

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

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

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

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

1 Abstract

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3 Antibiotics are a major risk factor for *Clostridioides difficile* infections (CDIs) because of their impact

4 on the intestinal microbiome. However, non-antibiotic medications such as the ubiquitous osmotic

5 laxative polyethylene glycol (PEG) 3350, also alter the microbiota, but whether PEG impacts CDI

6 susceptibility and clearance is unclear. To examine how PEG impacts susceptibility, we treated

7 C57Bl/6 mice with 5-day and 1-day doses of 15% PEG in the drinking water and then challenged

8 the mice with *C. difficile* 630 spores. We used clindamycin-treated mice as a control because they

9 consistently clear *C. difficile* within 10 days post-challenge (dpc). To examine how PEG treatment

10 impacts clearance, we administered PEG for 1 day to clindamycin-treated, *C. difficile*-challenged

11 mice either immediately following challenge or 3 dpc. We collected longitudinal stool samples to

12 examine *C. difficile* levels in the stool via anaerobic culture and profiled the microbiota by 16S

13 rRNA sequencing. PEG treatment alone was sufficient to render mice susceptible to CDI and

14 5-day PEG-treated mice remain colonized for up to 30 dpc. Additionally, 5-day PEG treated

15 mice remained susceptible to CDI 10-days post treatment. In contrast, 1-day PEG treated mice

16 were transiently colonized, clearing *C. difficile* within 7 dpc. Although 5-day PEG-treated mice

17 exhibited prolonged *C. difficile* colonization, we saw no difference in histological inflammation

18 between PEG- and clindamycin-treated mice. Additionally, administering PEG to mice after *C.*

19 *difficile* challenge prolonged colonization up to 30 dpc in mice that received PEG immediately after

20 challenge and 15 dpc in mice that received PEG 3 dpc. When we examined microbiota composition

21 across our different treatment groups, we found decreased richness in the PEG-treated mice that

22 exhibited prolonged *C. difficile* colonization. Importantly, there were increased Bacteroides and

23 Enterobacteriaceae and decreased Lachnospiraceae and Oscillibacter in most of the PEG-treated

24 mice with prolonged *C. difficile* colonization. Our findings suggest the osmotic laxative PEG 3350

25 alters the mouse microbiota and disrupts colonization resistance to *C. difficile*, as well as clearance

26 in mice with a CDI. Considering that most hospitals recommend not performing *C. difficile* testing

27 on 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 human microbiota colonization resistance.

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 microbiome
36 is 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 controls are separated into two groups based on stool consistency, there are shared microbiota
42 features such as lower alpha diversity in samples from CDI patients and control patients with
43 diarrhea compared to control samples that were *C. difficile* negative with non-diarrheal consistency
44 (12). These results led to a hypothesis that bacterial communities from patients experiencing
45 diarrhea are susceptible to developing CDIs.

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

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

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

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

71 **Results**

72 **5-day laxative treatment led to prolonged *C. difficile* colonization in mice.** Building off of
73 previous work that showed treating mice with the osmotic laxative, PEG 3350, rendered mice
74 susceptible to *C. difficile* colonization (15, 17), we decided to test how long *C. difficile* colonization
75 is sustained and how long PEG-treated mice remain susceptible to *C. difficile*. We compared three
76 groups of mice treated with PEG 3350 to one group of mice treated with our standard 10 mg/kg
77 clindamycin treatment, which temporarily renders the mice susceptible to *C. difficile* colonization,
78 with mice typically clearing *C. difficile* within 10 days post-challenge (9, 26). All three groups
79 of PEG-treated mice were administered a 15% PEG solution in the drinking water for 5-days, 1.
80 received no additional treatment, 2. was also treated with clindamycin, and 3. was allowed to
81 recover for 10 days prior to challenge (Fig. 1A). PEG treatment resulted in weight loss in all 3 groups
82 of PEG-treated mice relative to their baseline weights, with the greatest change in weight observed
83 on the fifth day of PEG treatment. The mice recovered most of the lost weight by five days after
84 treatment (Fig. 1B). After either the PEG, clindamycin, or PEG and clindamycin treatment all mice
85 were challenged with 10^5 *C. difficile* 630 spores (Fig. 1A). All treatments rendered mice susceptible

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

91 Notably, we also found PEG-treated mice were still susceptible to *C. difficile* colonization after a
92 10-day recovery period, although *C. difficile* was not detectable in most of the group in the initial
93 five days post-challenge (Fig. 1C, S1A). One mouse was found dead on the 6th day post-challenge,
94 presumable due to *C. difficile* as the bacteria became detecable in that mouse on the 4th day
95 post-challenge (Fig. S1A, mouse 10). From 8 days post-challenge onward, the density of *C. difficile*
96 stabilized in the 10-day recovery group and remained high through 20-30 days post-challenge
97 (Fig. 1C). Thus, osmotic laxative treatment alone was sufficient to render mice susceptible to
98 prolonged *C. difficile* colonization and PEG-treated mice remained susceptible through ten days
99 post-treatment.

100 **5-day laxative treatment differentially disrupted the fecal microbiota compared to**
101 **clindamycin treatment.** Since osmotic laxatives and clindamycin have previously been
102 shown to disrupt the murine microbiota (14–17), we hypothesized the different *C. difficile*
103 colonization dynamics between mice treated with the osmotic laxative or clindamycin were due to
104 the two drugs having differential effects on the microbiota. We profiled the stool microbiota over
105 time by sequencing the V4 region of the 16S rRNA gene to compare changes across treatment
106 groups. We found time and treatment group explained half of the observed variation between fecal
107 communities with most of the remaining variation explained by interactions between treatment
108 group and other experimental variables including time, cage effects, and sequencing preparation
109 plate (PERMANOVA combined $R^2 = 0.95$, $P < 0.001$, Fig. 2A, Data Set S1, Sheet X). Cage effects
110 refer to the well-documented phenomenon that mice housed in the same cages have similar
111 microbial communities due to coprophagy (27). We tried to minimize the impact of cage effects on
112 our experiment by breaking up cagemates when assigning mice to treatment groups. Importantly,
113 although we conducted a total of 5 separate experiments, the experiment number and its interaction
114 with treatment group was not one of the variables that significantly explained the observed variation

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

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

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

144 period and this corresponded to mouse 10, which died on the sixth day post-challenge and mouse
145 11, where *C. difficile* was present at a high density from the 4th day post-challenge onward (Fig.
146 S1B). While we did not identify a clear signal to explain the delayed appearance of *C. difficile* in the
147 5-day PEG mice that were allowed to recover for 10 days prior to challenge, the delay is striking
148 and could reflect changes in microbial activity or metabolites that were not examined in this study.

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

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

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

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

202 with shorter treatments resulting in a transient loss of *C. difficile* colonization resistance.

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

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

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

230 **C. difficile colonization.** After identifying bacteria associated with the 5-day, 1-day and post-CDI
231 1-day PEG treatments, we decided to examine the bacteria that influenced prolonged *C. difficile*
232 colonization. We trained 3 types of machine learning models (random forest, logistic regression,
233 and support vector machine) with bacterial community data from 5 days post-challenge to predict
234 whether the mice were still colonized with *C. difficile* 10 days post-challenge. We chose 5 days
235 post-challenge because that was the earliest time point where we would see a treatment effect
236 in the mice that were given 1-day PEG treatment three days after challenge. The random forest
237 model had the highest performance (AUROC = 0.90, Data Set S1, Sheet X), so we next performed
238 permutation importance to examine the bacteria that were the top contributors to the random forest
239 model predicting prolonged *C. difficile* colonization. We selected the top 10 bacteria contributing to
240 our models performance (Fig. 7A) and examined their relative abundance at 5 days post-challenge,
241 the time point used to predict *C. difficile* colonization status on day 10 (Fig. 7B). Next, we focused
242 on the 5 genera that had a greater than 1 % relative abundance in either the cleared or colonized
243 mice and examined how the bacteria changed over time. We found *Enterobacteriaceae* and
244 *Bacteroides* tended to have a higher relative abundance, the relative abundance of *Akkermansia*
245 was initially decreased and then increased, and *Porphyromonadaceae* and *Lachnospiraceae* had a
246 lower relative abundance in the mice with prolonged colonization compared to the mice that cleared
247 *C. difficile* (Fig. 7C). Together these results suggest a combination of low and high abundance
248 bacterial genera influence the prolonged colonization observed in 5-day PEG and post-CDI PEG
249 treated mice.

