first serial dilution. CFU
738 quantification data was not available for each mouse due to stool sampling difficulties (particularly
739 the day the mice came off of the PEG treatment) or early deaths. For B-C, lines represent the
740 median for each treatment group and circles represent samples from individual mice. Asterisks
741 indicate time points where the weight change or CFU/g was significantly different ($P < 0.05$)
742 between groups by the Kruskal-Wallis test with Benjamini-Hochberg correction for testing multiple
743 time points. The data presented are from a total of 5 separate experiments.



744

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

746 **compared to clindamycin-treated mice.** A. Principal Coordinate analysis (PCoA) of Bray-Curtis
747 distances from stool samples collected throughout the experiment. Each circle represents a
748 sample from an individual mouse and the transparency of the symbol corresponds to the day
749 post-challenge. See Data Set S1, sheet 1 for PERMANOVA results. B. Bray-Curtis distances of
750 stool samples collected on either day 10 or 30 post-challenge relative to the baseline sample
751 collected for each mouse (before any drug treatments were administered). The symbols represent
752 samples from individual mice and the line indicates the median value for each treatment group.
753 C. Shannon diversity in stool communities over time. The line indicates the median value for
754 each treatment group (Data Set S1, sheet 2). D. 14 of the 33 genera affected by PEG treatment
755 (Data Set S1, sheet 3). The symbols represent the median relative abundance for a treatment
756 group at either baseline (open circle) or 1-day post treatment (closed circle). Relative abundance
757 data from paired baseline and 1-day post treatment stool samples from the 5-day PEG and
758 5-day PEG plus 10-day recovery groups were analyzed by paired Wilcoxon signed-rank test with
759 Benjamini-Hochberg correction for testing all identified genera. The clindamycin and 5-day PEG
760 plus clindamycin treatment groups are shown on the plot for comparison. E. 6 of the 24 genera
761 that were significantly different between the treatment groups over multiple time points (see Data
762 Set S1, sheet 4 for complete list). The 5-day PEG plus clindamycin treatment group was only
763 followed through 6-days post-challenge. Differences between treatment groups were identified by
764 Kruskal-Wallis test with Benjamini-Hochberg correction for testing all identified genera (*, $P < 0.05$).
765 The gray vertical line (D) and horizontal vertical lines (E) indicate the limit of detection.

