

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

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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-infection (dpi). 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 dpi. We collected longitudinal stool samples

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

13 16S rRNA sequencing. PEG treatment alone was sufficient to render mice susceptible to CDI

14 and 5-day PEG-treated mice remain colonized for up to 30 dpi. 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 dpi. 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 dpi in mice that received PEG immediately after

20 challenge and 15 dpi in mice that received PEG 3 dpi. 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. Our group has shown that when *C. difficile* negative controls are
41 separated into two groups based on stool consistency, there are shared microbiota features such
42 as lower alpha diversity in samples from CDI patients and control patients with diarrhea compared
43 to control samples that were *C. difficile* negative with non-diarrheal consistency (12). These results
44 led to a hypothesis that bacterial communities from patients experiencing diarrhea are susceptible
45 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). However, whether laxative administration results in more severe
54 CDIs in 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 In the past, our group has used C57BL6 mice to characterize how antibiotics including clindamycin
63 disrupt the microbiota and influence *C. difficile* susceptibility and clearance (24–26). Although, two
64 groups have now shown PEG treatment alone renders mice susceptible to *C. difficile* (15, 17), these
65 studies have raised additional questions regarding the dynamics and severity of infection as well as
66 the role of laxative treatment in *C. difficile* clearance that should be addressed to better inform how
67 we think about laxatives in the context of CDIs. Here, we used our C57BL/6 clindamycin model
68 as a control group to characterize how long PEG-treated mice remain susceptible, whether PEG
69 treatment results in more severe CDI and sustained *C. difficile* colonization, and if PEG treatment
70 post-CDI can promote *C. difficile* clearance.

71 **Results**

72 **5-day laxative treatment leads to prolonged *C. difficile* colonization in mice.** We compared
73 mice treated with the osmotic laxative PEG 3350 to our standard 10 mg/kg clindamycin treatment,
74 which temporarily renders the mice susceptible to *C. difficile*, with mice typically clearing *C. difficile*
75 within 10 days post-infection (9, 26). All PEG-treated mice were administered a 15% PEG solution
76 in the drinking water for 5-days, one group was also treated with clindamycin, and one group was
77 allowed to recover for 10 days prior to challenge (Fig. 1A). PEG treatment resulted in weight loss in
78 all 3 groups of PEG-treated mice, with the greatest change in weight observed on the fifth day of
79 PEG treatment and the mice recovered most of the weight five days after treatment (Fig. 1B). After
80 either PEG, clindamycin, or PEG and clindamycin treatment all mice were challenged with 10^5 *C.*
81 *difficile* 630 spores (Fig. 1A). All treatments rendered mice susceptible to *C. difficile* colonization.
82 However, PEG-treated mice remained colonized with *C. difficile* at a high level through thirty days
83 post-infection (Fig. 1C). In contrast, the clindamycin-treated mice cleared *C. difficile* within ten
84 days post-infection. Surprisingly, PEG-treated mice were still susceptible to *C. difficile* infection
85 after 10-days of recovery from treatment, although *C. difficile* was not detectable in most of the

86 group in the initial five days post-infection (Fig. 1C). From 8 days post-infection onward, the median
87 *C. difficile* CFU stabilized for the 10-day recovery group of PEG-treated mice and remained high
88 through 30 days post-infection (Fig. 1C). Thus, osmotic laxative treatment alone was sufficient
89 to render mice susceptible to prolonged *C. difficile* colonization and PEG-treated mice remained
90 susceptible up to ten days post-treatment.

91 **5-day laxative treatment differentially disrupts the fecal microbiota compared to**
92 **clindamycin treatment.** Since osmotic laxatives and clindamycin have previously been
93 shown to disrupt the murine microbiota (14–17), we hypothesized the different *C. difficile*
94 colonization dynamics between mice treated with the osmotic laxative or clindamycin were due to
95 the two drugs having differential effects on the microbiota. We profiled the stool microbiota over
96 time by sequencing the V4 region of the 16S rRNA gene to compare changes across treatment
97 groups. We found time and treatment group explained half of the observed variation between fecal
98 communities with most of the remaining variation explained by interactions between treatment
99 group and other experimental variables including time, cage effects, and sequencing preparation
100 plate (PERMANOVA combined $R^2 = 0.95$, $P < 0.001$, Fig. 2A, Data Set S1, Sheet X). Cage
101 effects refer to the well-documented phenomenon that mice housed in the same cages have
102 similar microbial communities due to coprophagy (27). We tried to minimize the impact of cage
103 effects on our experiment by breaking up cagemates when assigning mice to treatment groups
104 and primarily housing only two mice per cage. Importantly, although we conducted a total of 5
105 separate experiments, the experiment number and its interaction with treatment group was not one
106 of the variables that significantly explained the observed variation in fecal communities (Data Set
107 S1, Sheet X). Interestingly, none of the treatment groups recovered to their baseline community
108 structure either 10 or 30 days post-infection suggesting other community features besides recovery
109 to baseline were responsible for the prolonged *C. difficile* colonization in PEG-treated mice (Fig.
110 2B).

111 Next, we examined alpha diversity by looking at Shannon diversity over time. Although both
112 clindamycin and PEG treatments decreased diversity, Shannon diversity was lower in the groups of
113 mice that received PEG treatment compared to those that received clindamycin through thirty days
114 post-infection (Fig. 2C). We next examined the bacterial genera that shifted after PEG treatment by

115 comparing the baseline samples of mice treated with only PEG to samples from the same mice one
116 day post-treatment. We found 18 genera that were altered by PEG treatment (Data Set S1, Sheet
117 X). The majority of the bacterial relative abundances decreased after PEG, but *Enterobacteriaceae*
118 and *Bacteroides* increased and the increase in *Bacteroides* was unique to PEG treatment, as
119 *Bacteroides* actually decreased in clindamycin treated mice (Fig. 2D). Finally, we examined the
120 bacteria that differed across treatment groups over multiple time points. We found 24 genera were
121 different over multiple time points out of the 33 genera that were different between treatment groups
122 (Fig. 2E, Data Set S1, Sheet X). Thus, PEG had a significant impact on the fecal microbiota that
123 was maintained over time and was distinct from clindamycin treatment.

124 Interestingly, *C. difficile* was not immediately detectable in the stools of the PEG-treated mice
125 that were allowed to recover for 10 days prior to challenge. We decided to examine the
126 bacteria that changed during the post-infection period when the group median *C. difficile* shifted
127 from undetectable at 1 day post-infection to detectable in the stool samples with the median
128 stabilizing around 8 days post-infection (Fig. S1A). Interestingly, we found *Erysipelotrichaceae*,
129 *Enterobacteriaceae*, and *Akkermansia* were changing during the time period when *C. difficile* was
130 becoming detectable in the stools (Fig. S1B), although none of the bacteria were significant after
131 multiple hypothesis correction (Data Set S1, Sheet X). While we did not identify a clear signal to
132 explain the delayed appearance of *C. difficile* in the 5-day PEG mice that were allowed to recover
133 for 10 days prior to challenge, the delay is striking and could reflect changes in microbial activity or
134 metabolites that were not examined in this study.

135 **5-day laxative treatment does not promote more severe CDIs despite altering the mucosal**
136 **microbiota.** Given the findings from a previous study that demonstrated PEG treatment disrupts
137 the mucus layer and alters the immune response in mice (16), we decided to examine the impact of
138 PEG treatment on the mucosal microbiota and CDI severity. To evaluate the mucosal microbiota,
139 we sequenced snips of tissue collected from the cecum, proximal colon, and distal colon. Similar
140 to what was observed with the stool samples, alpha diversity was lower in the PEG-treated mice
141 compared to clindamycin treatment (Fig. 3A). Although alpha diversity continued to increase over
142 time based on the communities from PEG-treated mice collected at 20 and 30 days post-infection
143 (Fig. 3A, Data Set S1, Sheet X). Group, time point, and their interactions with other variables

144 (cage, experiment number, and sample type) explained the majority of the variation observed in
145 mucosal communities (PERMANOVA combined $R^2 = 0.83$, $P < 0.05$, Fig. 3B, Data Set S1, Sheet
146 X). We saw the greatest difference in the mucosal microbiota between treatment groups at 6 days
147 post-infection with 16 genera that were significantly different in all three of the tissue types we
148 collected (cecum, proximal colon, and distal colon; Fig. S3A, Data Set S1, Sheet X). However, at
149 30 days post-infection only *Bacteroides*, *Clostridiales*, *Firmicutes*, and *Ruminococcaceae* were
150 different between treatment groups and only in the cecum tissues (Fig. 3C, Fig. 2E, Data Set S1,
151 Sheet X). Interestingly, one of the bacteria that was different between treatment groups at 6 days
152 post-infection was *Peptostreptococcaceae* (the genera that contains a sequence that matches *C.*
153 *difficile*). *Peptostreptococcaceae* was primarily only present in the 5-day PEG treatment group
154 of mice and decreased in the proximal and distal colon tissues over time (Fig. S2B). Thus, PEG
155 treatment had a significant impact on the mucosal microbiota and we detected *C. difficile* sequences
156 in the cecum, proximal colon, and distal colon tissue communities.

157 Next, we examined CDI severity by evaluating cecum and colon histopathology (28) and found there
158 was no difference in cecum and colon scores between clindamycin and PEG-treated mice that were
159 challenged with *C. difficile* at 4 days post-infection (Fig. 3D), the time point typically examined in
160 *C. difficile* 630 challenged mice (29). We also looked at 6 days post-infection because that was
161 when we started to see a large difference in *C. difficile* colonization levels between PEG- and
162 clindamycin-treated mice (Fig. 1C). Although, there was a slight difference in the colon between
163 PEG and clindamycin-treated mice, there was no difference in the cecum and the overall score
164 is still relatively low given that the max possible summary score is twelve (Fig. 3E). Therefore,
165 although PEG treatment had a disruptive effect on the mucosal microbiota, the impact of *C. difficile*
166 630 infection on the cecum and colon was similar between PEG and clindamycin treated mice.