250 Previous work examining the impact of PEG on the murine microbiota found that PEG
251 treatment resulted in the permanent loss of *Muribaculum intestinalis* (16), which is
252 classified as *Porphyromonadaceae* by the Ribosomal Database Project (RDP) database
253 (31). *Porphyromonadaceae* was a top feature in the random forest model predicting prolonged *C.*
254 *difficile* colonization. We identified 4 OTUs that had 92-96% identity to *Muribaculum intestinalis*
255 and examined their abundance in mice that either cleared or were still colonized with *C. difficile*
256 at 10 days post-challenge. While all of the OTUs, were decreased by PEG and clindamycin
257 treatment, there was some recovery in the mice that cleared (Fig. S4A). We also examined
258 other *Porphyromonadaceae* and *Lachnospiraceae* OTUs since these were the 2 genera that

259 were important to our classification model and contained multiple OTUs that were different at
260 5 days post-challenge between mice that either cleared or remained colonized with *C. difficile*
261 by 10 days post-challenge (Data Set S1, sheet X). While individual *Porphyromonadaceae* and
262 *Lachnospiraceae* OTUs tended to be more abundant in the mice that clear *C. difficile* relative
263 to the mice that exhibit prolonged colonization (Fig. S4B-C), there is no single OTU that fits the
264 pattern we observed at the genus level (Fig. 7C), suggesting multiple *Porphyromonadaceae* and
265 *Lachnospiraceae* OTUs influenced *C. difficile* clearance. Overall, our results suggest that specific
266 bacterial community differences explain the prolonged *C. difficile* colonization we observed in 5-day
267 PEG and post-CDI 1-day PEG treated mice.

268 Discussion

- 269 • Summary of major findings (Fig. 8A)
- 270 • Discussion of prolonged persistence. *C. difficile* sequences detected in tissue samples.
271 Association with mucin-degrading bacteria suggested by recent papers.
- 272 • Discuss why we might not have observed more severe histology in PEG mice relative to
273 clindamycin-treated mice
 - 274 – Antibiotics may also impact mucus layer
 - 275 – Strain of bacteria used
- 276 • Protective bacteria missing in PEG-treated mice
- 277 • Discuss what these findings might mean for human patients (Fig. 8B)
 - 278 – What's known regarding laxatives and susceptibility to CDIs
 - 279 – Clinical trial of PEG, results never posted (32)
 - 280 – Relevance to human FMTs? Unclear what the best administration route is because there
281 have been no studies designed to evaluate the best administration route for FMTs.

282 **Conclusions**

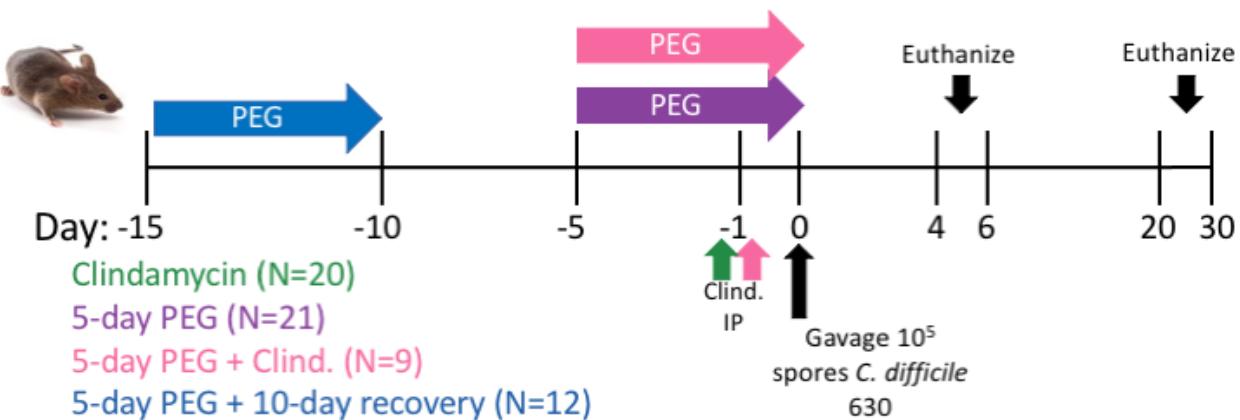
283 **Acknowledgements**

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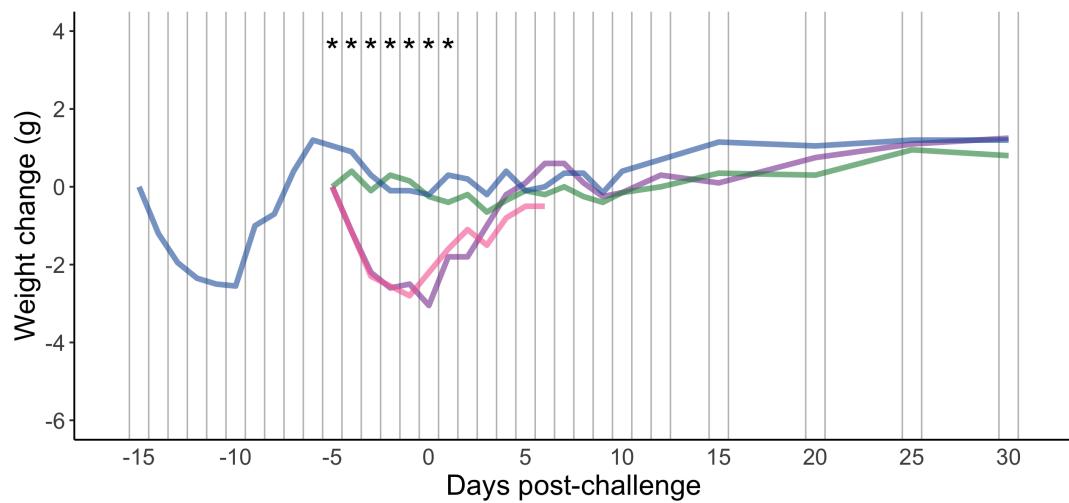
294 **Materials and Methods**

- 295 • Histopathology (33)

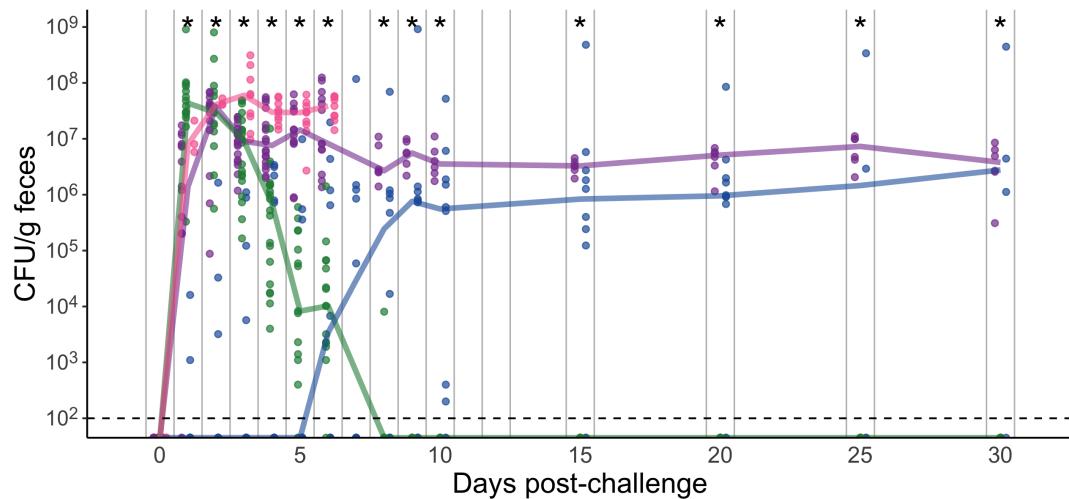
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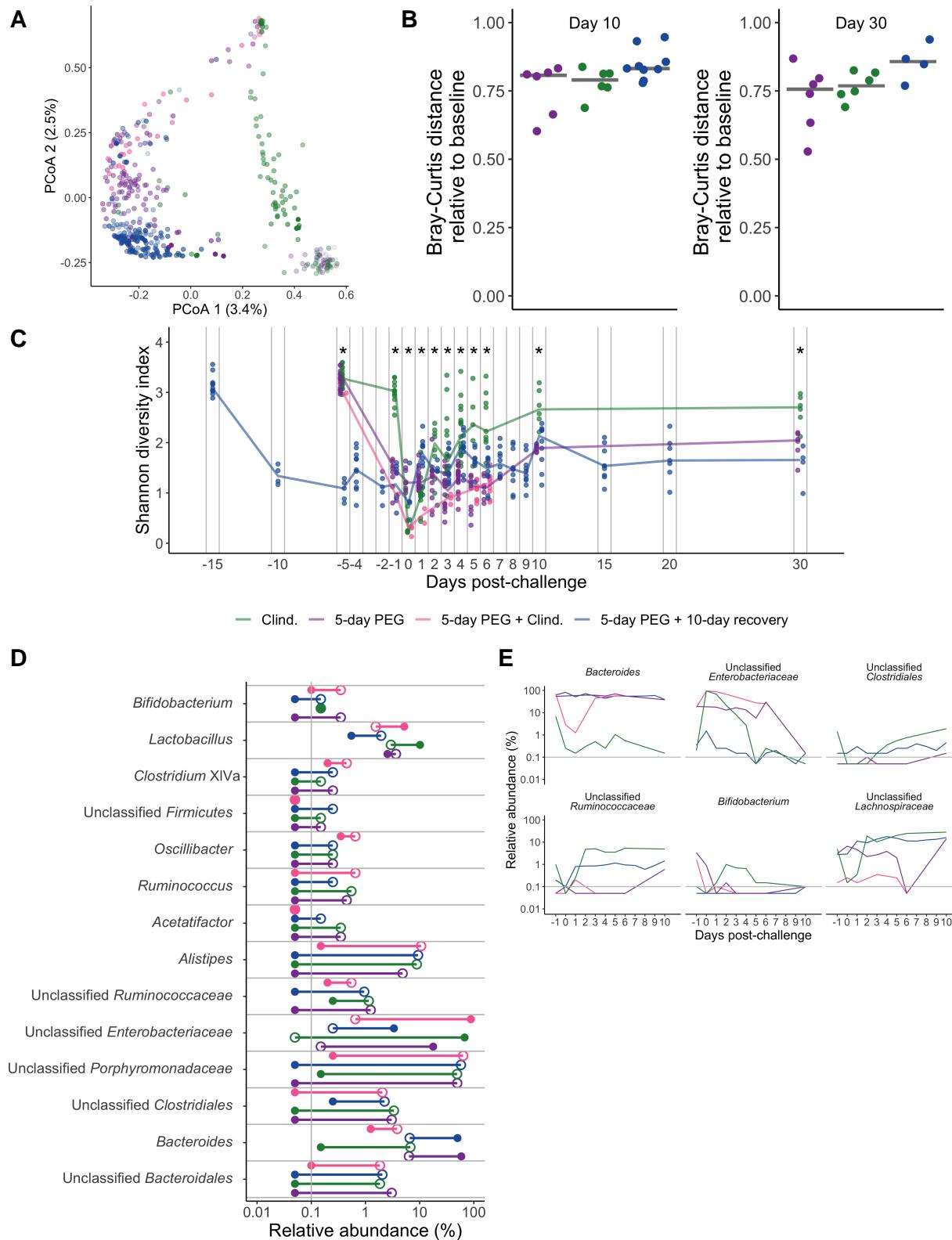
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296