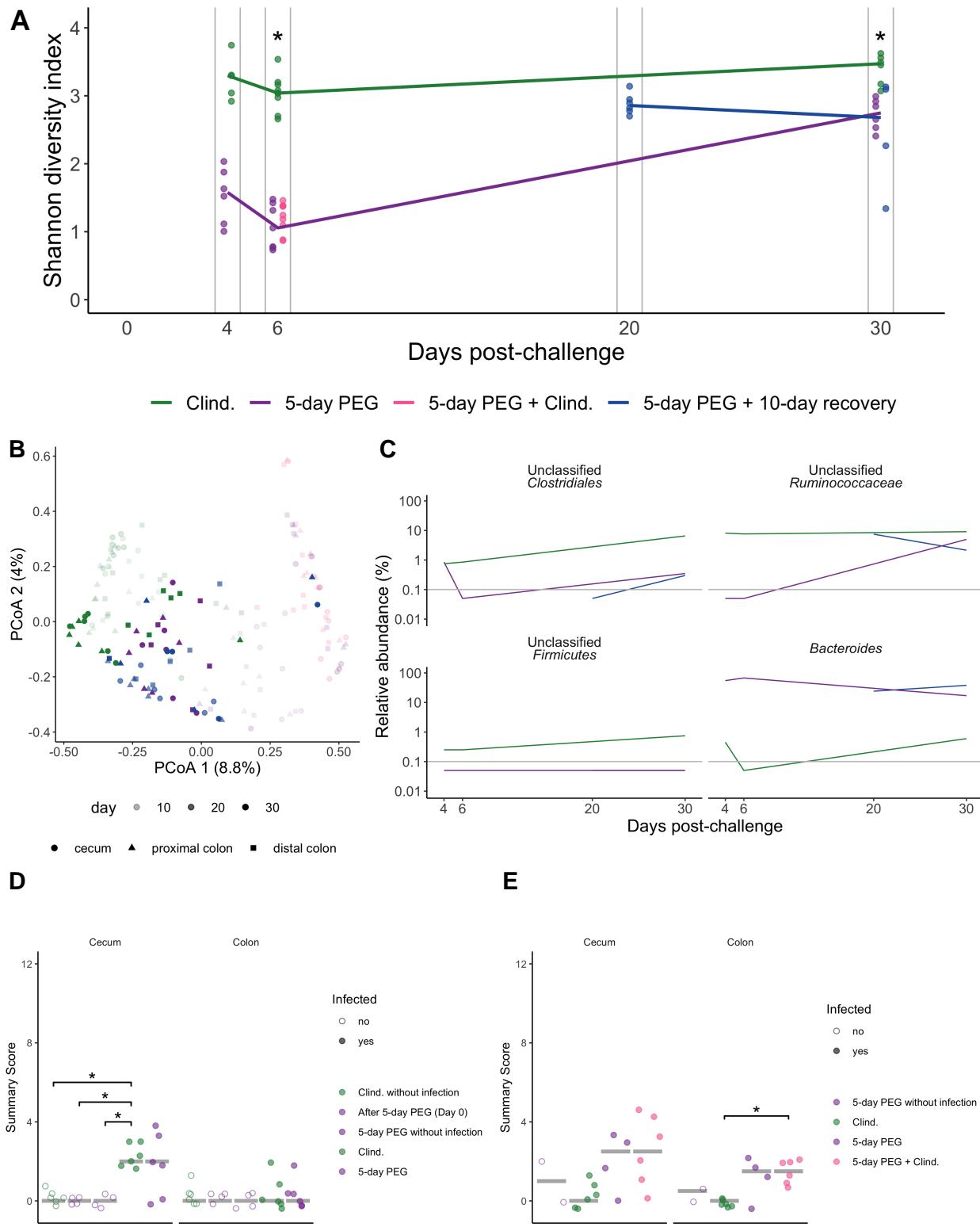
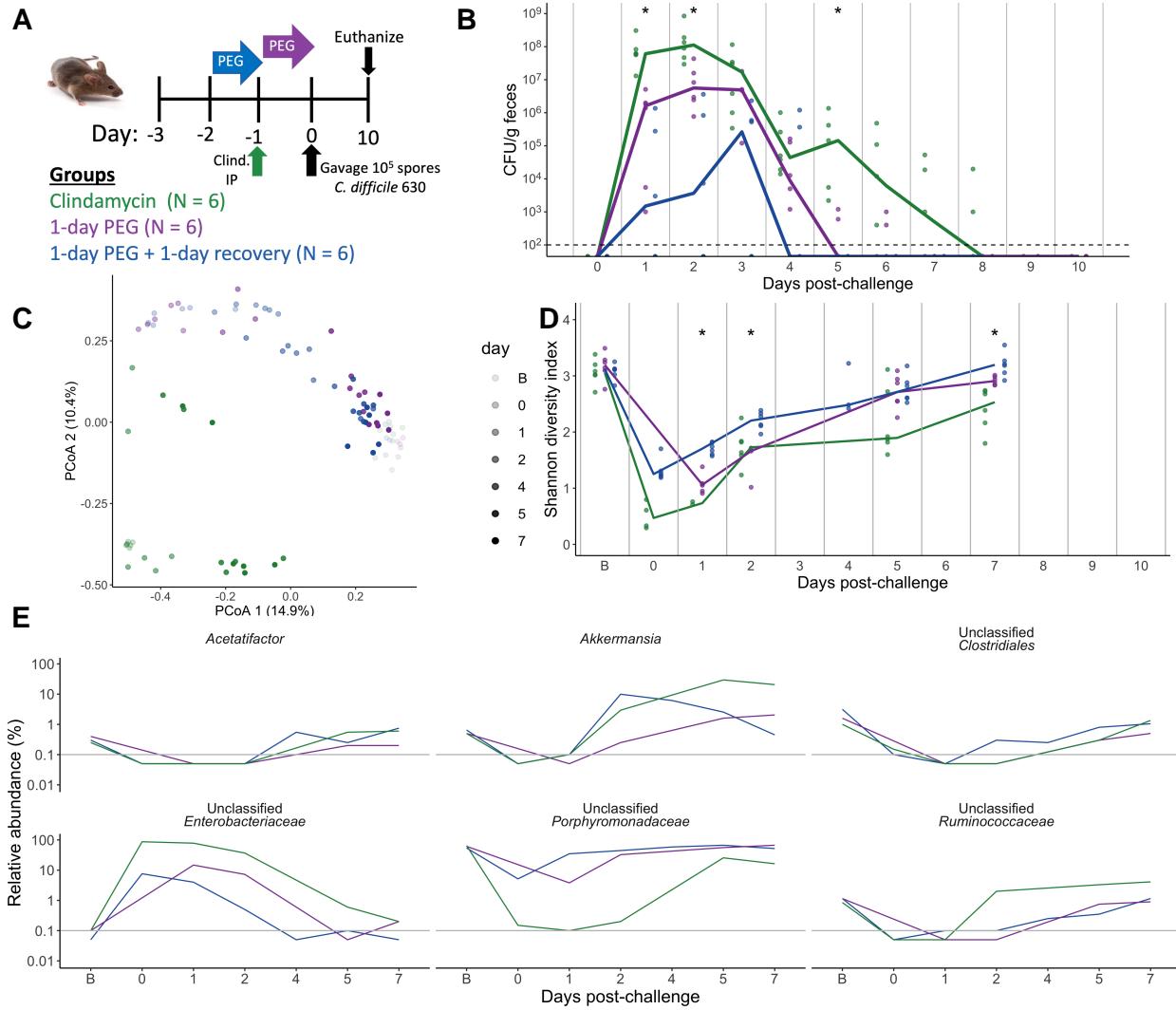


Figure 3. 5-day PEG treatment does not result in more severe CDIs, although mucosal

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



785

786 **Figure 4. 1-day PEG treatment renders mice susceptible to transient *C. difficile* 787 colonization.** A. Setup of the experimental time line for the 1-day PEG treated mice 788 consisting of 3 treatment groups. 1. Clindamycin was administered at 10 mg/kg by intraperitoneal 789 injection. 2. 15% PEG 3350 was administered in the drinking water for 1 day. 3. 1-day PEG 790 plus 1-day recovery. The three treatment groups were then challenged with 10^5 *C. difficile* 630 791 spores. B. *C. difficile* CFU/gram stool measured over time (N = 12-18 mice per time point) by serial 792 dilutions. The black dashed horizontal line represents the limit of detection for the first serial dilution. 793 For B and D, asterisks indicate time points where there was a significant difference ($P < 0.05$) 794 between treatment groups by Kruskall-Wallis test with Benjamini-Hochberg correction for testing 795 multiple time points. For B-D, each symbol represents a sample from an individual mouse and