167 ***C. difficile* challenge does not have a synergistic disruptive effect on the microbiota of**
168 **PEG-treated mice**

Because *C. difficile* itself can have an impact on the microbiota (30), we also
sequenced the tissue and stools of mock-challenged clindamycin and 5-day PEG treated mice.
Examining the stools of the mock-challenged mice revealed similar bacterial disruptions as the *C.*
difficile challenged mice (Fig. S2A-C). Similarly, there was no difference between the tissues of
mock and *C. difficile* challenged mice (Fig. S2D-F). Thus, most of the microbiota alterations we

173 observed in the PEG-treated mice were a result of the laxative and not an interaction between the
174 laxative and *C. difficile*.

175 **1-day laxative treatment results in transient *C. difficile* colonization and minor microbiota**
176 **disruption** Next, we decided to examine how a shorter osmotic laxative perturbation would impact
177 the microbiome and susceptibility to *C. difficile*. We administered either a 1-day PEG treatment,
178 a 1-day PEG treatment with a 1-day recovery period, or clindamycin to mice before challenging
179 them with *C. difficile* (Fig. 3A). In contrast to the 5-day PEG treated mice, the 1-day PEG treated
180 mice were only transiently colonized and cleared *C. difficile* by 7 days post-infection (Fig. 3B). The
181 stool communities of PEG-treated mice were also only transiently disrupted, with Shannon diversity
182 recovering close to baseline by 7 days post-infection (Fig. 3C-D). We found 14 bacteria were
183 impacted by treatment, but recovered close to baseline levels by 7 days post-infection including
184 *Enterobacteriaceae*, *Clostridiales*, *Porphyromonadaceae*, and *Ruminococcaceae* (Fig. 3E, Data
185 Set S1, Sheet X). These findings suggest the duration of treatment matters when considering the
186 impact of the laxatives, with shorter treatments resulting in a transient loss of *C. difficile* colonization
187 resistance.

188 **Post-CDI laxative treatment disrupts clearance in clindamycin-treated mice regardless of**
189 **whether an FMT is also administered** Since 1-day PEG treatment resulted in a more mild
190 microbiota perturbation, we decided to use the 1-day treatment to examine the hypothesis that
191 PEG helps to flush *C. difficile* spores from the intestine. We were also interested in exploring
192 whether PEG might help with engraftment in the context of FMTs. To examine the impact of PEG
193 treatment on *C. difficile* clearance and FMT treatment, we treated 4 groups of mice with clindamycin
194 and then challenged all mice with *C. difficile* before administering the following treatments: no
195 additional treatment, 1-day PEG immediately after challenge, and 1-day PEG treatment 3 days after
196 challenge followed by either administration of an FMT or PBS solution by oral gavage (Fig. 5A).
197 Contrary to our hypothesis, all groups of mice that received PEG exhibited prolonged *C. difficile*
198 colonization (Fig. 5B). Next we examined how post-CDI PEG treatment impacted the microbiota.
199 Alpha diversity was lower in the PEG-treated mice with the exception of the PEG-treated mice that
200 were administered an FMT (Fig 5C-D). The FMT appeared to partially restore Shannon diversity
201 but not richness (Fig. 5C-D). Similarly, we saw some overlap between the communities of mice that

202 received FMT and the mice treated with only clindamycin after 5 days post-infection (Fig. 6A). The
203 increase in Shannon diversity suggests that the FMT did have an impact on the microbiota, despite
204 seeing prolonged *C. difficile* colonization in the FMT treated mice. However, only *Bacteroidales*
205 and *Porphyromonadaceae* consistently differed between the mice received either an FMT or PBS
206 gavage (Fig. 6B), suggesting the FMT only restored a couple of genera. Overall, we found
207 there were 24 bacteria that differed over multiple time points between treatment groups including
208 increased *Akkermansia* and decreased *Ruminococcaceae*, *Clostridiales*, *Lachnospiraceae*, and
209 *Oscillibacter* in mice that received PEG after *C. difficile* challenge (Fig. 6C). In sum, administering
210 PEG actually prolonged *C. difficile* colonization, including in mice that received an FMT, which only
211 restored a couple of bacterial genera.

212 **Five-day post-infection community data can predict which mice that will have prolonged *C.***
213 ***difficile* colonization** After identifying bacteria associated with the 5-day, 1-day and post-CDI
214 1-day PEG treatments, we decided to examine the bacteria that influenced prolonged *C. difficile*
215 colonization. We trained 3 types of machine learning models (random forest, logistic regression,
216 and support vector machine) with input bacterial community data from 5 days post-infection to
217 predict whether the mice were still colonized with *C. difficile* 10 days post-infection. We chose
218 5 days post-infection because that was the earliest time point where we would see a treatment
219 effect in the mice that were given 1-day PEG treatment three days after *C. difficile* challenge and
220 then administered an FMT or PBS gavage. The random forest model had the highest performance
221 (AUROC = 0.90, Data Set S1, Sheet X), so we next performed permutation importance to examine
222 the bacteria that were the top contributors to the random forest model predicting prolonged *C.*
223 *difficile* colonization. We selected the top 10 bacteria contributing to our models performance
224 (Fig. 7A) and examined their relative abundance at 5 days post-infection, the time point used to
225 predict *C. difficile* colonization status on day 10 (Fig. 7B). Next, we focused on the 5 genera that
226 had a greater than 1 % relative abundance in either the cleared or colonized mice and examined
227 how the bacteria changed over time. We found *Enterobacteriaceae* and *Bacteroides* tended to
228 have a higher relative abundance, *Akkermansia* was initially decreased and then increased, and
229 *Porphyromonadaceae* and *Lachnospiraceae* had a lower relative abundance in the mice with
230 prolonged colonization compared to the mice that cleared *C. difficile* (Fig. 7C). Together these

231 results suggest a combination of low and high abundance bacterial genera influence the prolonged
232 colonization observed in 5-day PEG and post-CDI PEG treated mice.

233 Previous work examining the impact of PEG on the murine microbiota found that PEG treatment
234 resulted in the permanent loss of S24-7, also known as *Muribaculum intestinale* (16). We decided
235 to check our *Porphyromonadaceae* operational taxonomic units (OTUs) because *Muribaculum*
236 *intestinale* is classified as *Porphyromonadaceae* by the Ribosomal Database Project (RDP)
237 database (31) and *Porphyromonadaceae* was a top feature in the random forest model predicting
238 prolonged *C. difficile* colonization. We identified 4 OTUs that had at least 92% identity to
239 *Muribaculum intestinale* and examined their abundance in mice that either cleared or were still
240 colonized with *C. difficile* at 10 days post-infection. While all of the OTUs, were decreased by
241 PEG and clindamycin treatment, there was some recovery in the mice that cleared (Fig. S4A).
242 We also examined other *Porphyromonadaceae* and *Lachnospiraceae* OTUs since these were the
243 2 genera that were important to our classification model and contained multiple OTUs that were
244 different at 5 days post-infection between mice that either cleared or remained colonized with *C.*
245 *difficile* by 10 days post-infection (Data Set S1, sheet X). While individual *Porphyromonadaceae*
246 and *Lachnospiraceae* OTUs tended to be more abundant in the mice that clear *C. difficile* relative
247 to the mice that exhibit prolonged colonization (Fig. S4B-C), there is no single OTU that fits the
248 pattern we observed at the genus level (Fig. 7C), suggesting multiple *Porphyromonadaceae* and
249 *Lachnospiraceae* OTUs influenced *C. difficile* clearance. Overall, our results suggest that specific
250 bacterial community differences explain the prolonged *C. difficile* colonization we observed in 5-day
251 PEG and post-CDI 1-day PEG treated mice.

252 Discussion

- 253 • Summary of major findings (Fig. 8A)
- 254 • Discussion of prolonged persistence. *C. difficile* sequences detected in tissue samples.
255 Association with mucin-degrading bacteria suggested by recent papers.
- 256 • Discuss why we might not have observed more severe histology in PEG mice relative to
257 clindamycin-treated mice

- 258 – Antibiotics may also impact mucus layer
- 259 – Strain of bacteria used
- 260 • Protective bacteria missing in PEG-treated mice
- 261 • Discuss what these findings might mean for human patients (Fig. 8B)
- 262 – What's known regarding laxatives and susceptibility to CDIs
- 263 – Clinical trial of PEG, results never posted (32)
- 264 – Relevance to human FMTs? Unclear what the best administration route is because there
- 265 have been no studies designed to evaluate the best administration route for FMTs.