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

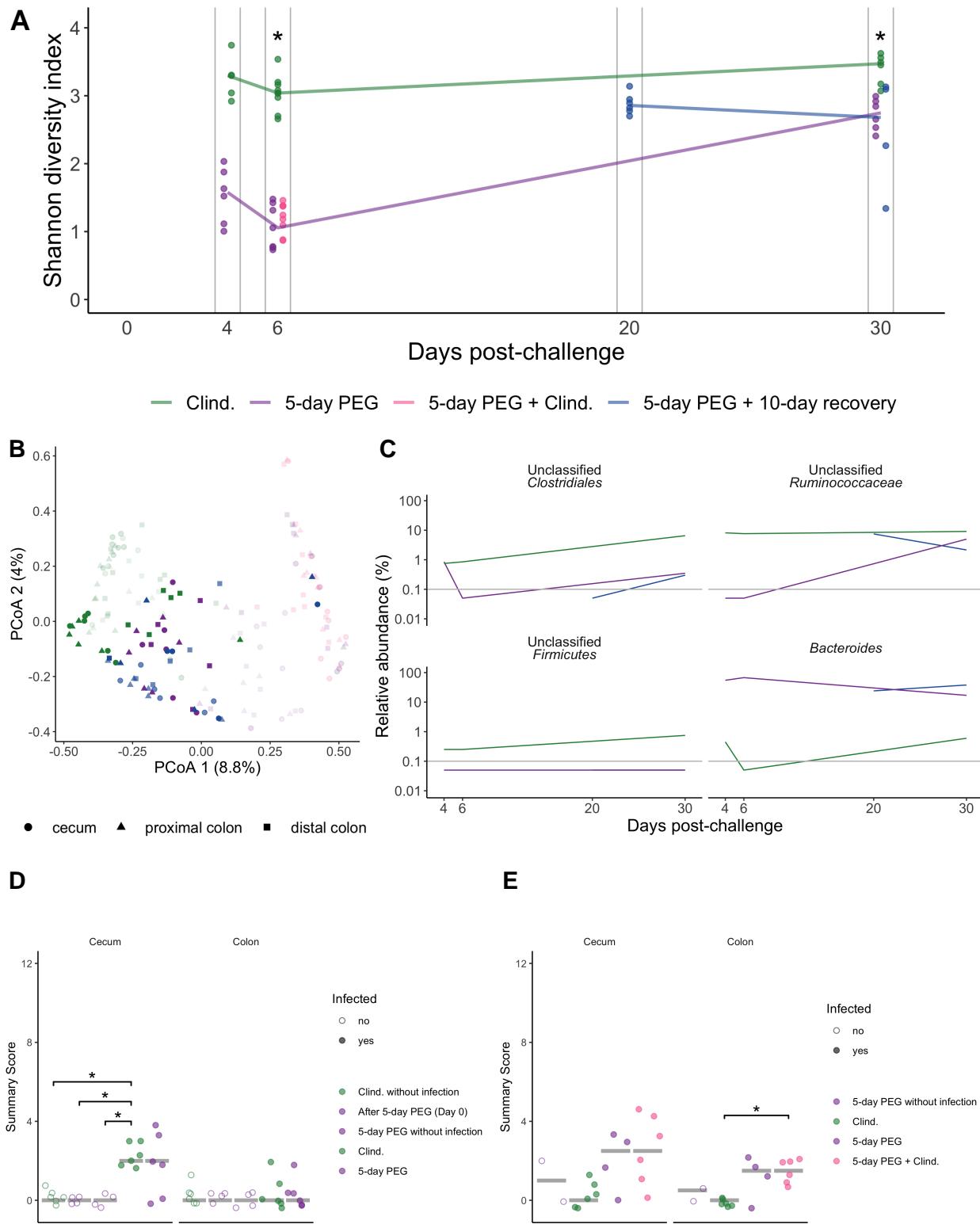
300 intraperitoneal injection. 2. 15% PEG 3350 was administered in the drinking water for five days. 3.
301 5-day PEG plus clindamycin treatment. 4. 5-day PEG plus 10-day recovery treatment. All treatment
302 groups were then challenged with 10^5 *C. difficile* 630 spores. A subset of mice were euthanized
303 on either 4 or 6 days post-challenge and tissues were collected for histopathology analysis, the
304 remaining mice were followed through 20 or 30 days post-challenge. B. Weight change from
305 baseline weight in groups after treatment with PEG and/or clindamycin, followed by *C. difficile*
306 challenge. C. *C. difficile* CFU/gram stool measured over time (N = 10-59 mice per time point) via
307 serial dilutions. The black line represents the limit of detection for the first serial dilution. CFU
308 quantification data was not available for each mouse due to stool sampling difficulties (particularly
309 the day the mice came off of the PEG treatment) or early deaths. For B-C, lines represent the
310 median for each treatment group and circles represent samples from individual mice. Asterisks
311 indicate time points where the weight change or CFU/g was significantly different between groups
312 by the Kruskal-Wallis test with Benjamini-Hochberg correction for testing multiple time points. The
313 data presented are from a total of 5 separate experiments.