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

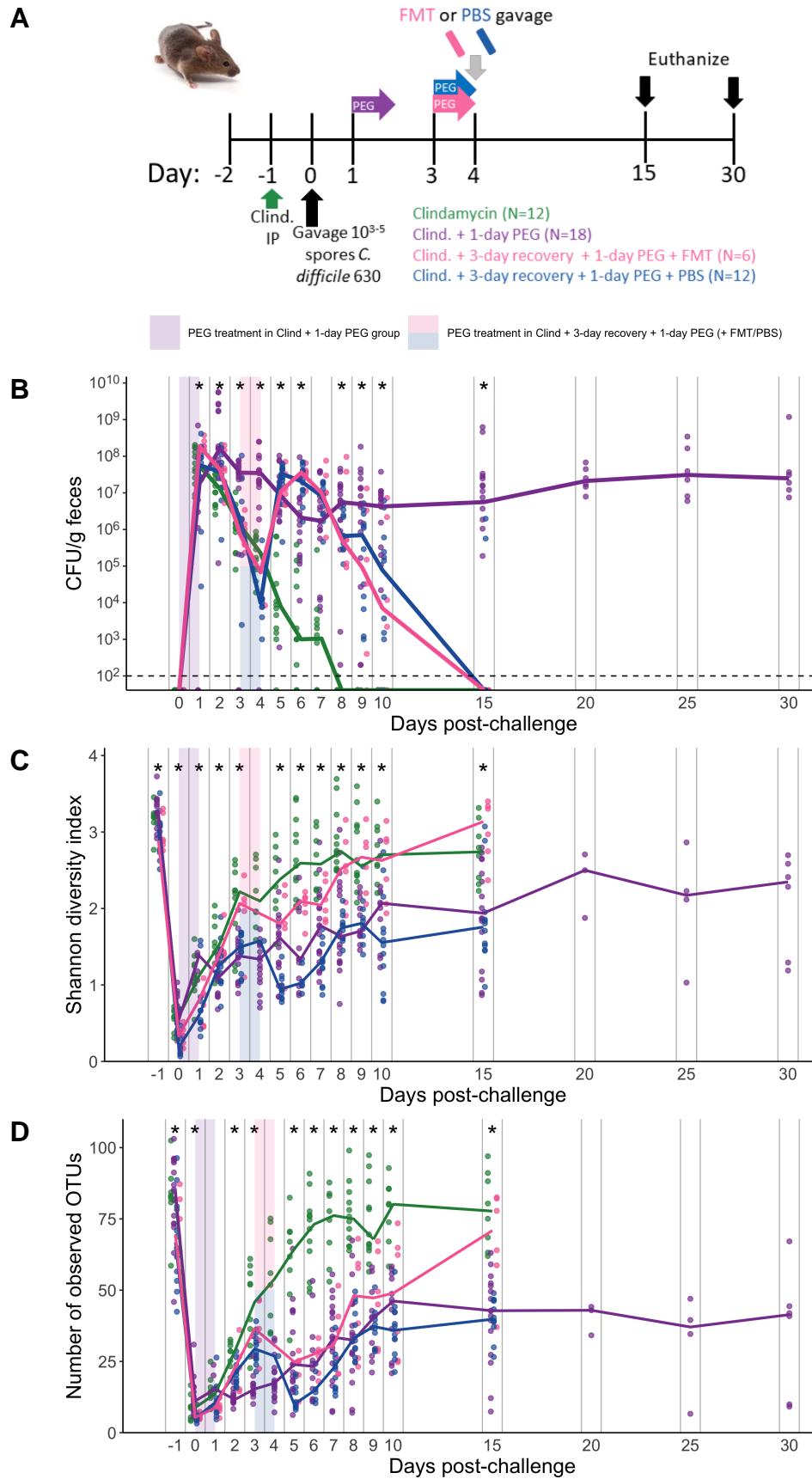
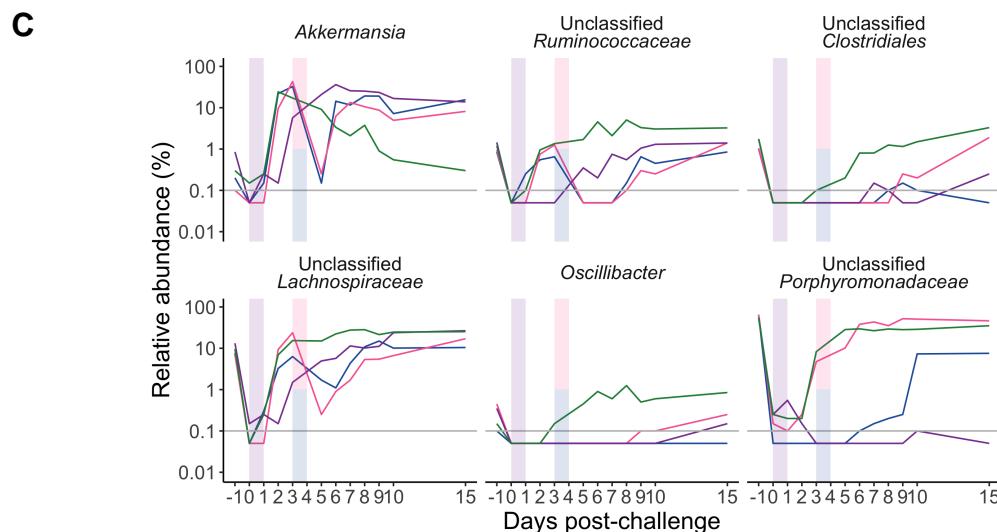