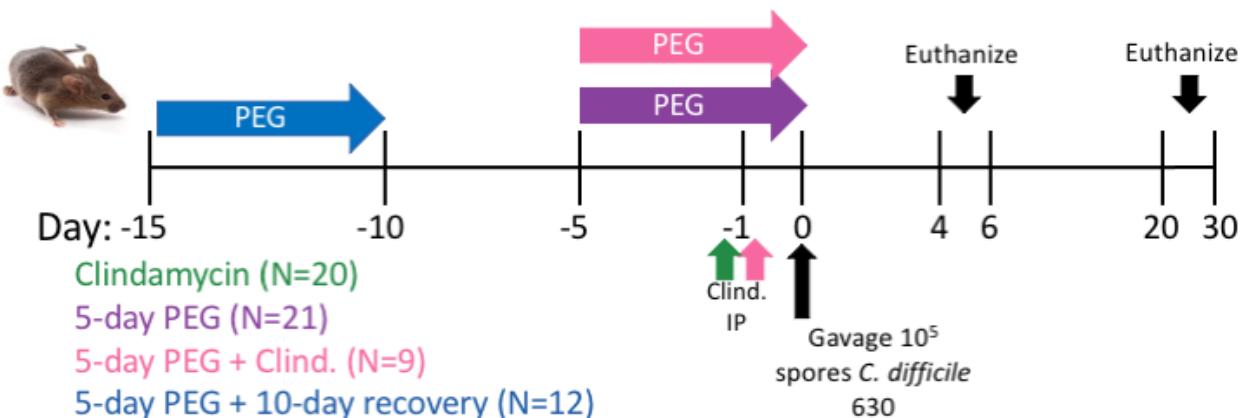
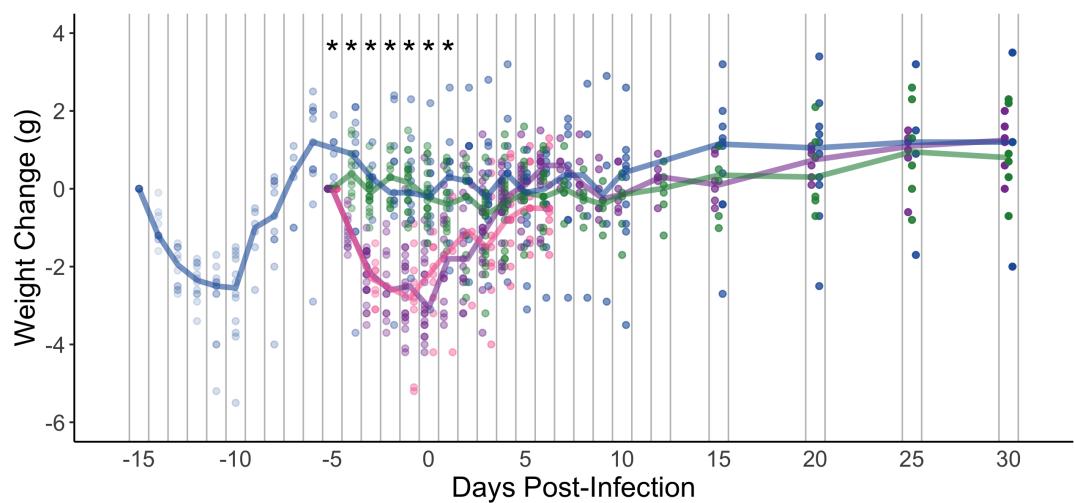
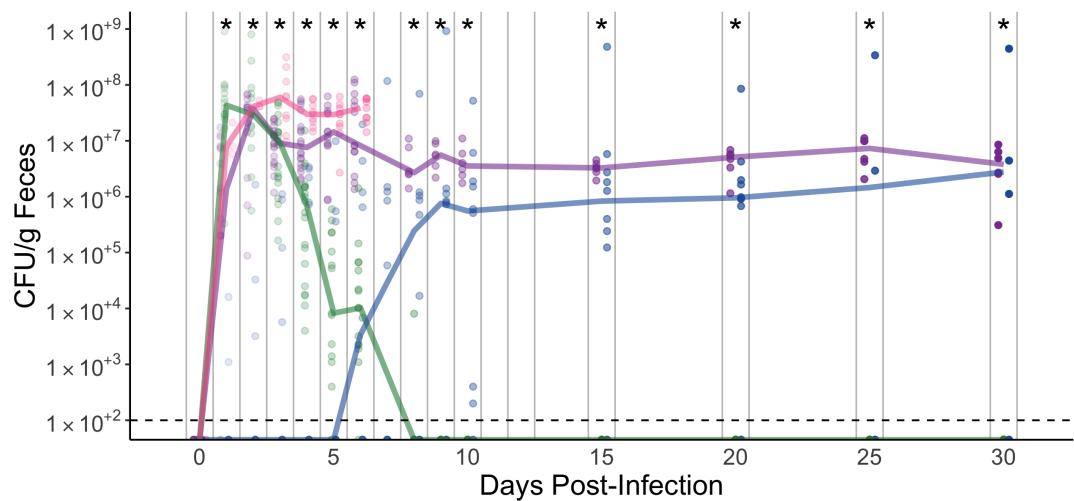
266 **Conclusions**

267 **Acknowledgements**

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278 **Materials and Methods**

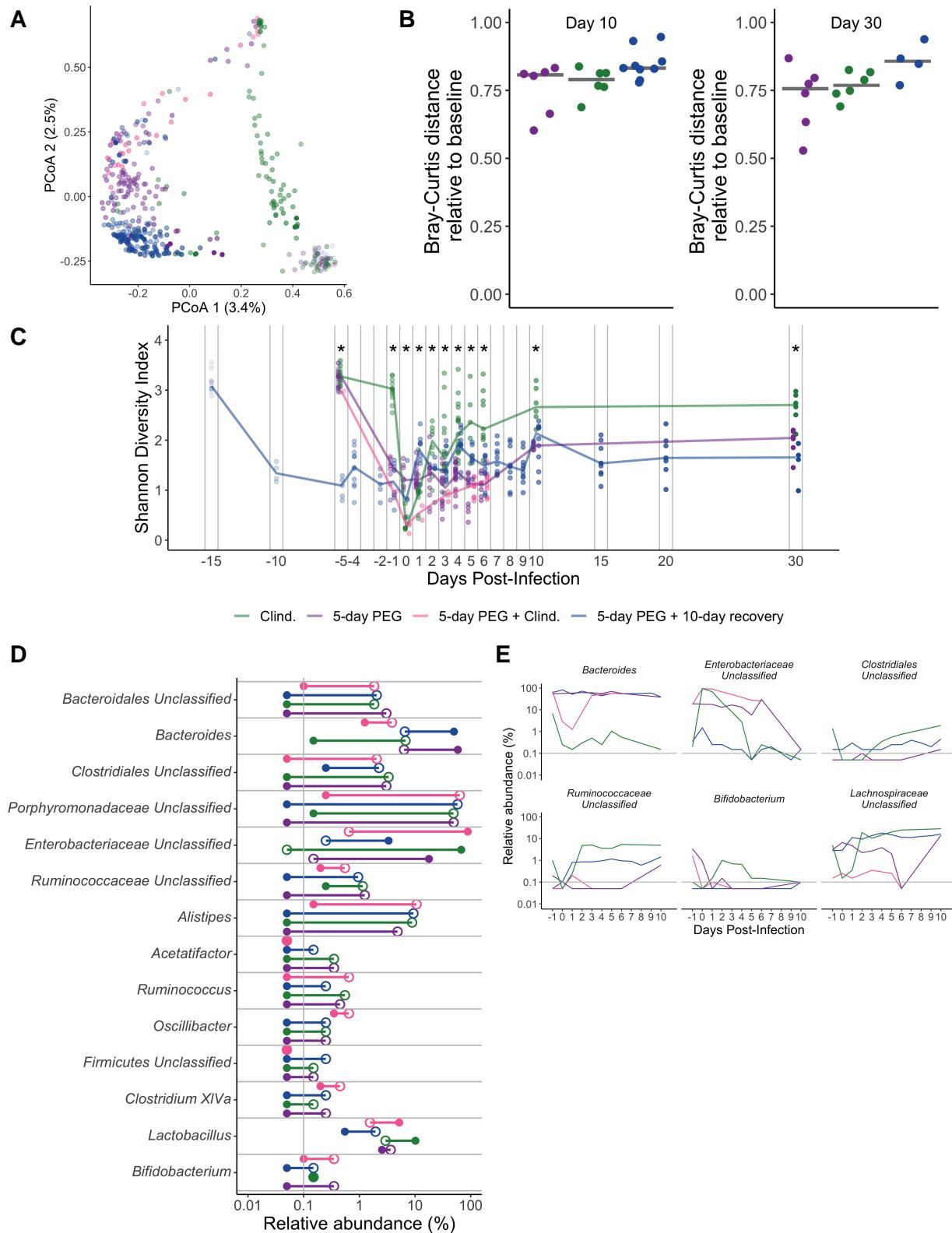
- 279 • Histopathology (33)

