

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* (Ref). However, whether laxative results in more severe CDIs in mice and
54 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. For FMTs that are delivered via colonoscopy, patients typically undergo bowel

58 preparation by taking an osmotic laxative prior to the procedure. Many of the FMT studies to date
59 rationalize the use of laxatives (Ref) based on a 1996 case study with 2 pediatric patients where the
60 authors suggested in the discussion that the laxative may help flush *C. difficile* spores and toxins
61 from the intestine (18).

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 [ref]. Although, two
64 groups have now shown PEG treatment alone renders mice susceptible to *C. difficile*, these studies
65 have raised additional questions regarding the dynamics and severity of infection as well as the
66 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, 19). 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 regained 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 10^3 *C.*
81 *difficile* 630 spores. All treatments rendered mice susceptible to *C. difficile* colonization (Fig. 1C),
82 however PEG-treated mice remained colonized at a high level through thirty days post-infection.
83 In contrast, the clindamycin-treated mice that cleared *C. difficile* within ten days post-infection.
84 Surprisingly, PEG-treated mice were still susceptible to *C. difficile* infection after 10-days of recovery
85 from treatment although *C. difficile* was not detectable in most of the group in the initial five days

86 post-infection (Fig. 1C). From 9 days post-infection onward, the median *C. difficile* stabilized for the
87 5-day PEG plus 10-day recovery group of mice and remained high through 30 days post-infection
88 (Fig. 1C). Thus, osmotic laxative treatment alone was sufficient to render mice susceptible to
89 prolonged *C. difficile* colonization and PEG-treated mice remained susceptible for up to ten days
90 post-treatment.

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

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

115 mice one day post-treatment. We found 18 OTUs that were altered by PEG, the majority of these
116 OTUs decreased after PEG, but *Enterobacteriaceae* and *Bacteroides* increased and the increase
117 in *Bacteroides* was unique to PEG treatment as *Bacteroides* actually decreased in clindamycin
118 treated mice (Fig. 2D, Data Set S1, Sheet X). Finally, we examined the bacteria that differ across
119 treatment groups over multiple timepoints. We found 24 were different over multiple timepoints out
120 of the 33 that were different between treatment groups (Fig. 2E, Data Set S1, Sheet X). Thus, PEG
121 has a significant impact on the fecal microbiota that was maintained over time and distinct from
122 clindamycin treatment.

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

134 **5-day laxative treatment does not promote more severe CDIs despite altering the mucosal**
135 **microbiota.** Given the findings from a previous study that demonstrated that PEG treatment
136 disrupts the mucus layer and alters the immune response in mice (16), we decided to examine the
137 impact of PEG treatment on the mucosal microbiota and CDI severity. To evaluate the mucosal
138 microbiota, we sequenced snips of tissue collected from the cecum, proximal colon, and distal
139 colon. Similar to what was observed with the stool samples, alpha diversity was lower in the
140 PEG-treated mice compared to clindamycin treatment (Fig. 3A). Although alpha diversity continued
141 to increase over time based on the communities from PEG-treated mice collected at 20 and 30 days
142 post-infection (Fig. 3A). Group, day, and the interactions with other variables (cage, experiment
143 number, and sample type) explained the majority of the variation observed in mucosal communities

¹⁴⁴ (PERMANOVA combined $R^2 = 0.83$, $P < 0.05$, Fig. 3B, Data Set S1, Sheet X). *Bacteroides*,
¹⁴⁵ *Butyricoccus*, *Clostridiales*, *Clostridium Cluster XIVb*, *Firmicutes*, and *Ruminococcaceae* were
¹⁴⁶ consistently different between treatment groups in both the tissue and stool communities (Fig.
¹⁴⁷ 3C, Fig. 2E, Data Set S1, Sheet X). Next, we examined CDI severity by evaluating cecum and
¹⁴⁸ colon histopathology (20) and found there was no difference in cecum and colon scores between
¹⁴⁹ clindamycin and PEG-treated mice that were challenged with *C. difficile* at 4 days post-infection
¹⁵⁰ (Fig. 3D), the timepoint typically examined in *C. difficile* 630 challenged mice (ref). We also looked
¹⁵¹ at 6 days post-infection because that was when we started to see a large difference in *C. difficile*
¹⁵² colonization levels between PEG- and clindamycin-treated mice (Fig. 1C). Although, there was a
¹⁵³ slight difference in the colon between PEG and clindamycin-treated mice, there was no difference
¹⁵⁴ in the cecum and the overall score is still relatively low given that the max possible summary score
¹⁵⁵ is 12 (Fig. 3E). Thus, although PEG treatment had a profound impact on the mucosal microbiota,
¹⁵⁶ the impact of *C. difficile* on the cecum and colon was similar between PEG and clindamycin treated
¹⁵⁷ mice.