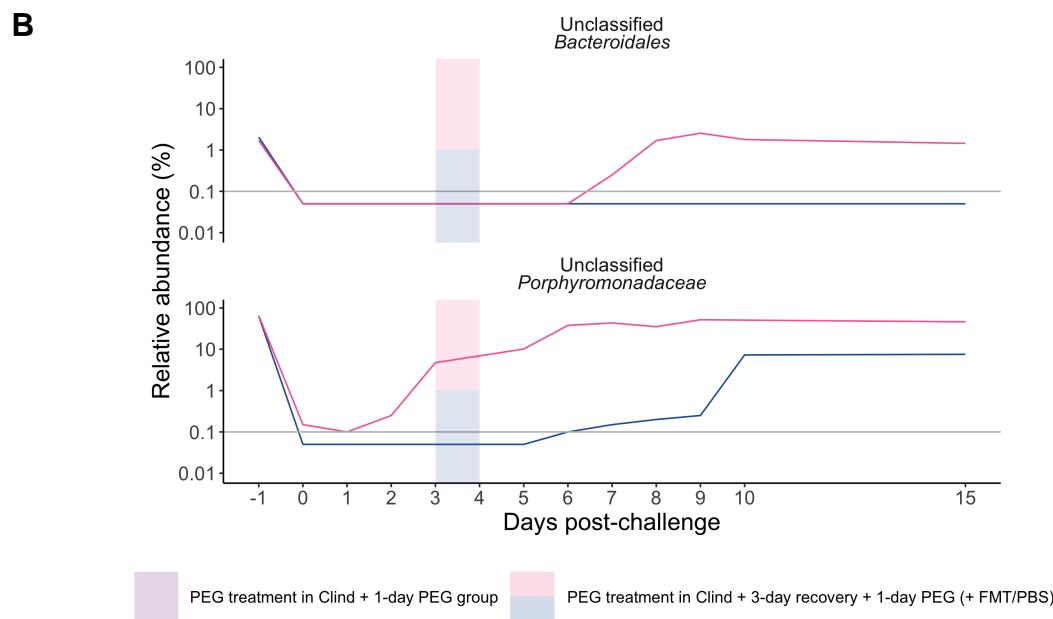
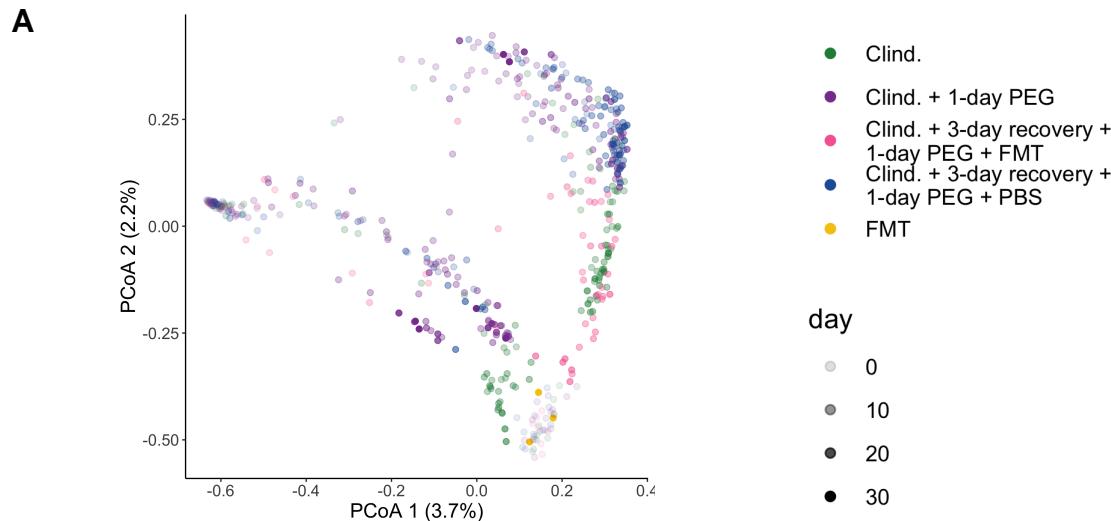
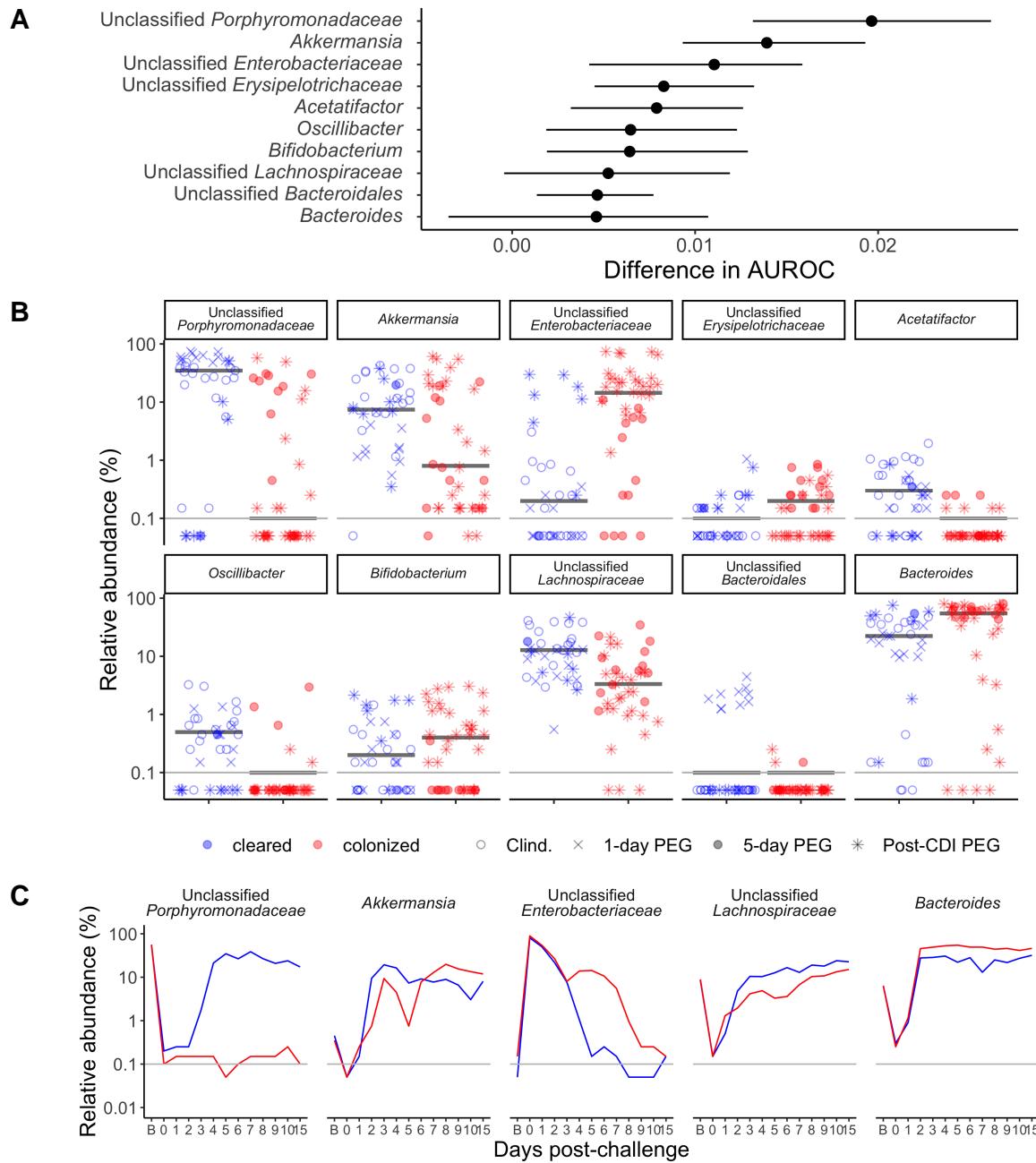