A**B****C**

280

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

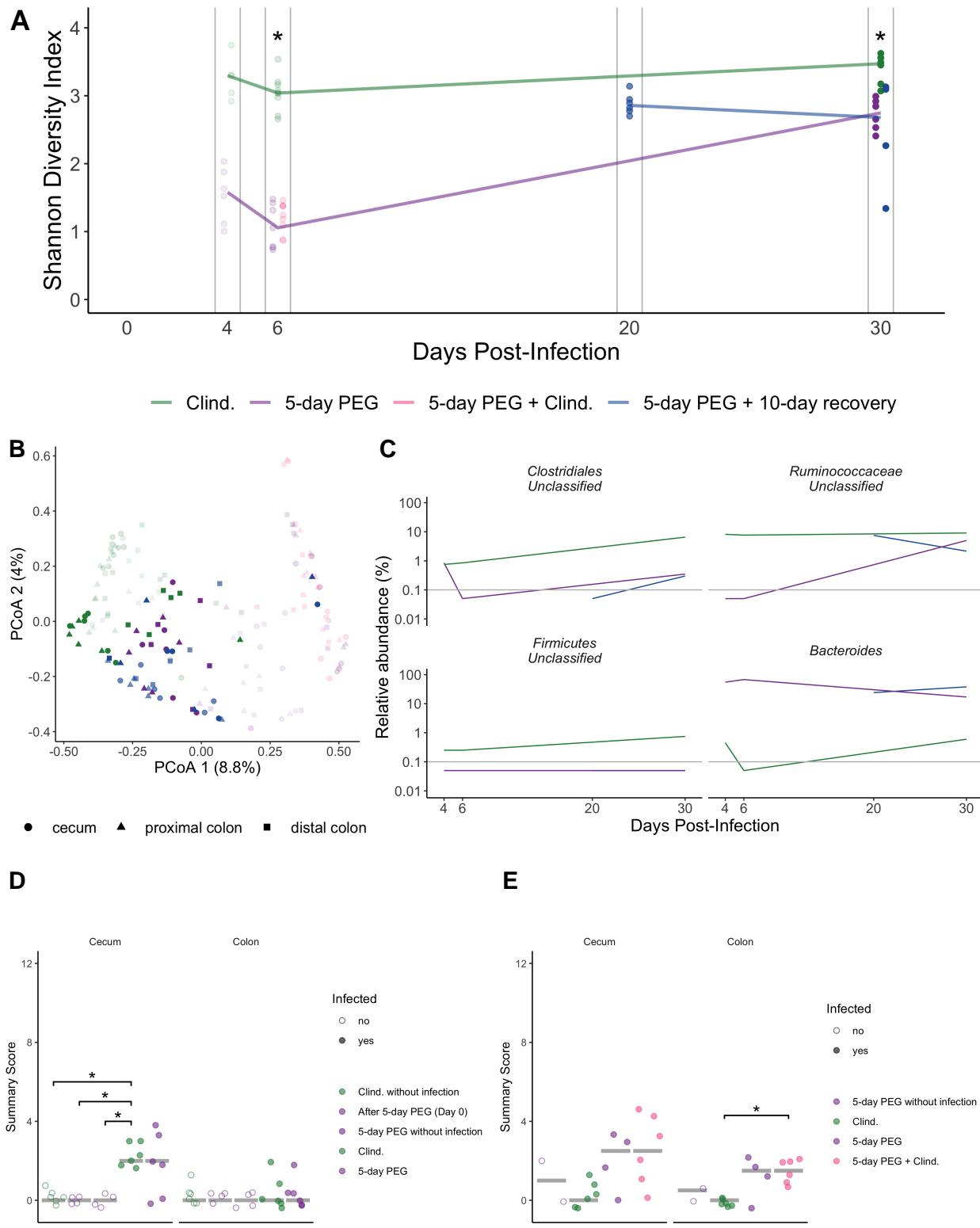
284 by intraperitoneal injection. 2. 15% PEG 3350 was administered in the drinking water for five
285 days. 3. 5-day PEG plus clindamycin treatment. 4. 5-day PEG plus 10-day recovery treatment. All
286 treatment groups were then challenged with 10^5 *C. difficile* 630 spores. A subset of mice were
287 euthanized on either 4 or 6 days post-infection and tissues were collected for histopathology
288 analysis, the remaining mice were followed through 20 or 30 days post-infection. B. Weight change
289 from baseline weight in groups after treatment with PEG and/or clindamycin, followed by *C. difficile*
290 challenge. C. *C. difficile* CFU/gram stool measured over time (N = 16-59 mice per time point) via
291 serial dilutions. The black line represents the limit of detection for the first serial dilution. CFU
292 quantification data was not available for each mouse due to stool sampling difficulties (particularly
293 the day the mice came off of the PEG treatment) or early deaths. For B-C, lines represent the
294 median for each treatment group and circles represent samples from individual mice. Asterisks
295 indicate time points where the weight change or CFU/g was significantly different between groups
296 by the Kruskal-Wallis test with Benjamini-Hochberg correction for testing multiple time points. The
297 data presented are from a total of 5 separate experiments.



298

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

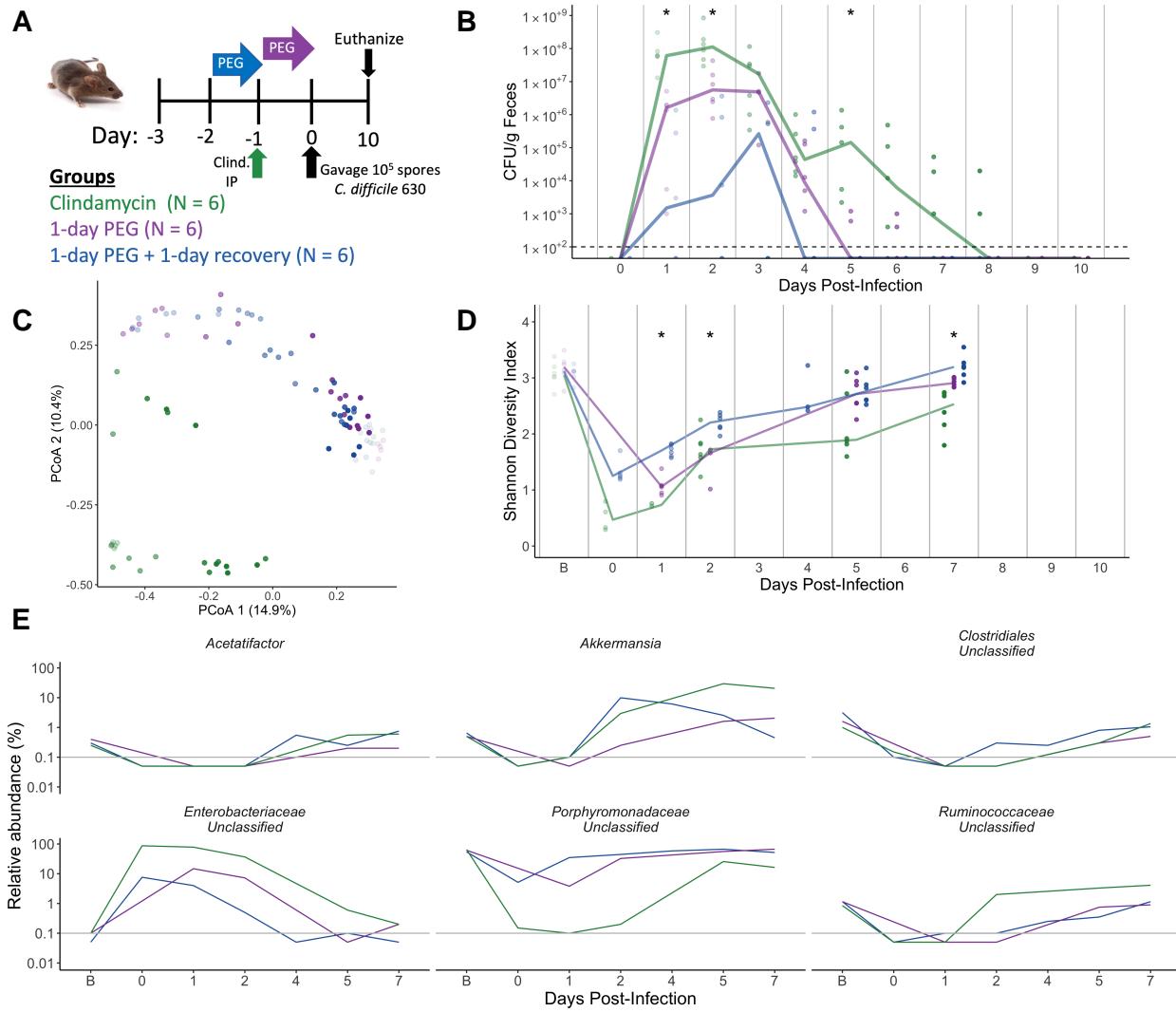
300 **compared to clindamycin-treated mice.** A. Principal Coordinate analysis (PCoA) of Bray-Curtis
301 distances from stool samples collected throughout the experiment. For A and C, each circle
302 represents a sample from an individual mouse and the transparency of the symbol corresponds to
303 the day post-infection. B. Bray-Curtis distances of stool samples collected on either day 10 or 30
304 post-infection relative to the baseline sample collected for each mouse (before any drug treatments
305 were administered). C. Shannon diversity in stool communities over time. The line indicates the
306 median value for each treatment group. The colors of the symbols and lines represent the four
307 treatment groups. D. 14 of the 33 genera affected by PEG treatment (Data Set S1, sheet X). The
308 symbols represent the median relative abundance for a treatment group at either baseline (open
309 circle) or 1-day post treatment (closed circle). Data from the 5-day PEG and 5-day PEG plus 10-day
310 recovery groups were analyzed by paired Wilcoxon signed-rank test with Benjamini-Hochberg
311 correction for testing all identified genera. The clindamycin and 5-day PEG plus clindamycin
312 treatment groups are shown for comparison. E. 6 of the 24 genera that were significantly different
313 between the four treatment groups over multiple time points. Differences between treatment groups
314 were identified by Kruskal-Wallis test with Benjamini-Hochberg correction for testing all identified
315 genera. The gray vertical line (D) and horizontal vertical lines (E) indicate the limit of detection.



316

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

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



334

335 **Figure 4. 1-day PEG treatment renders mice susceptible to transient *C. difficile***
 336 **colonization.** A. Setup of the experimental time line for the 1-day PEG treated mice
 337 consisting of 3 treatment groups. 1. Clindamycin was administered at 10 mg/kg by intraperitoneal
 338 injection. 2. 15% PEG 3350 was administered in the drinking water for 1 day. 3. 1-day PEG plus
 339 1-day recovery. The three treatment groups were then challenged with 10^5 *C. difficile* 630 spores.
 340 B. *C. difficile* CFU/gram stool measured over time (N = 12-18 mice per time point) via several
 341 dilutions. The black dotted line represents the limit of detection for the first serial dilution. Asterisks
 342 indicate time points where the CFU/gram was significantly different between treatment groups by
 343 Kruskall-Wallis test with Benjamini-Hochberg correction for testing multiple time points. C. PCoA
 344 of Bray-Curtis distances from stool communities collected from the three treatment groups over

345 time (day: $R^2 = 0.43$; group: $R^2 = 0.19$). D. Shannon diversity in stool communities over time
346 with colored lines representing the median value for each treatment group. For B-D, each symbol
347 represents a sample from an individual mouse and symbol transparency corresponds to the day
348 post-infection that the sample was collected. E. Median relative abundances per treatment group
349 for 6 out of the 14 genera that were affected by treatment, but recovered close to baseline levels by
350 7 days post-infection (Fig. 3E, Data Set S1, Sheet X). Stool samples from either baseline and day 1
351 or baseline and day 7 were analyzed by paired Wilcoxon signed-rank test with Benjamini-Hochberg
352 correction for testing all identified genera. The gray horizontal line represents the limit of detection.