314

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

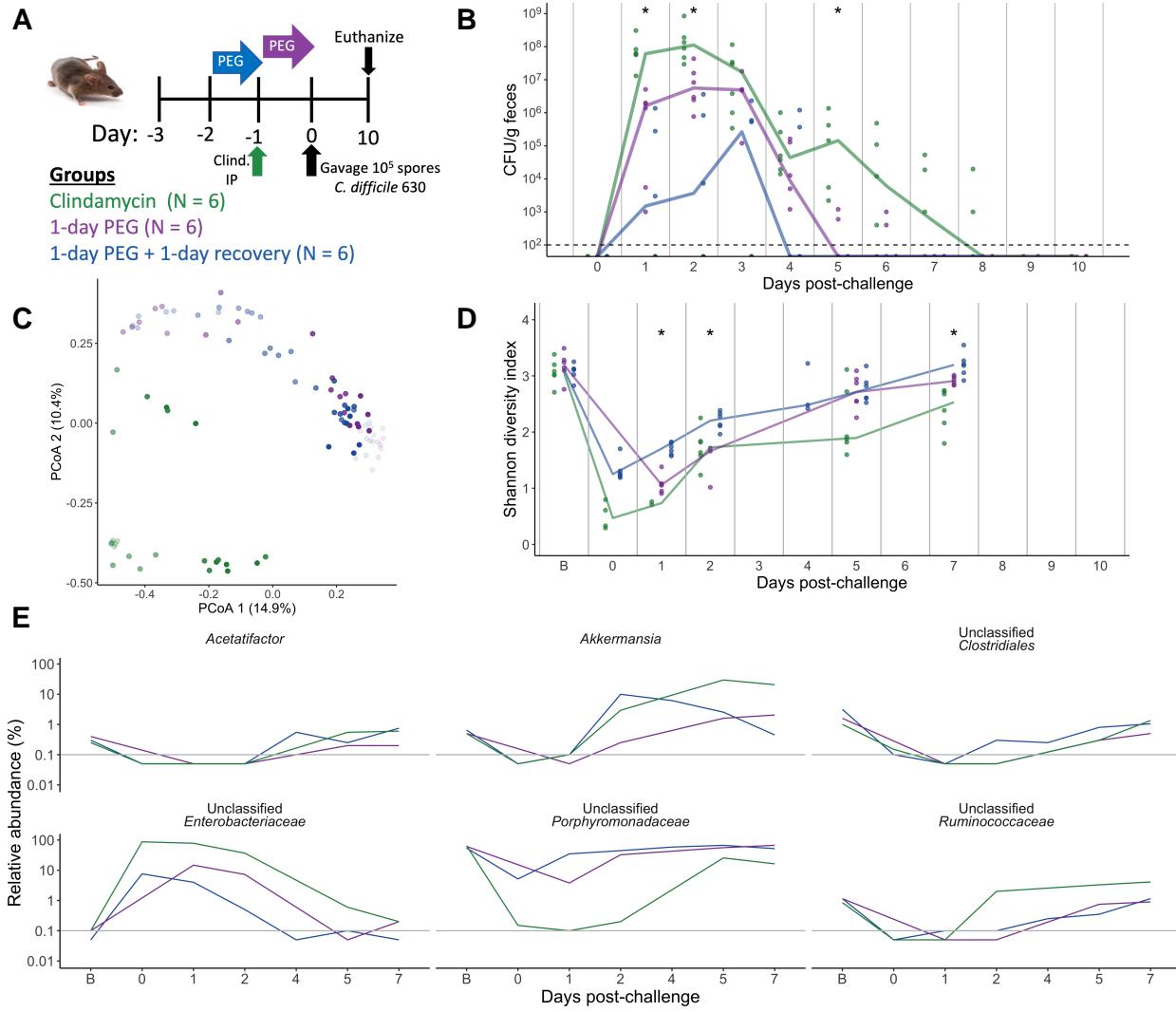
316 **compared to clindamycin-treated mice.** A. Principal Coordinate analysis (PCoA) of Bray-Curtis
317 distances from stool samples collected throughout the experiment. For A and C, each circle
318 represents a sample from an individual mouse and the transparency of the symbol corresponds to
319 the day post-challenge. B. Bray-Curtis distances of stool samples collected on either day 10 or 30
320 post-challenge relative to the baseline sample collected for each mouse (before any drug treatments
321 were administered). C. Shannon diversity in stool communities over time. The line indicates the
322 median value for each treatment group. The colors of the symbols and lines represent the four
323 treatment groups. D. 14 of the 33 genera affected by PEG treatment (Data Set S1, sheet X). The
324 symbols represent the median relative abundance for a treatment group at either baseline (open
325 circle) or 1-day post treatment (closed circle). Data from the 5-day PEG and 5-day PEG plus 10-day
326 recovery groups were analyzed by paired Wilcoxon signed-rank test with Benjamini-Hochberg
327 correction for testing all identified genera. The clindamycin and 5-day PEG plus clindamycin
328 treatment groups are shown for comparison. E. 6 of the 24 genera that were significantly different
329 between the four treatment groups over multiple time points. Differences between treatment groups
330 were identified by Kruskal-Wallis test with Benjamini-Hochberg correction for testing all identified
331 genera. The gray vertical line (D) and horizontal vertical lines (E) indicate the limit of detection.



332

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

334 **microbiota is altered.** A. Shannon diversity in cecum communities over time. The line indicates
335 the median value for each treatment group. The colors of the symbols and lines represent the four
336 treatment groups. A similar pattern was observed with the proximal and distal colon communities
337 (Data Set S1, sheet X-X). B. PCoA of Bray-Curtis distances from mucosal samples collected
338 throughout the experiment. Circles, triangles, and squares indicate cecum, proximal colon, and
339 distal colon communities, respectively. For A-B, transparency of the symbol corresponds to the day
340 post-challenge that the sample was collected. C. The median relative abundance of the 4 genera
341 that were significantly different between the cecum communities of different treatment groups on
342 day 6 and day 30 (Data Set S1, sheet X). The gray vertical line indicate the limit of detection. D-E.
343 The histopathology summary scores from cecum and colon H&E stained slides. The summary
344 score is the total score based on evaluation of edema, cellular infiltration, and inflammation. Each
345 category is given a score ranging from 0-4, thus the maximum possible summary score is 12. The
346 tissue for histology was collected at either 4 (D) or 6 (E) days post-challenge with the exception that
347 one set of 5-day PEG treated mock-challenged mice were collected on day 0 post-challenge (first
348 set of open circles in D). Histology data were analyzed with the Kruskal-Wallis test followed by
349 pairwise Wilcoxon comparisons with Benjamini-Hochberg correction.



350

351 **Figure 4. 1-day PEG treatment renders mice susceptible to transient *C. difficile***
 352 **colonization.** A. Setup of the experimental time line for the 1-day PEG treated mice consisting of 3
 353 treatment groups. 1. Clindamycin was administered at 10 mg/kg by intraperitoneal injection. 2.
 354 15% PEG 3350 was administered in the drinking water for 1 day. 3. 1-day PEG plus 1-day recovery.
 355 The three treatment groups were then challenged with 10^5 *C. difficile* 630 spores. B. *C. difficile*
 356 CFU/gram stool measured over time (N = 12-18 mice per time point) via several dilutions. The
 357 black dotted line represents the limit of detection for the first serial dilution. Asterisks indicate time
 358 points where the CFU/gram was significantly different between treatment groups by Kruskall-Wallis
 359 test with Benjamini-Hochberg correction for testing multiple time points. C. PCoA of Bray-Curtis
 360 distances from stool communities collected from the three treatment groups over time (R^2 =

361 0.43; group: $R^2 = 0.19$). D. Shannon diversity in stool communities over time with colored lines
362 representing the median value for each treatment group. For B-D, each symbol represents a
363 sample from an individual mouse and symbol transparency corresponds to the day post-challenge
364 that the sample was collected. E. Median relative abundances per treatment group for 6 out of
365 the 14 genera that were affected by treatment, but recovered close to baseline levels by 7 days
366 post-challenge (Fig. 3E, Data Set S1, Sheet X). Stool samples from either baseline and day 1 or
367 baseline and day 7 were analyzed by paired Wilcoxon signed-rank test with Benjamini-Hochberg
368 correction for testing all identified genera. The gray horizontal line represents the limit of detection.