Figure 5.

809 **1-day PEG treatment post *C. difficile* challenge prolongs colonization regardless of**
810 **whether an FMT is also administered.** A. Setup of the experimental time line for experiments
811 with post-challenge PEG treated mice. There were a total of 4 different treatment groups. All mice
812 were administered 10 mg/kg clindamycin intraperitoneally (IP) 1 day before challenge with 10^{3-5}
813 *C. difficile* 630 spores. 1. Received no additional treatment (Clindamycin). 2. Immediately after
814 *C. difficile* challenge, mice received 15% PEG 3350 in the drinking water for 1 day. 3-4. 3-days
815 after challenge, mice received 1-day PEG treatment and then received either 100 microliters a
816 fecal microbiota transplant (3) or PBS (4) solution by oral gavage. Mice were followed through
817 15-30 days post-challenge (only the post-CDI 1-day PEG group was followed through 30 days
818 post-challenge). B. CFU/g of *C. difficile* stool measured over time via serial dilutions. The black line
819 represents the limit of detection for the first serial dilution. C-D. Shannon diversity (C) and richness
820 (D) in stool communities over time (Data Set S1, sheets 15 and 16). B-D. Each symbol represents
821 a stool sample from an individual mouse with the lines representing the median value for each
822 treatment group. Asterisks indicate time points with significant differences ($P < 0.05$) between
823 groups by the Kruskall-Wallis test with a Benjamini-Hochberg correction for testing multiple times
824 points. Colored rectangles indicates the 1-day PEG treatment period for applicable groups. The
825 data presented are from a total of 3 separate experiments.