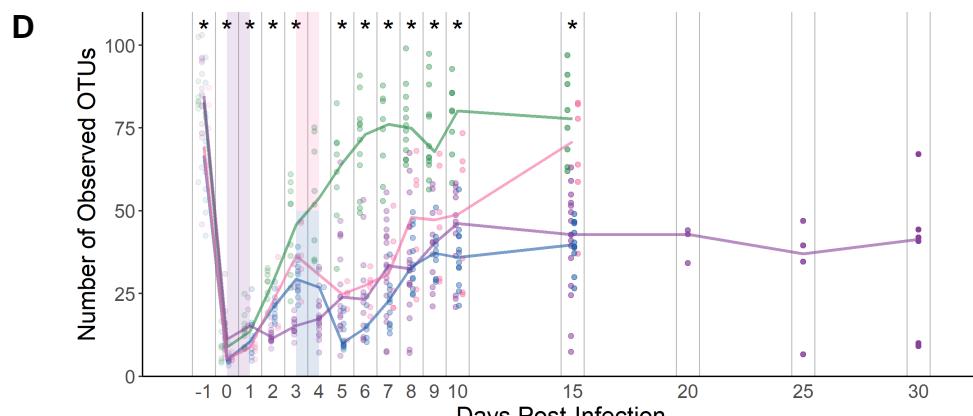
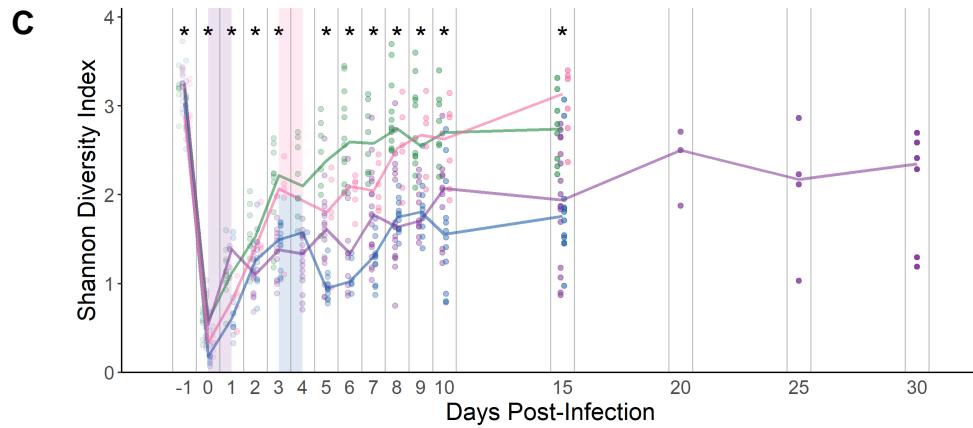
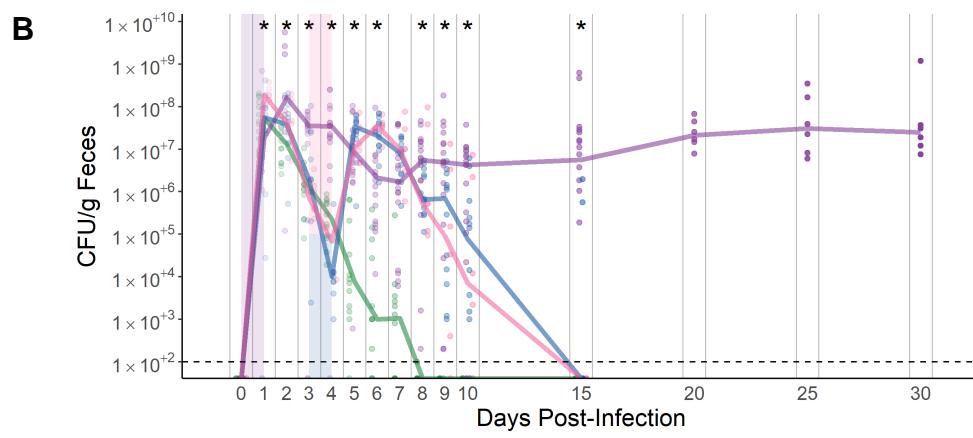
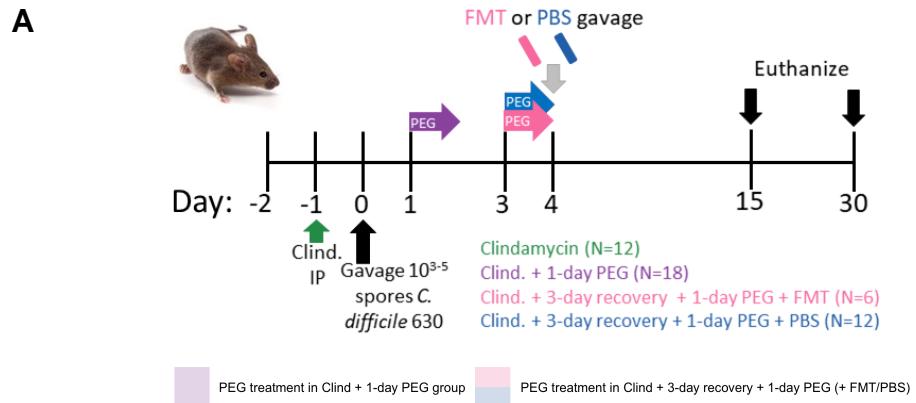
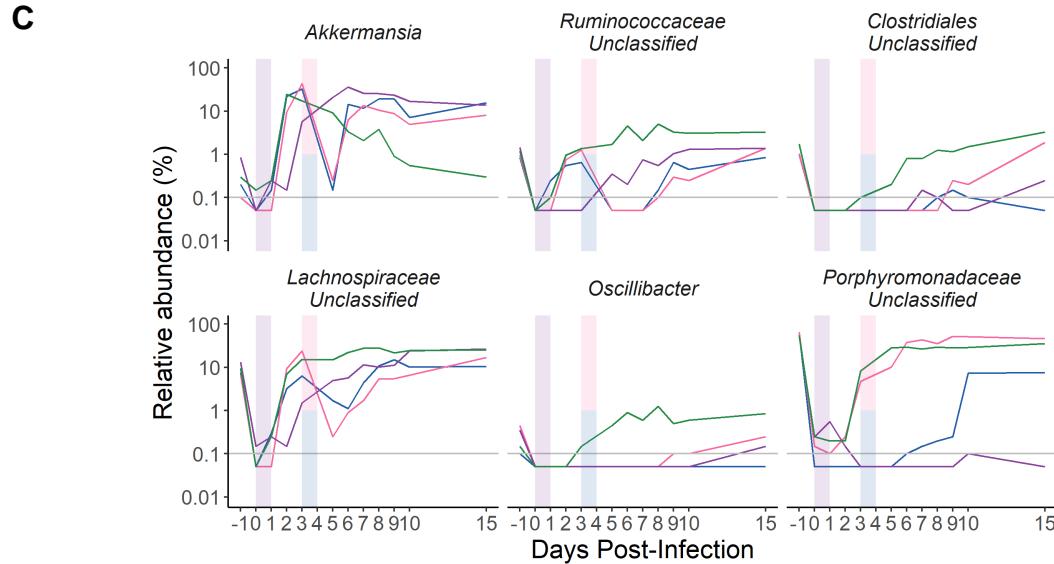
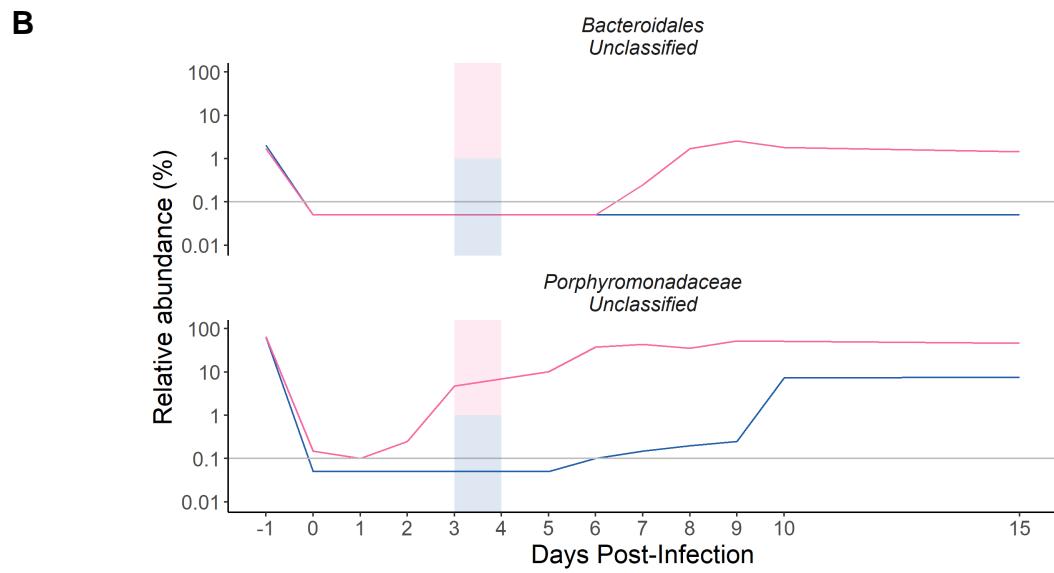
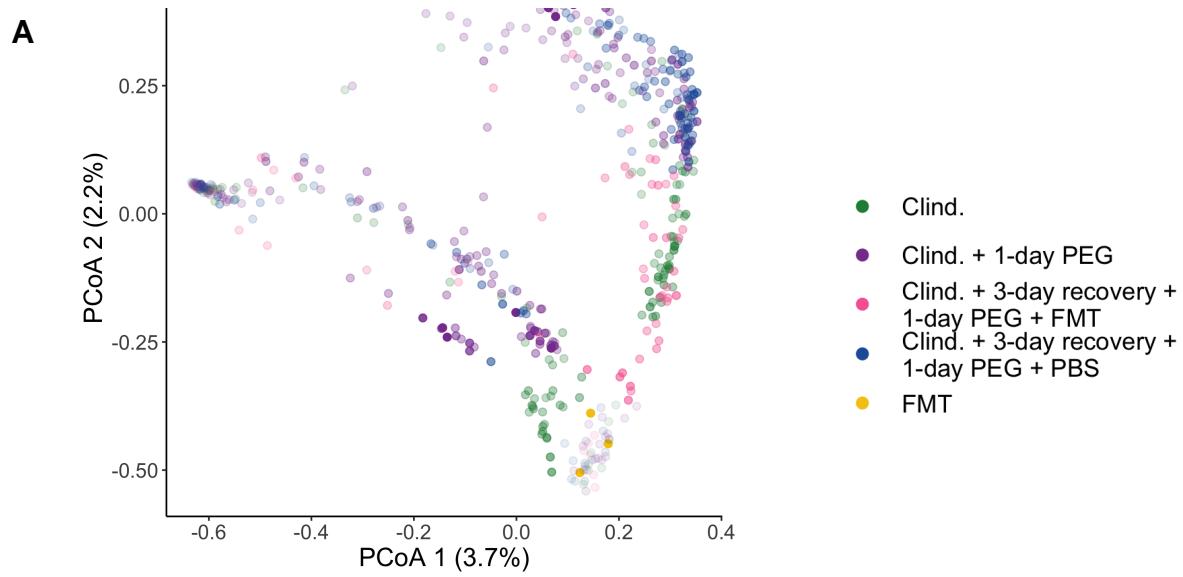
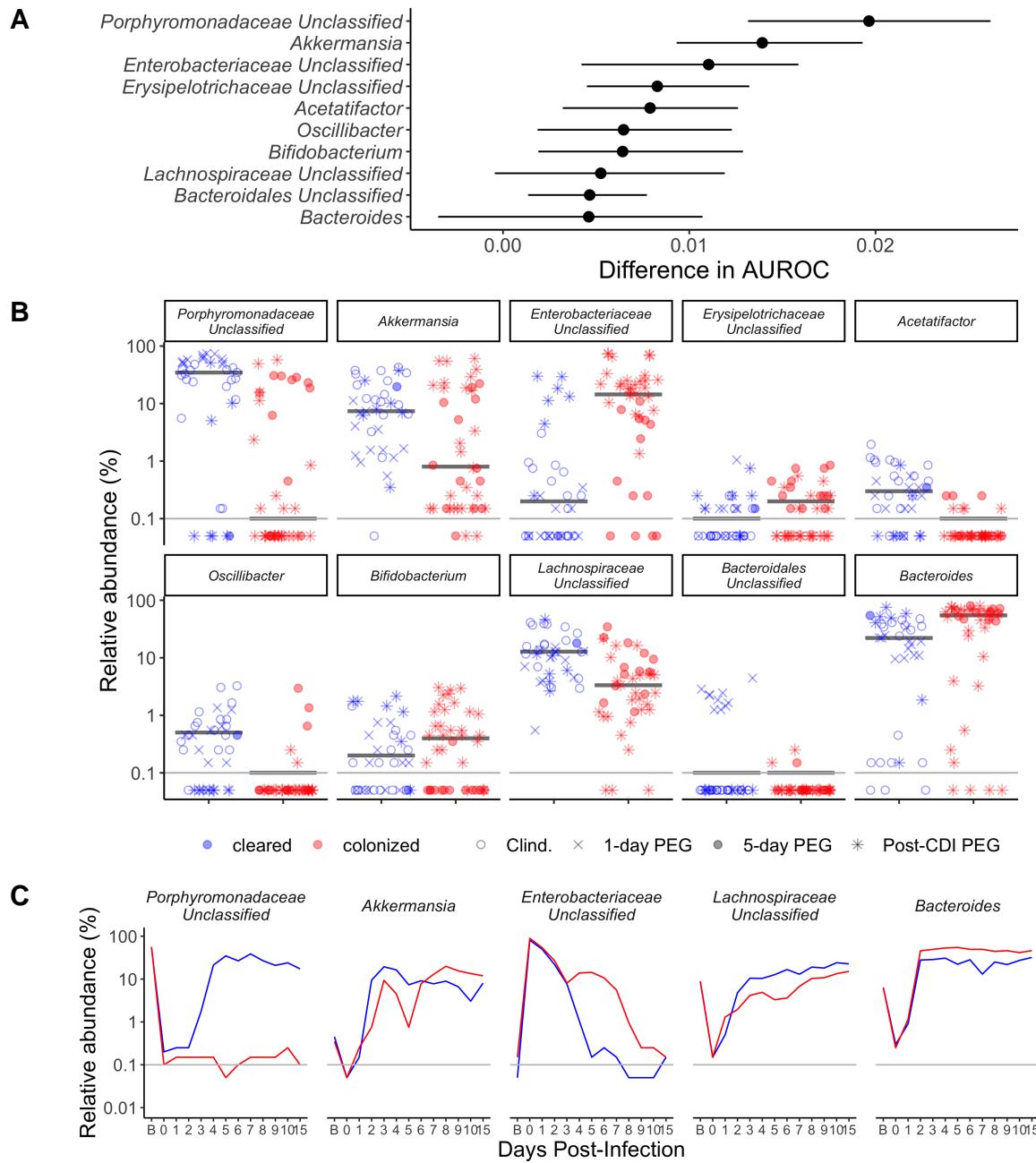