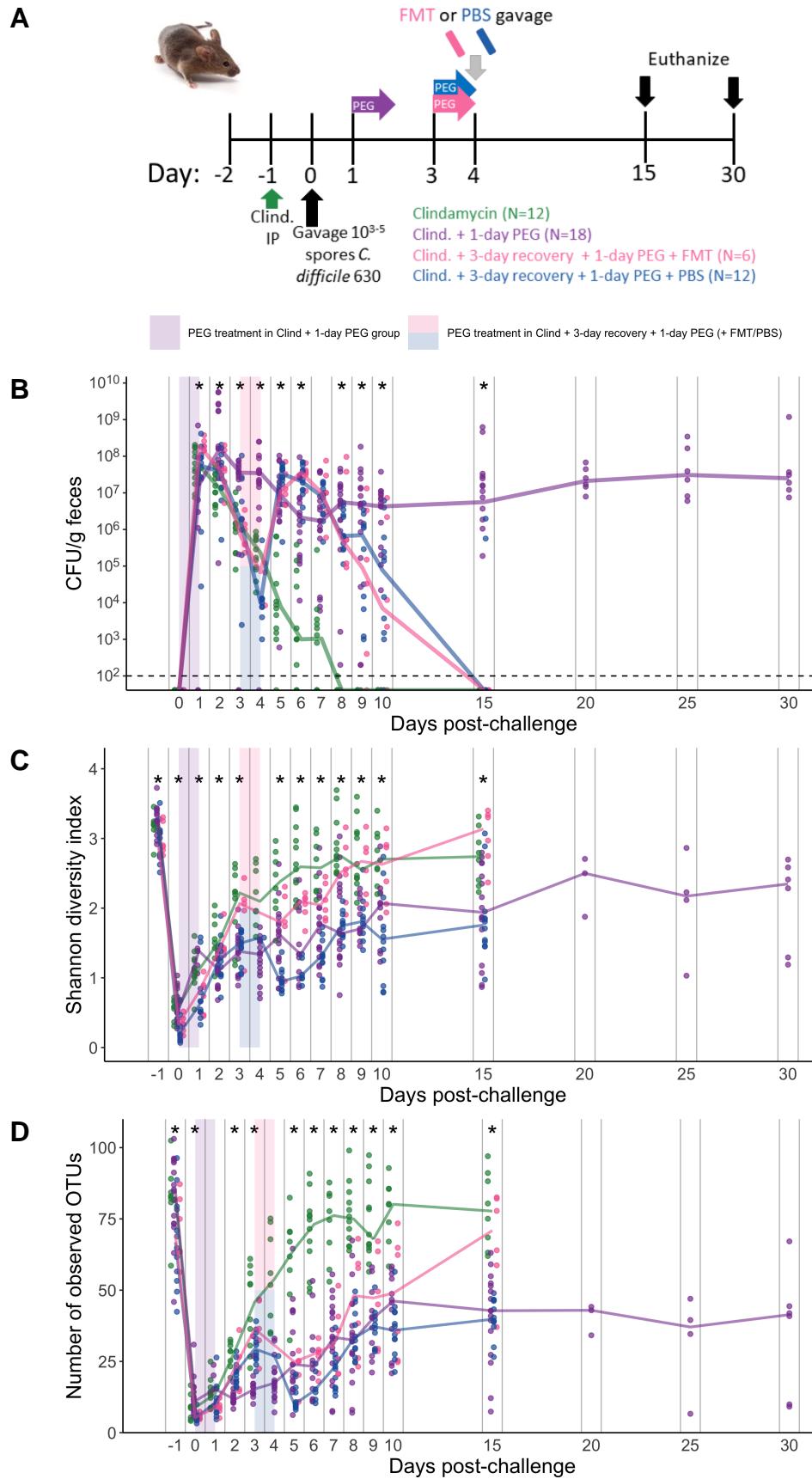
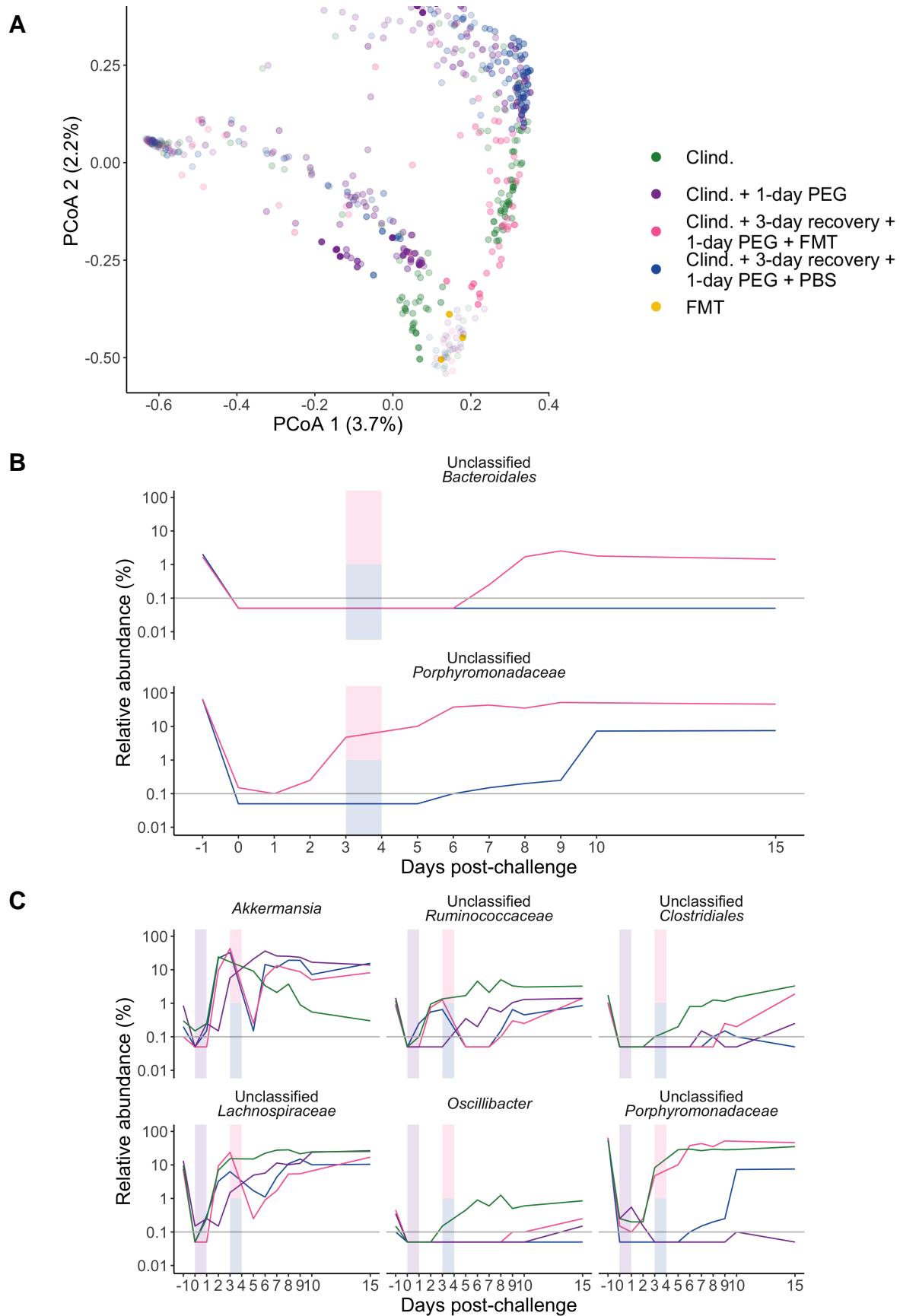
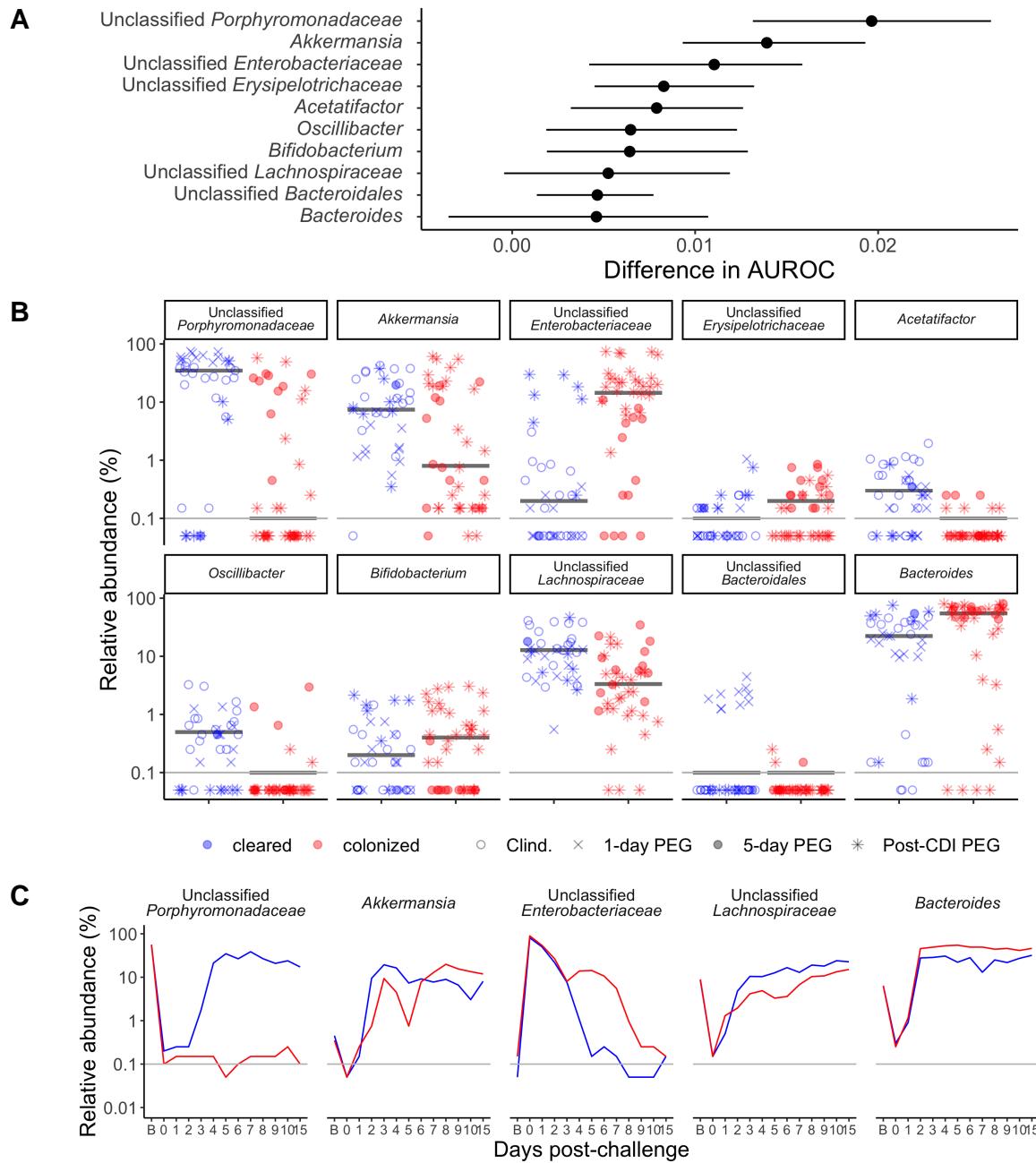