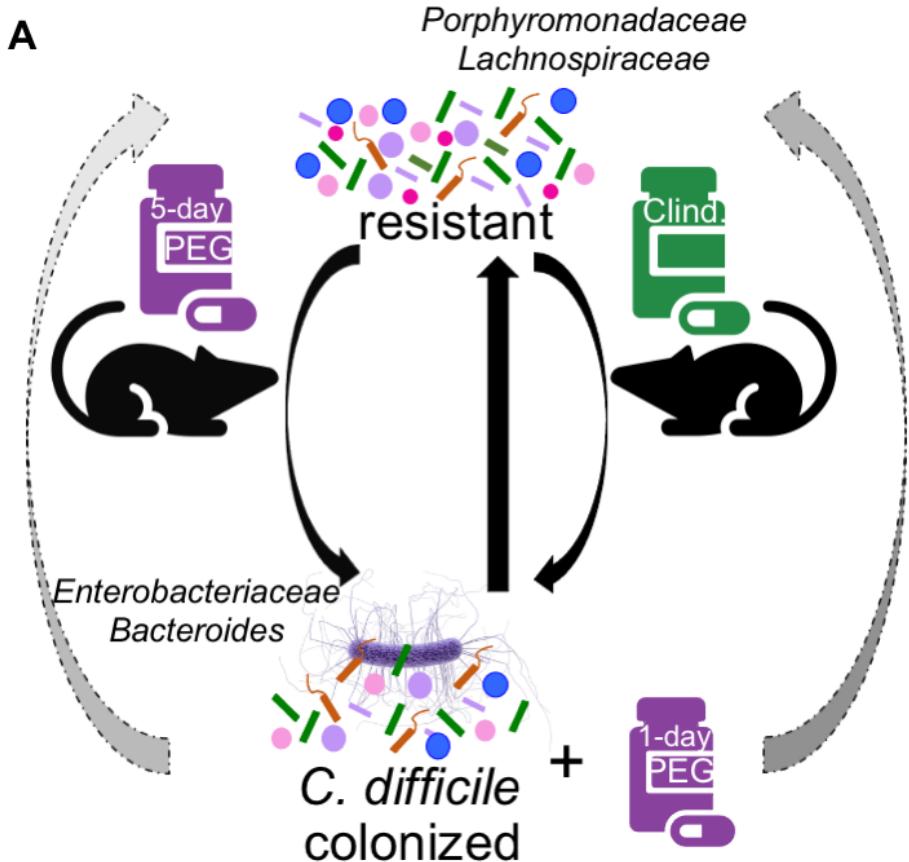
827 **6. For 1-day PEG treatment post *C. difficile* challenge mice that also receive an FMT only**
828 **some bacterial genera were restored.** A. PCoA of Bray-Curtis distances from stool samples
829 collected over time as well as the FMT solution that was administered to one of the treatment
830 groups.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



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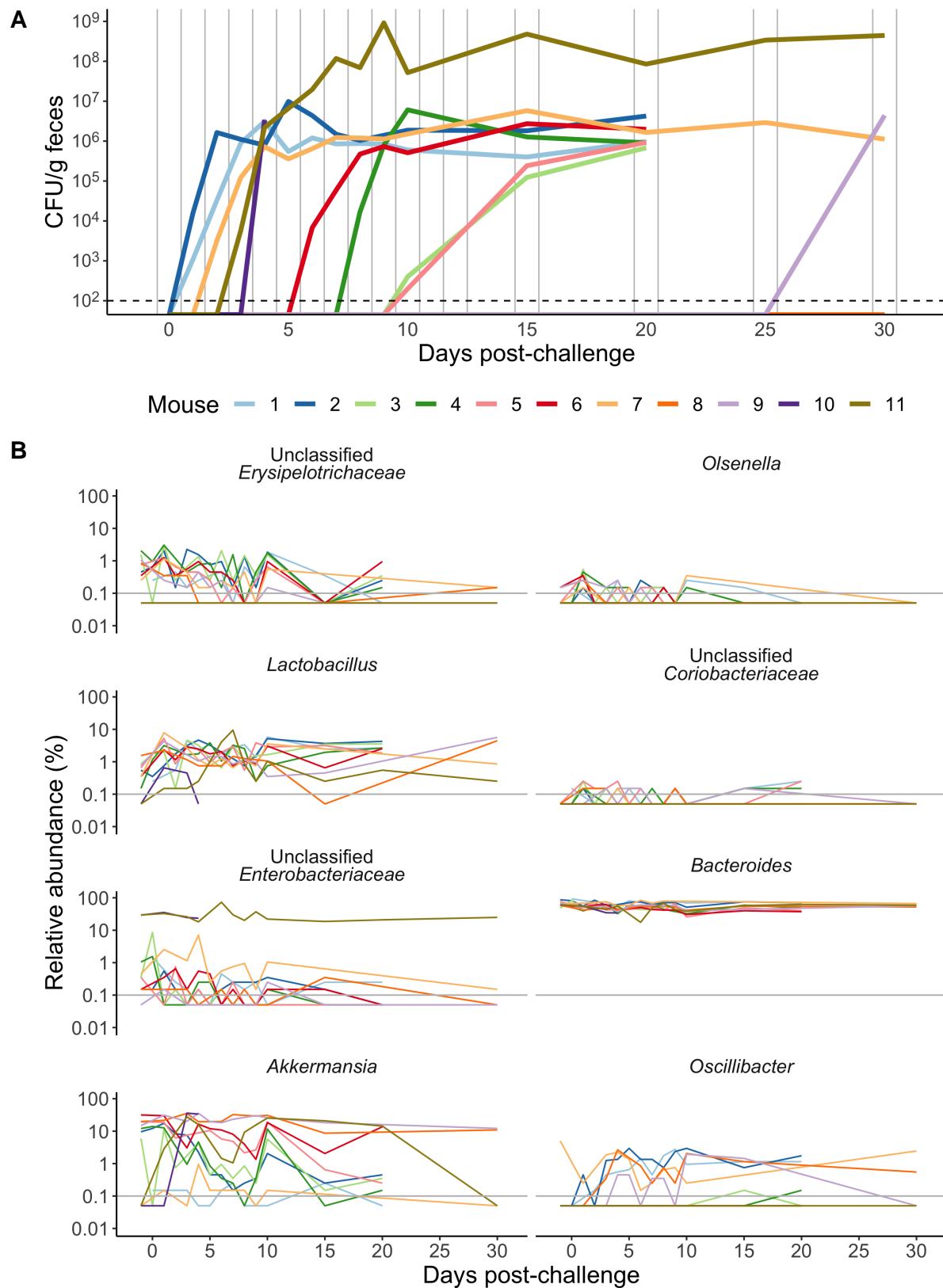
842 **Figure 7. Specific microbiota features associated with prolonged *C. difficile* colonization**
 843 **in PEG treated mice.** A. Top ten bacteria that contributed to the random forest model trained on
 844 5-day post-challenge community relative abundance data, predicting whether mice would still be
 845 colonized with *C. difficile* 10 days post-challenge. The median (point) and interquartile range (lines)
 846 change in AUROC when the bacteria were left out of the model by permutation feature importance
 847 analysis. B. The median relative abundances of the top ten bacteria that contributed to the random
 848 forest classification model at 5 days post-challenge . Red indicates the mice were still colonized