Figure 5.

354 **1-day PEG treatment post *C. difficile* challenge prolongs colonization regardless of**
355 **whether an FMT is also administered.** A. Setup off the experimental time line for experiments
356 with post-CDI PEG treated mice. There were a total of 4 different treatment groups. All mice were
357 administered 10 mg/kg clindamycin intraperitoneally (IP) 1 day before challenge with 10^{3-5} *C.*
358 *difficile* 630 spores. 1. Did not receive any additional treatment. 2. Immediately after *C. difficile*
359 challenge, mice received 15% PEG 3350 in the drinking water for 1 day. 3-4. 3-days after challenge,
360 mice received 1-day PEG treatment and then received either a fecal microbiota transplant (3) or
361 PBS (4) solution by oral gavage. Mice were followed through 15-30 days post-infection (only the
362 post-CDI 1-day PEG group was followed through 30 days post-infection). B. CFU/g of *C. difficile*
363 stool measured over time via serial dilutions. The black line represents the limit of detection for the
364 first serial dilution. C-D. Shannon diversity (C) and richness (D) in stool communities over time.
365 B-D. Each symbol represents a stool sample from an individual mouse with the lines representing
366 the median value for each treatment group. The transparency of the symbol corresponds to the
367 day post-infection. Asterisks indicate time points with significant differences between groups by a
368 Kruskall-Wallis test with a Benjamini-Hochberg correction for testing multiple times points. Colored
369 rectangles indicates the 1-day PEG treatment period for applicable groups. The data presented are
370 from a total of 3 separate experiments.



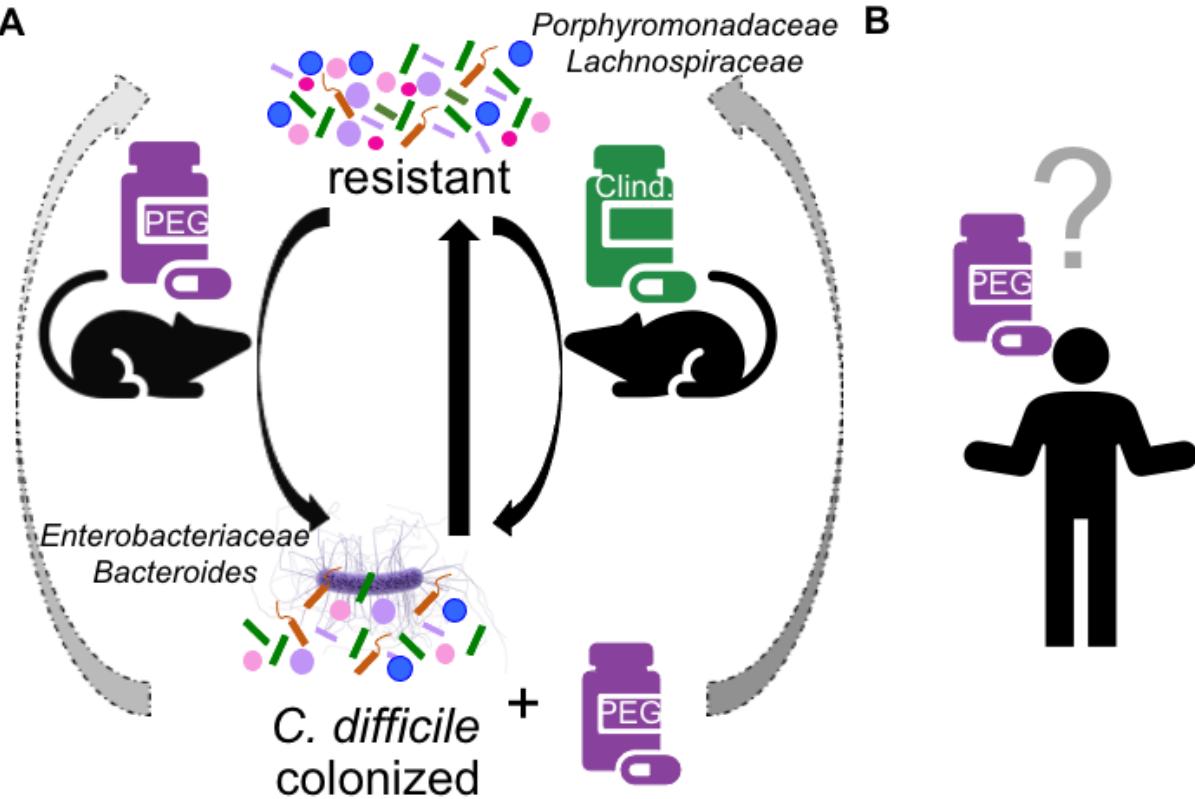
372 **Figure 6. For 1-day PEG treatment post *C. difficile* challenge mice that also receive an**
373 **FMT only some bacterial genera were restored.** A. PCoA of Bray-Curtis distances from stool
374 samples collected over time as well as the FMT solution that was administered to one treatment
375 group.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