Figure 5.

370 **1-day PEG treatment post *C. difficile* challenge prolongs colonization regardless of**
371 **whether an FMT is also administered.** A. Setup off the experimental time line for experiments
372 with post-CDI PEG treated mice. There were a total of 4 different treatment groups. All mice were
373 administered 10 mg/kg clindamycin intraperitoneally (IP) 1 day before challenge with 10^{3-5} *C.*
374 *difficile* 630 spores. 1. Did not receive any additional treatment. 2. Immediately after *C. difficile*
375 challenge, mice received 15% PEG 3350 in the drinking water for 1 day. 3-4. 3-days after challenge,
376 mice received 1-day PEG treatment and then received either a fecal microbiota transplant (3) or
377 PBS (4) solution by oral gavage. Mice were followed through 15-30 days post-challenge (only the
378 post-CDI 1-day PEG group was followed through 30 days post-challenge). B. CFU/g of *C. difficile*
379 stool measured over time via serial dilutions. The black line represents the limit of detection for the
380 first serial dilution. C-D. Shannon diversity (C) and richness (D) in stool communities over time.
381 B-D. Each symbol represents a stool sample from an individual mouse with the lines representing
382 the median value for each treatment group. The transparency of the symbol corresponds to the day
383 post-challenge. Asterisks indicate time points with significant differences between groups by a
384 Kruskall-Wallis test with a Benjamini-Hochberg correction for testing multiple times points. Colored
385 rectangles indicates the 1-day PEG treatment period for applicable groups. The data presented are
386 from a total of 3 separate experiments.



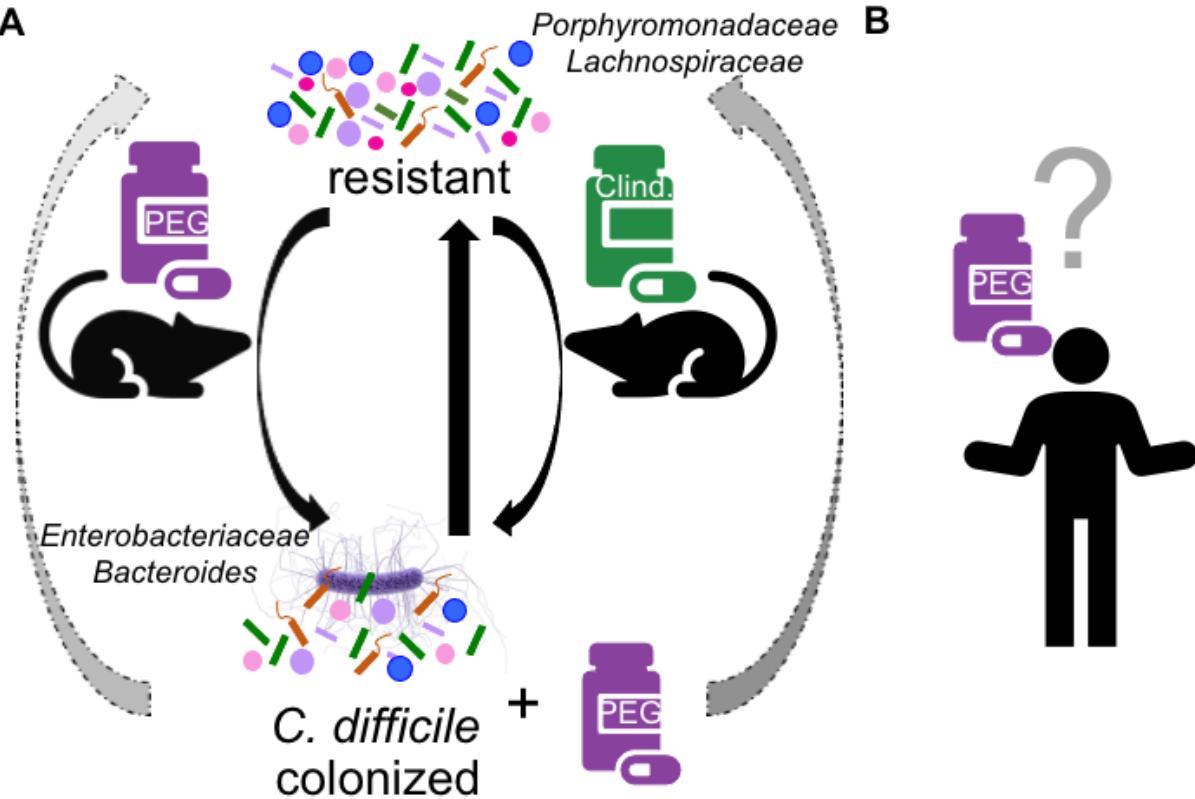
388 **Figure 6. For 1-day PEG treatment post *C. difficile* challenge mice that also receive an**
389 **FMT only some bacterial genera were restored.** A. PCoA of Bray-Curtis distances from stool
390 samples collected over time as well as the FMT solution that was administered to one treatment
391 group. Each circle represents an individual sample, the transparency of the circle corresponds
392 to day post-challenge as shown in Fig. 6C-D. B. Median relative abundances of 2 genera that
393 were significantly different over multiple time points in mice that were administered either FMT or
394 PBS solution via gavage C. Median relative abundances of the top 6 out of 24 genera that were
395 significant over multiple timepoints, plotted over time (Data Set S1, Sheet X). For B-C, colored
396 rectangles indicates the 1-day PEG treatment period for applicable groups. Gray horizontal lines
397 represent the limit of detection.