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



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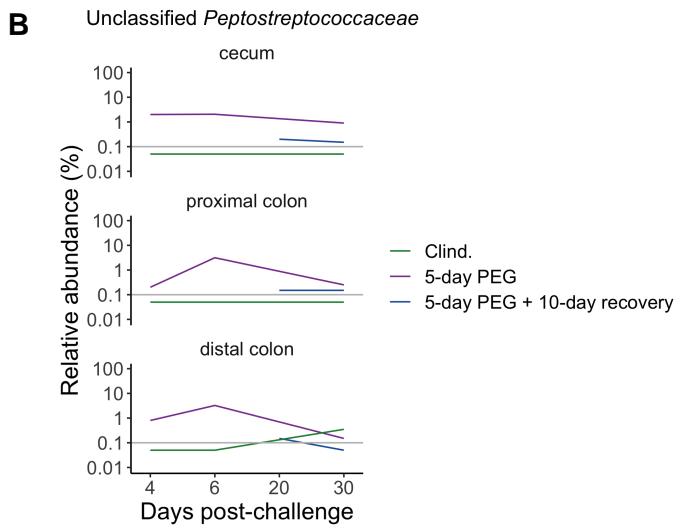
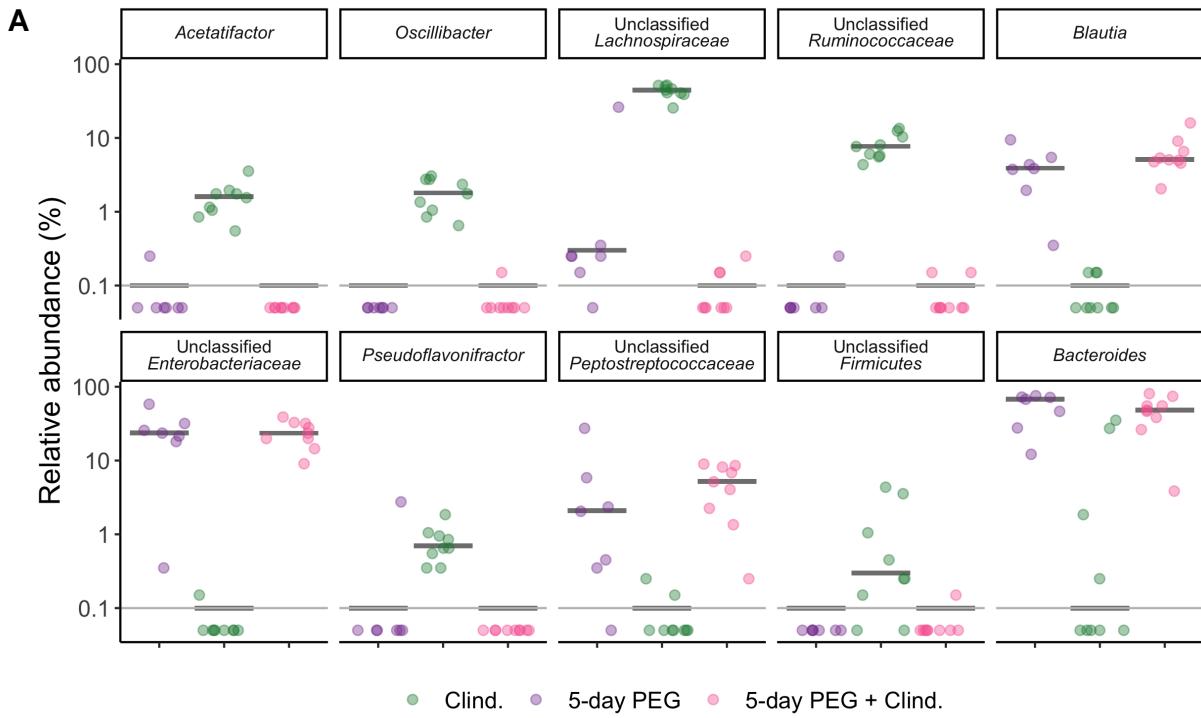
857 **Figure 8. Schematic summarizing findings.** The gut microbiota of our C57Bl/6 mice is resistant
 858 to *C. difficile* but treatment with either the antibiotic, clindamycin, or the osmotic laxative, PEG
 859 3350, renders the mice susceptible to *C. difficile* colonization. Recovery of colonization resistance
 860 in clindamycin-treated mice is relatively straightforward and the mice clear *C. difficile* within 10
 861 days post-challenge. However, for mice that received either a 5-day PEG treatment prior to *C.*
 862 *difficile* challenge or a 1-day PEG treatment post-challenge recovery of colonization resistance was
 863 delayed because most mice were still colonized with *C. difficile* 10 days post-challenge. We found
 864 increased relative abundances of *Porphyromonadaceae* and *Lachnospiraceae* were associated
 865 with recovery of colonization resistance, while increased relative abundances of *Enterobacteriaceae*
 866 and *Bacteroides* were associated with prolonged *C. difficile* colonization.