382

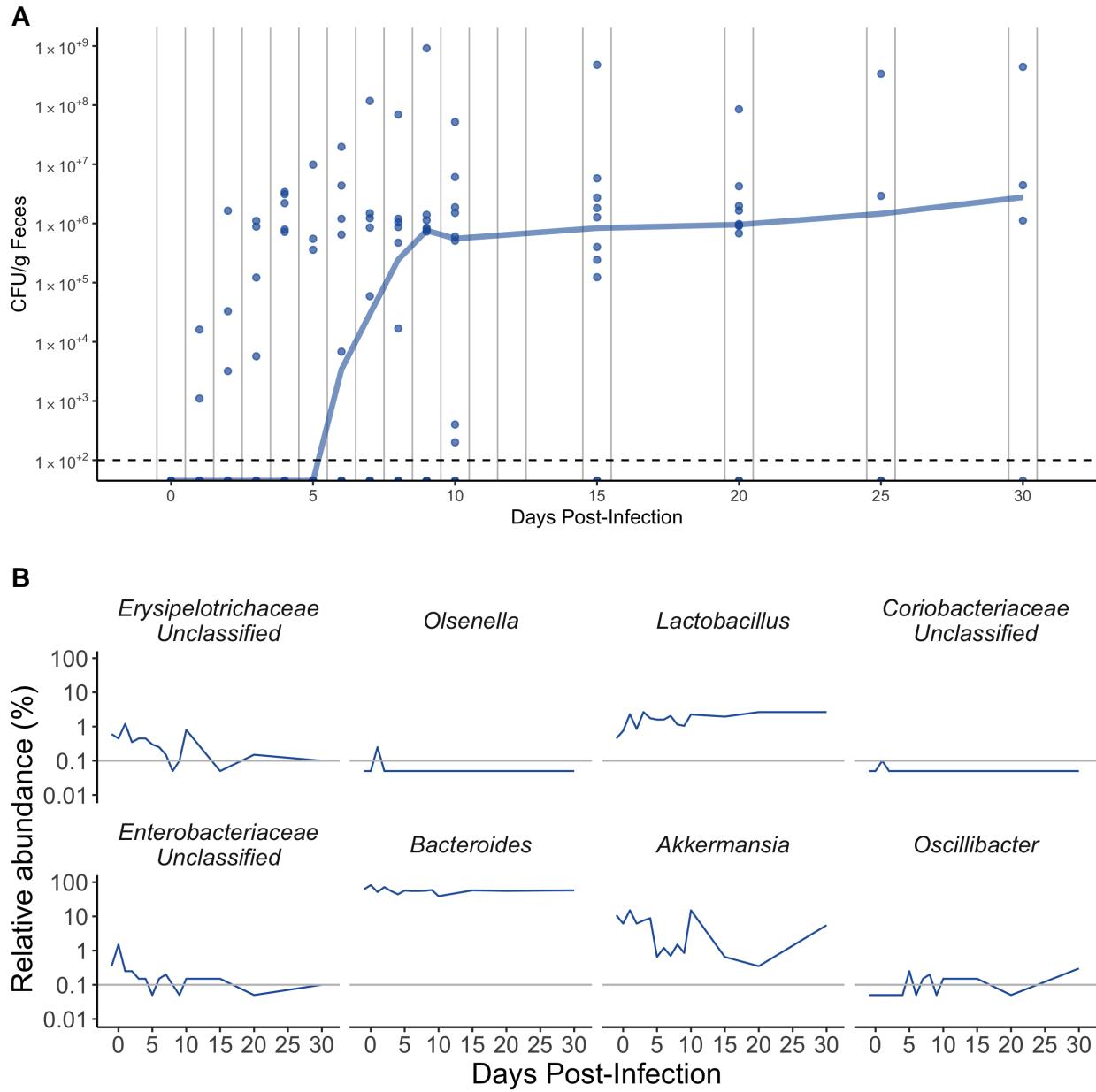
383 **Figure 7. Specific microbiota features associated with prolonged *C. difficile* colonization**
 384 **in PEG treated mice.** A. Top ten bacteria that contributed to the random forest model trained
 385 on five day post-infection community relative abundance data to predict whether mice would still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

390 days post-infection and the black horizontal line represents the median relative abundance. Each
391 symbol represents a stool sample from an individual mouse and the shape of the symbol indicates
392 whether the PEG-treated mice received a 5-day (Fig. 1-3), 1-day (Fig. 4) or post-CDI PEG (Fig.
393 5-6) treatment. C. The median relative abundances of the 5 genera with greater than 1% median
394 relative abundance in the stool community over time. For B-C, the gray horizontal line represents
395 the limit of detection.



396

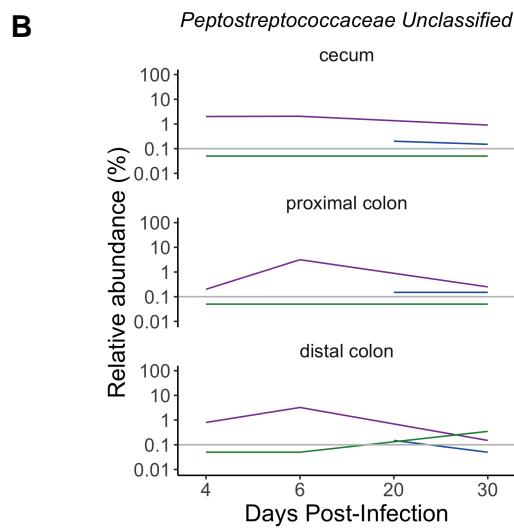
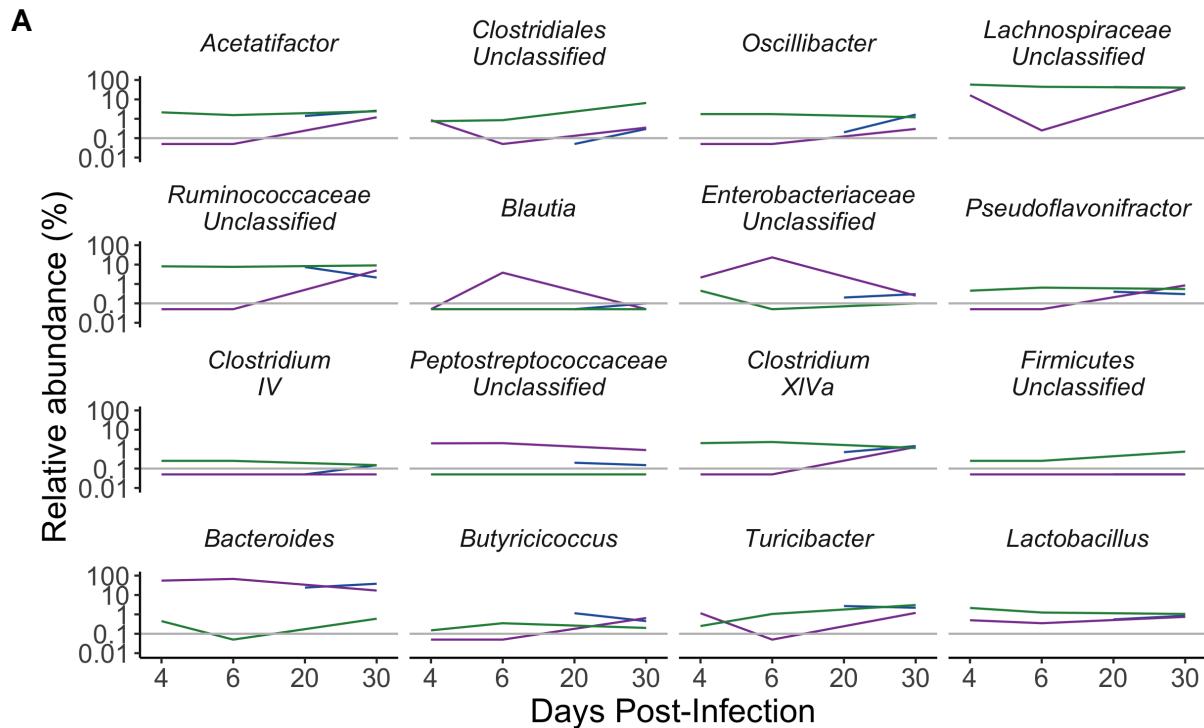
397 **Figure 8. Schematic summarizing findings.** A. The gut microbiota of our C57Bl/6 mice is resistant
 398 to *C. difficile* but treatment with either the antibiotic, clindamycin, or the osmotic laxative, PEG 3350
 399 renders the mice susceptible to *C. difficile* colonization. Recovery of colonization resistance in
 400 clindamycin-treated mice is relatively straightforward and the mice clear *C. difficile* within 10 days
 401 post-infection. However, for mice that received a 5-day PEG treatment or a 1-day PEG treatment
 402 after *C. difficile* challenge recovery of colonization resistance is more uncertain because mice were
 403 still colonized with *C. difficile* 30 days post-infection in the case of several PEG treatments. We
 404 found increased *Porphyromonadaceae* and *Lachnospiraceae* were associated with recovery of
 405 colonization resistance, while increased *Enterobacteriaceae* and *Bacteroides* were associated with
 406 prolonged *C. difficile* colonization. B. Considering that most hospitals recommend not performing
 407 *C. difficile* testing on patients taking laxatives and laxatives are used when administering fecal
 408 microbiota transplants via colonoscopy to patients with recurrent CDIs, further studies are needed
 409 to evaluate if laxatives impact human microbiota colonization resistance. Further studies are
 410 needed to understand the impact of osmotic laxatives on *C. difficile* colonization resistance and
 411 clearance in human patients.