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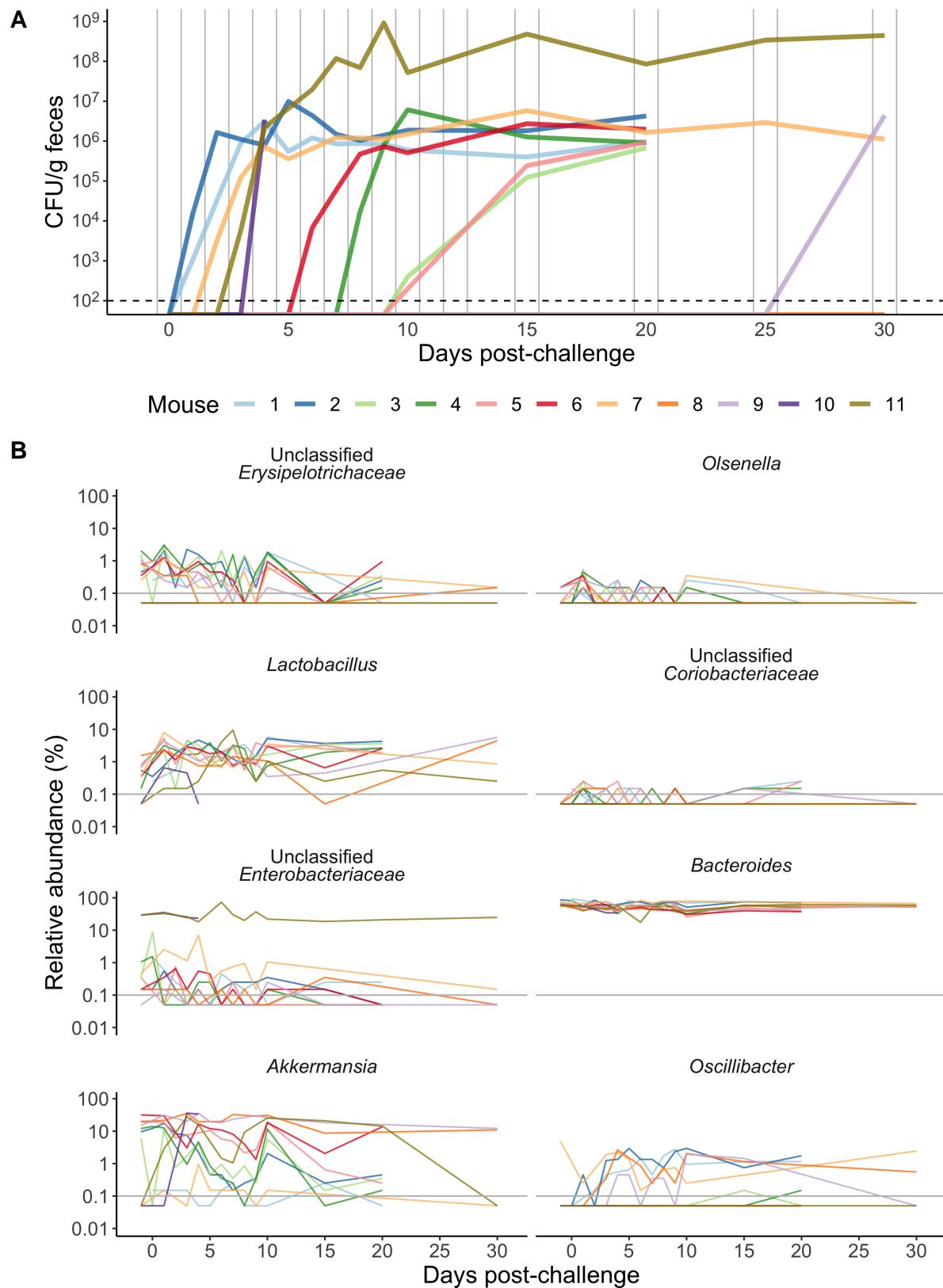
399 **Figure 7. Specific microbiota features associated with prolonged *C. difficile* colonization**
400 **in PEG treated mice.** A. Top ten bacteria that contributed to the random forest model trained on
401 five day post-challenge community relative abundance data to predict whether mice would still be
402 colonized with *C. difficile* 10 days post-challenge. The median (point) and interquartile range (lines)
403 change in AUROC when the bacteria is left out of the model is shown. B. The median relative
404 abundance at 5 days post-challenge of the top ten bacteria that contributed to the random forest
405 classification model. Color indicates whether the mice were still colonized with *C. difficile* 10 days

406 post-challenge and the black horizontal line represents the median relative abundance. Each
407 symbol represents a stool sample from an individual mouse and the shape of the symbol indicates
408 whether the PEG-treated mice received a 5-day (Fig. 1-3), 1-day (Fig. 4) or post-CDI PEG (Fig.
409 5-6) treatment. C. The median relative abundances of the 5 genera with greater than 1% median
410 relative abundance in the stool community over time. For B-C, the gray horizontal line represents
411 the limit of detection.



412

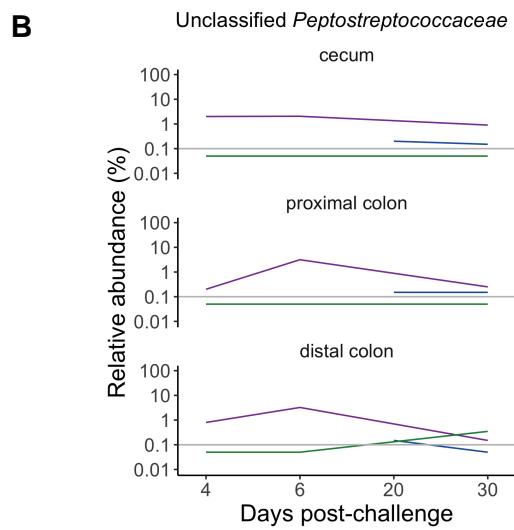
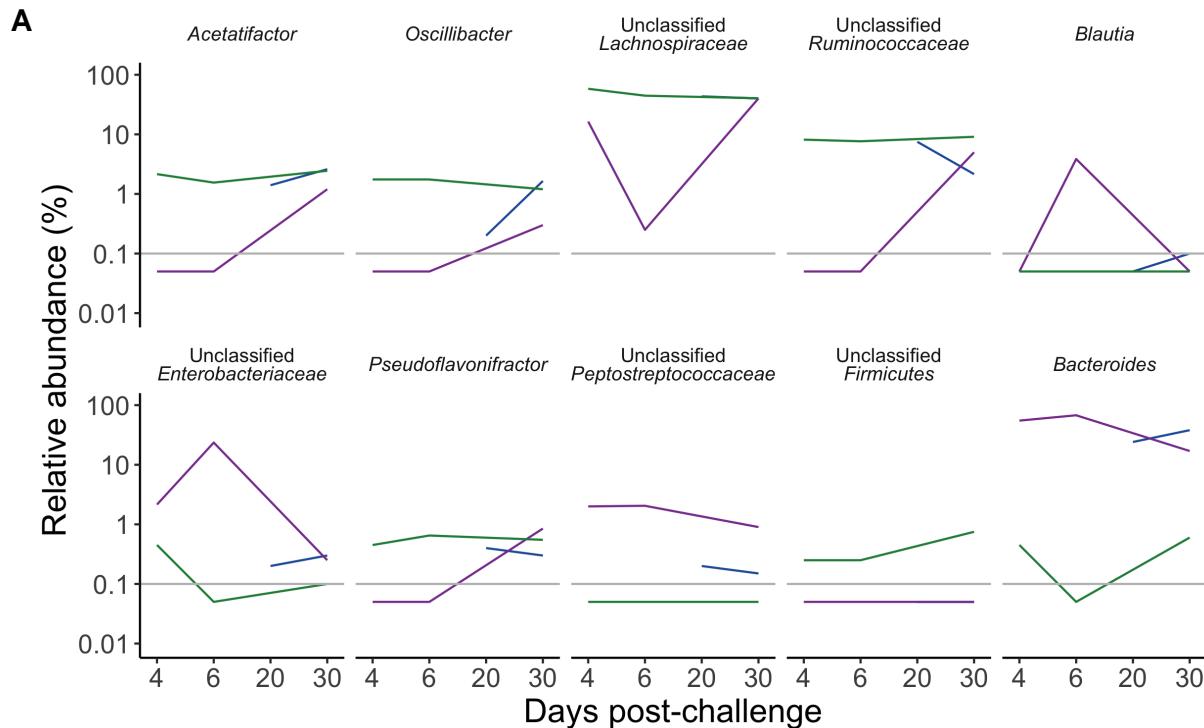
413 **Figure 8. Schematic summarizing findings.** A. The gut microbiota of our C57Bl/6 mice is resistant
 414 to *C. difficile* but treatment with either the antibiotic, clindamycin, or the osmotic laxative, PEG 3350
 415 renders the mice susceptible to *C. difficile* colonization. Recovery of colonization resistance in
 416 clindamycin-treated mice is relatively straightforward and the mice clear *C. difficile* within 10 days
 417 post-challenge. However, for mice that received a 5-day PEG treatment or a 1-day PEG treatment
 418 after *C. difficile* challenge recovery of colonization resistance is more uncertain because mice were
 419 still colonized with *C. difficile* 30 days post-challenge in the case of several PEG treatments. We
 420 found increased *Porphyromonadaceae* and *Lachnospiraceae* were associated with recovery of
 421 colonization resistance, while increased *Enterobacteriaceae* and *Bacteroides* were associated with
 422 prolonged *C. difficile* colonization. B. Considering that most hospitals recommend not performing
 423 *C. difficile* testing on patients taking laxatives and laxatives are used when administering fecal
 424 microbiota transplants via colonoscopy to patients with recurrent CDIs, further studies are needed
 425 to evaluate if laxatives impact human microbiota colonization resistance. Further studies are
 426 needed to understand the impact of osmotic laxatives on *C. difficile* colonization resistance and
 427 clearance in human patients.