867

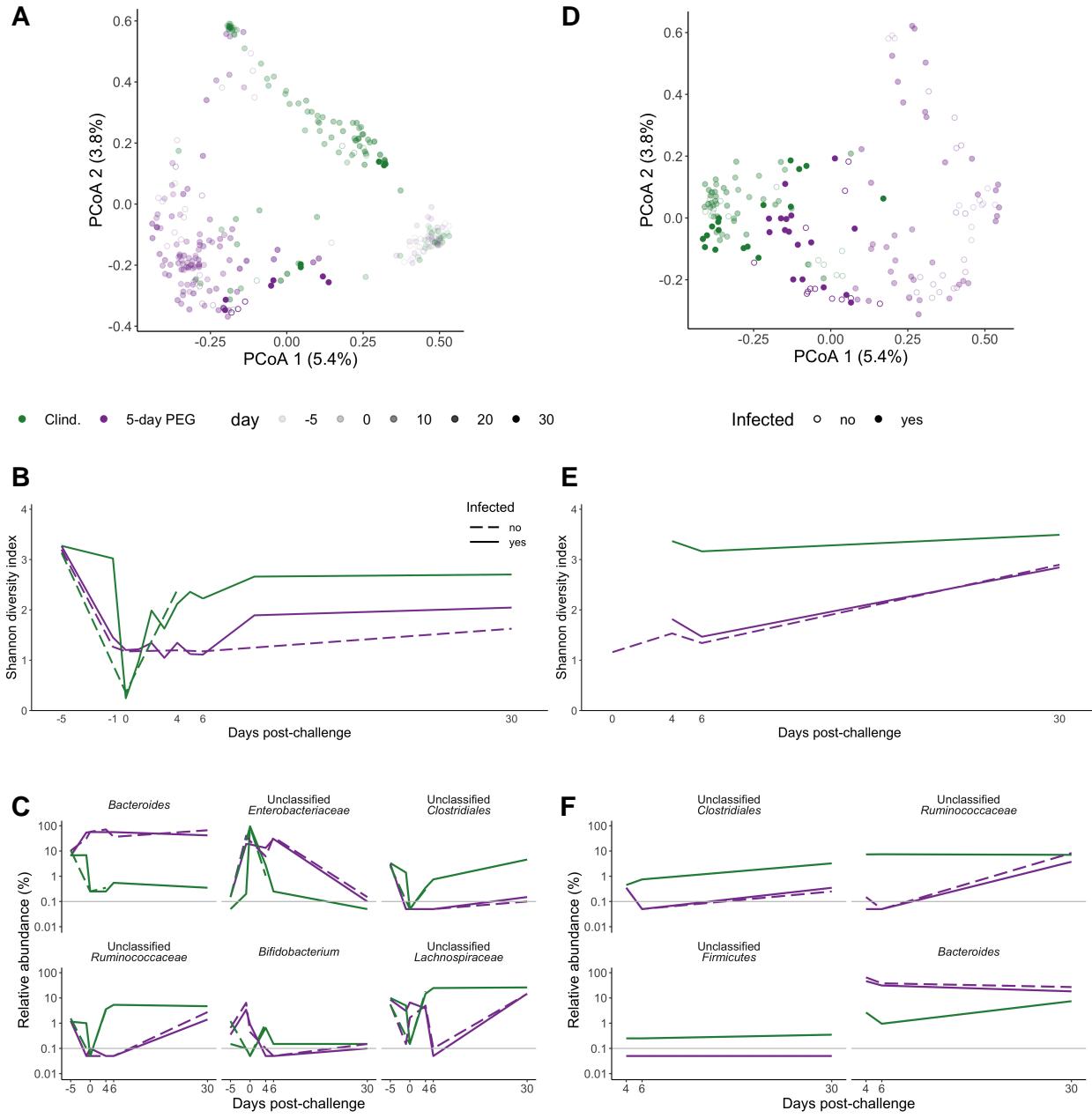
Figure S1. Microbiota dynamics post-challenge in the 5-day PEG treatment plus 10-day

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



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

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



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

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

899 **Data Set S1**

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

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

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

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

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

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

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

914 **Data Set S1, Sheet 7. PERMANOVA results for the tissue communities from mice in the
915 5-day PEG subset.**

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

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

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

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

- 923 **of 1-day PEG subset.**
- 924 **Data Set S1, Sheet 12. Shannon diversity analysis for the stool communities from mice in**
925 **the 1-day PEG experiments.**
- 926 **Data Set S1, Sheet 13. Genera with different relative abundances between the baseline and**
927 **day 1 time points in the 1-day PEG subset.**
- 928 **Data Set S1, Sheet 14. Genera with different relative abundances between the baseline and**
929 **day 7 time points in the 1-day PEG subset..**
- 930 **Data Set S1, Sheet 15. Shannon diversity analysis for the stool communities from mice in**
931 **the post-challenge PEG experiments.**
- 932 **Data Set S1, Sheet 16. Richness analysis for the stool communities from mice in the**
933 **post-challenge PEG experiments.**
- 934 **Data Set S1, Sheet 17. PERMANOVA results for the stool communities from mice in the**
935 **post-challenge PEG subset.**
- 936 **Data Set S1, Sheet 18. Genera with relative abundances that vary between treatment groups**
937 **in the stool communities from mice in the post-challenge PEG subset.**
- 938 **Data Set S1, Sheet 19. AUROC results for the 100 different seeds from each of the 3 models**
939 **tested.**