412

413 **Figure S1. Microbiota dynamics post-infection in the 5-day PEG treatment plus 10-day**
 414 **recovery mice.** A. *C. difficile* CFU/g over time in the stool samples collected from 5-day PEG
 415 treatment plus 10-day recovery mice. Same data presented in Fig. 1C, but the data for the other 3
 416 treatment groups have been removed. B. Median relative abundances of 8 bacterial genera from
 417 day 0 post-infection onward from the 10-day recovery mice. We analyzed samples from day 0
 418 and day 8 post-infection, which represented the the time points where mice were challenged with
 419 *C. difficile* and when the median relative *C. difficile* CFU stabilized for the group using the paired

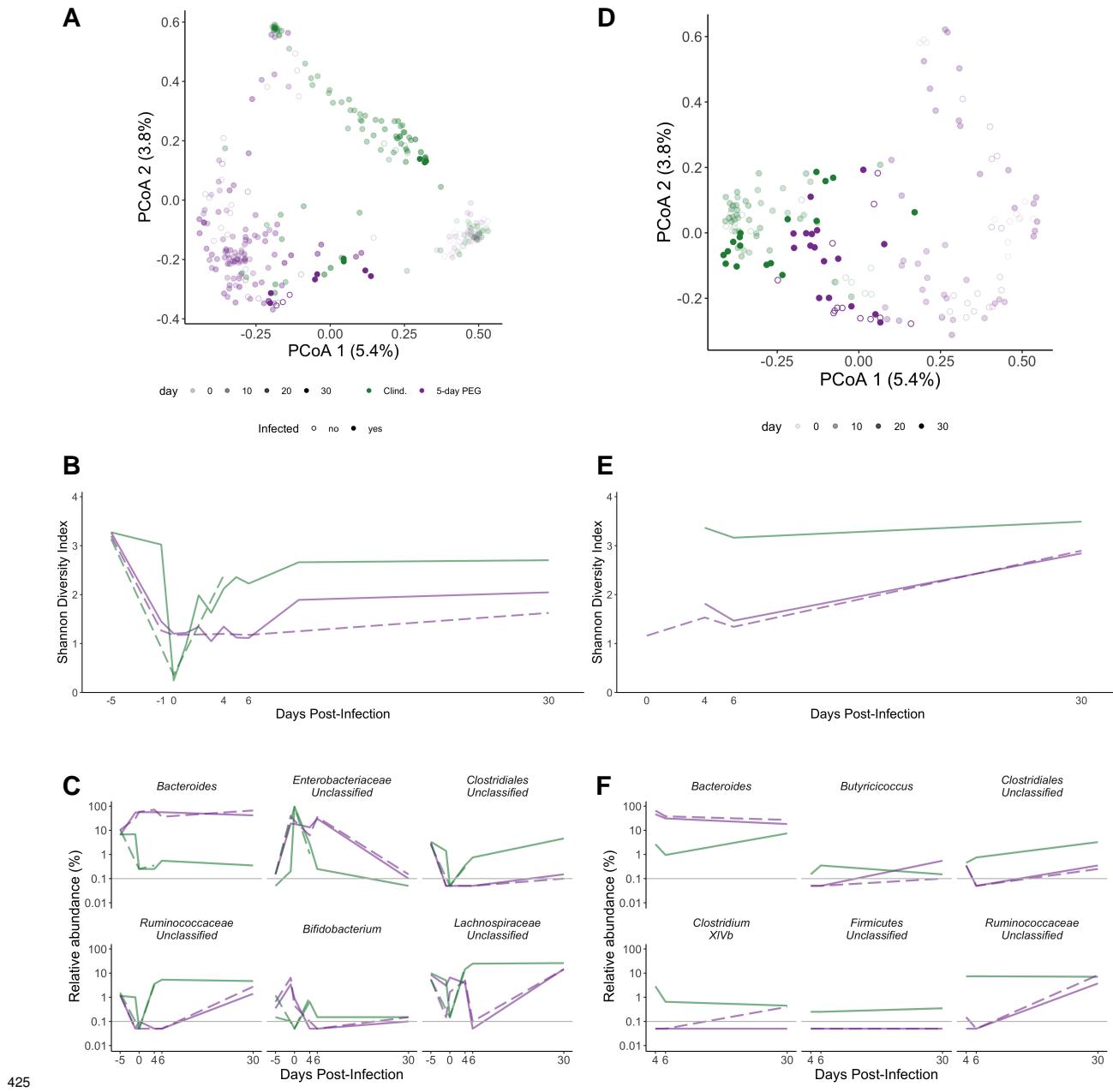
⁴²⁰ Wilcoxon signed-rank test, but no genera were significant after Benjamini-Hochberg correction.



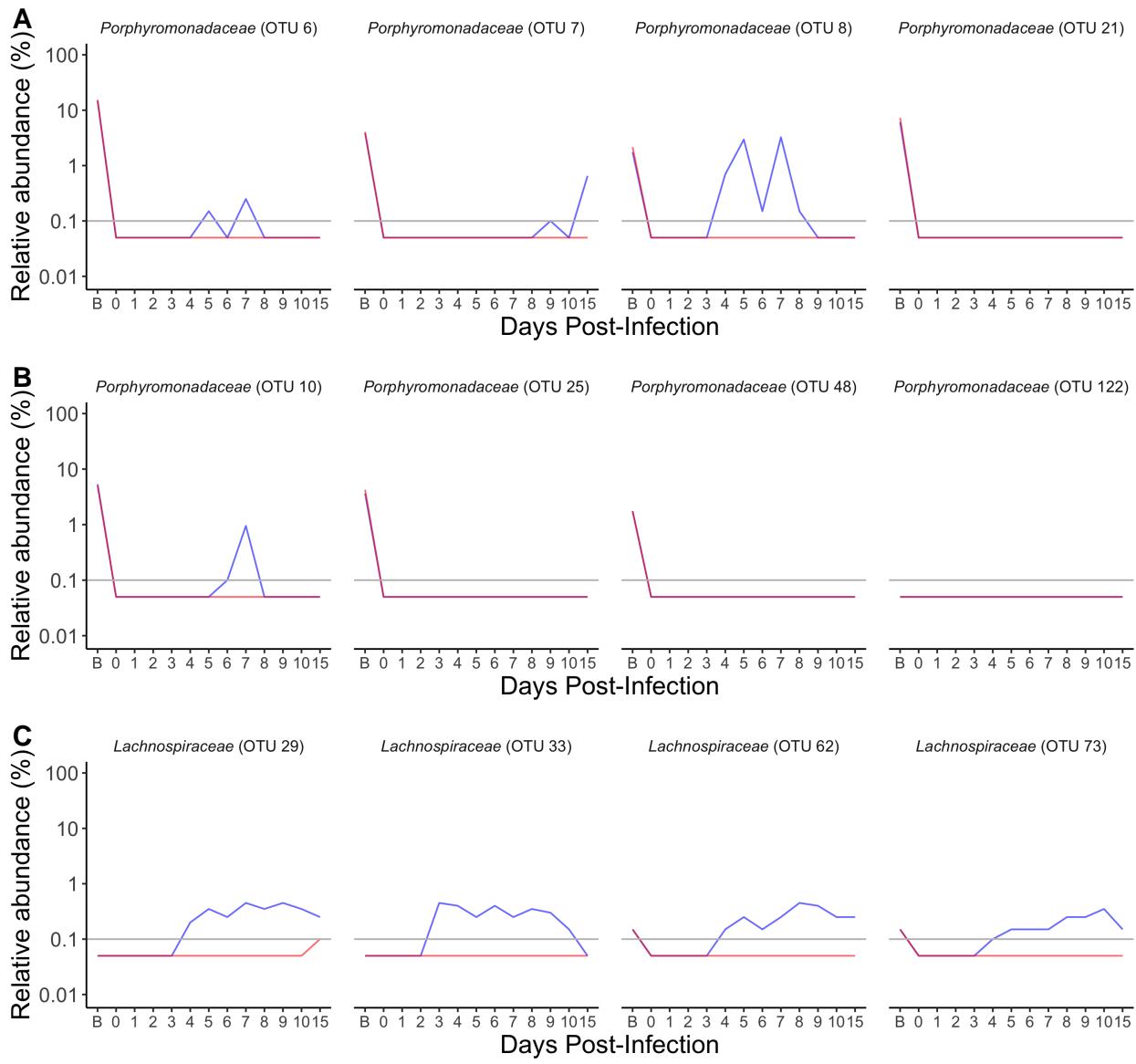
421

422 **Figure S2. PEG treatment still has a large impact on the mucosal microbiota 6 days
423 post-infection A.** The

424



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



428

429 **Figure S4. Specific OTUs associated with clearance by 10 days post-infection that are**
 430 **mostly absent in mice with prolonged *C. difficile* colonization. Ex. *Muribaculum intestinale*.**

431 A.

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