428

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

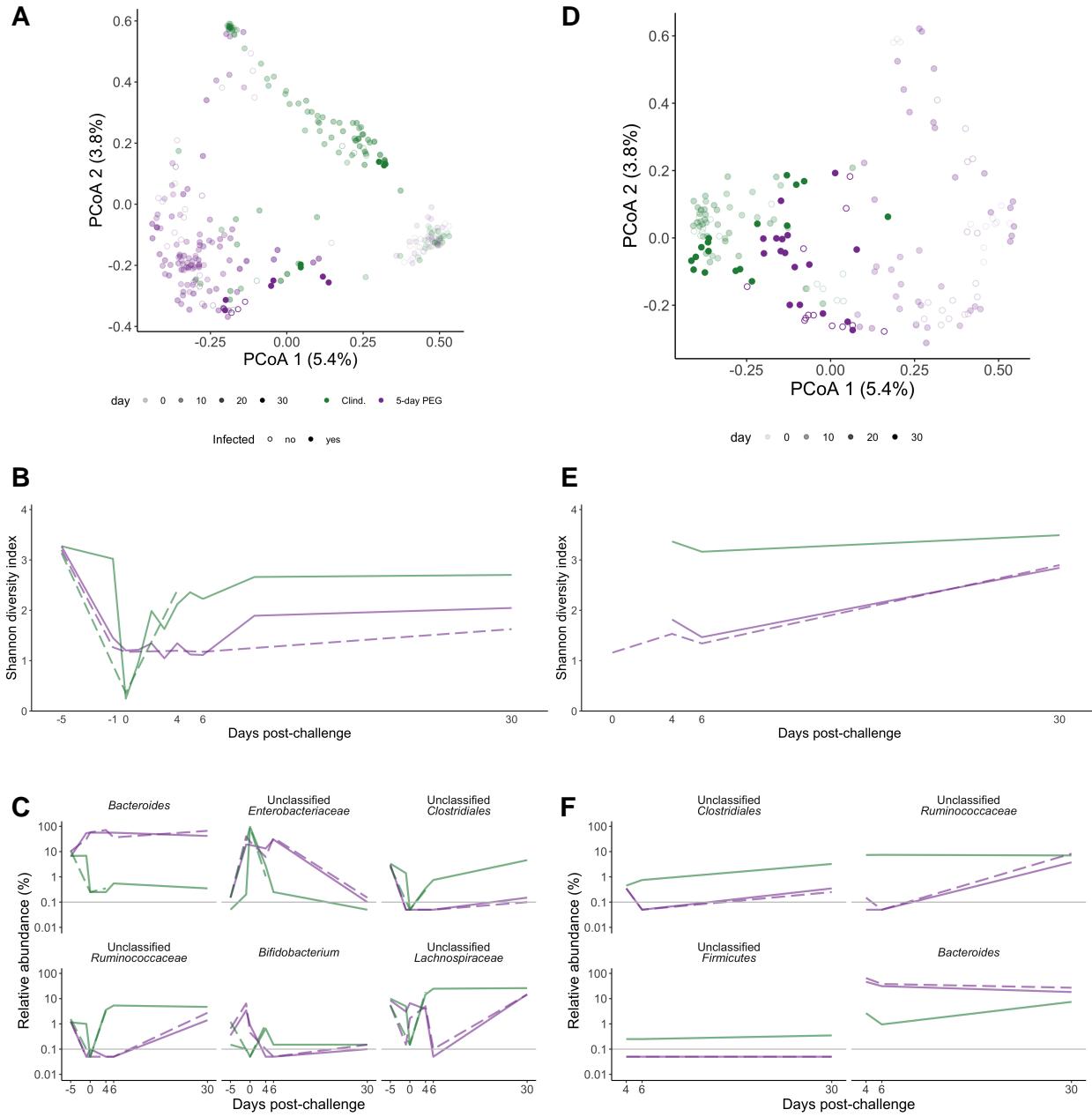
430 **recovery mice.** A. *C. difficile* CFU/g over time in the stool samples collected from 5-day PEG
431 treatment plus 10-day recovery mice. Same data presented in Fig. 1C, but the data for the other 3
432 treatment groups have been removed. B. Median relative abundances of 8 bacterial genera from
433 day 0 post-challenge onward from the 10-day recovery mice. We analyzed samples from day 0 and
434 day 8 post-challenge, which represented the the time points where mice were challenged with
435 *C. difficile* and when the median relative *C. difficile* CFU stabilized for the group using the paired
436 Wilcoxon signed-rank test, but no genera were signfcant after Benjamini-Hochberg correction.



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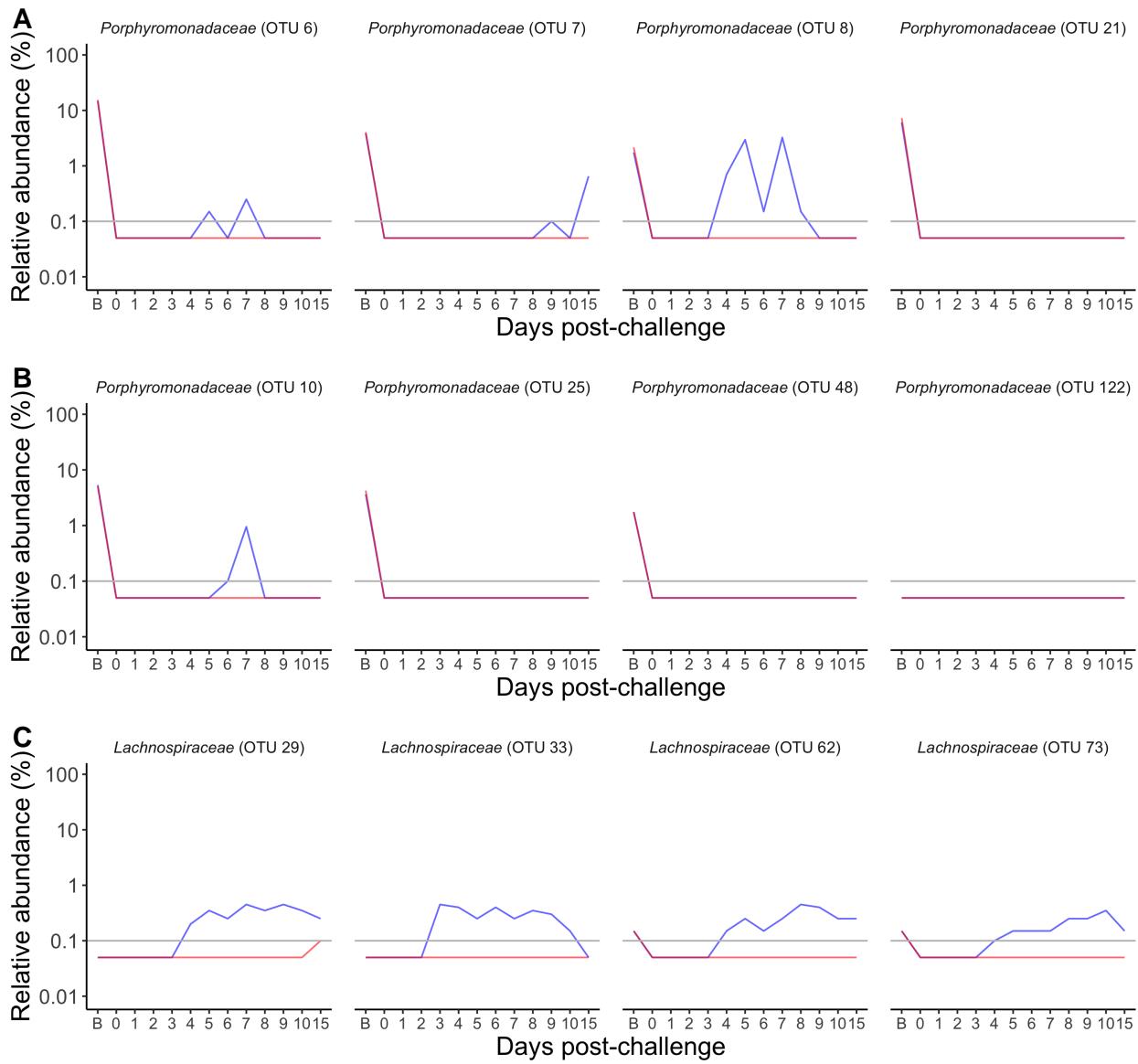
438 **Figure S2. PEG treatment still has a large impact on the mucosal microbiota 6 days
439 post-challenge A. The**

440



441

442 **Figure S3.** *C. difficile* challenge does not enhance the disruptive effect of PEG on the
443 microbiota. A.



444

445 **Figure S4. Specific OTUs associated with clearance by 10 days post-challenge that are**

446 **mostly absent in mice with prolonged *C. difficile* colonization. Ex. *Muribaculum intestinale*.**

447 A.

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