¹⁵⁸ ***C. difficile* challenge does not have a synergistic disruptive effect on the microbiota of**
¹⁵⁹ **PEG-treated mice** Because *C. difficile* itself can have an impact on the microbiota (21), we also
¹⁶⁰ sequenced the tissue and stools of mock-challenged clindamycin and 5-day PEG treated mice.
¹⁶¹ Examining the stools of the mock-infected PEG- and clindamycin-treated mice revealed similar
¹⁶² bacterial disruptions as the *C. difficile* challenged mice (Fig. S2A-C). Similarly, there was no
¹⁶³ difference between the tissues of mock and *C. difficile* challenged mice (Fig. S2D-F). Thus, most of
¹⁶⁴ the microbiota alterations we observed in the PEG-treated mice were a result of the laxative and
¹⁶⁵ not an interaction between the laxative and *C. difficile*.

¹⁶⁶ **1-day laxative treatment results in transient *C. difficile* colonization and minor microbiota**
¹⁶⁷ **disruption** + Explain motivation for experiment, set-up of experiment (Fig. 4A). + Transient *C.*
¹⁶⁸ *difficile* colonization in 1-day PEG treated mice with all PEG mice clearing by 7dpi (Fig. 4B) + PCoA
¹⁶⁹ results (Day has larger R2 than treatment group). PEG mice are closer to baseline communities
¹⁷⁰ after 7-day period (Fig. 4C) + Shannon results show alpha diversity is only transiently disrupted
¹⁷¹ (Fig. 4D) + Highlight bacteria that are disrupted by PEG or clindamycin treatment but recover within
¹⁷² 7 days (Fig. 4E)

173 **Post-CDI laxative treatment disrupts clearance in clindamycin-treated mice regardless of**
174 **whether an FMT is also administered** + Reiterate motivation for experiment. Saw only a transient
175 microbiota disruption with 1-day PEG treatment alone. Also, see if adding FMT helps with clearance,
176 mention groups have been using PEG or Abx/PEG treatments as a tool to engraft mice with human
177 bacteria communities. Set up of experiment (Fig. 5A). + Prolonged *C. difficile* colonization in
178 post-CDI PEG treated mice (Fig. 5B) + FMT appears to partially restore alpha diversity (Shannon,
179 but not richness Fig. 5C-D) + PCoA (necessary?) Could comment on the clustering of Clindamycin
180 and PEG-treated mice that received FMT (Fig. 5E) + Only 2 genera significantly impacted by FMT
181 treatment, likely not as important for clearance (Fig. 5F) + Bacteria that consistently differ between
182 groups over time, associated with prolonged colonization (Fig. G)

183 **Five-day post-infection community data can predict mice that will have prolonged *C.***
184 ***difficile* colonization** After identifying

- 185 • Figure 6. Specific microbiota features associated with prolonged *C. difficile* colonization in
186 PEG treated mice.
- 187 • Figure S2. Specific OTUs associated with clearance that are mostly absent in mice with
188 prolonged *C. difficile* colonization.
 - 189 – Ex. *Muribaculum intestinale*.

190 **Discussion**

- 191 • Summary of major findings (Fig. 7A)
- 192 • Discussion of prolonged persistence. *C. difficile* sequences detected in tissue samples.
193 Association with mucin-degrading bacteria suggested by recent papers.
- 194 • Discuss why we might not have observed more severe histology in PEG mice relative to
195 clindamycin-treated mice
 - 196 – Antibiotics may also impact mucus layer
 - 197 – Strain of bacteria used

- 198 • Protective bacteria missing in PEG-treated mice
- 199 • Discuss what these findings might mean for human patients (Fig. 7B)
- 200 – What's known regarding laxatives and susceptibility to CDIs
- 201 – Relevance to human FMTs? Unclear what the best administration route is because there
- 202 have been no studies designed to evaluate the best administration route for FMTs.

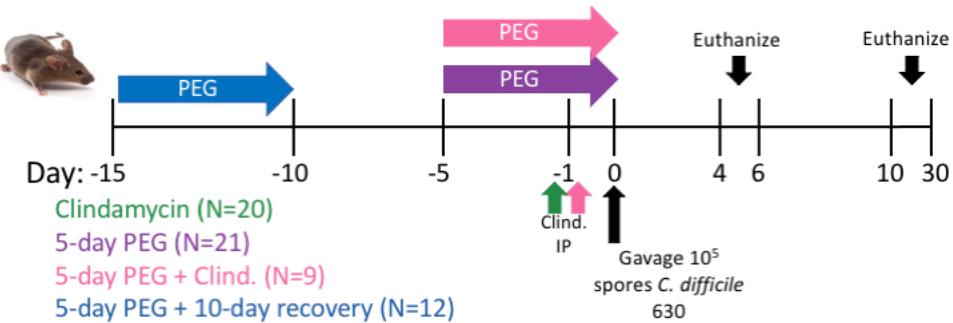
203 **Conclusions**

204 **Acknowledgements**

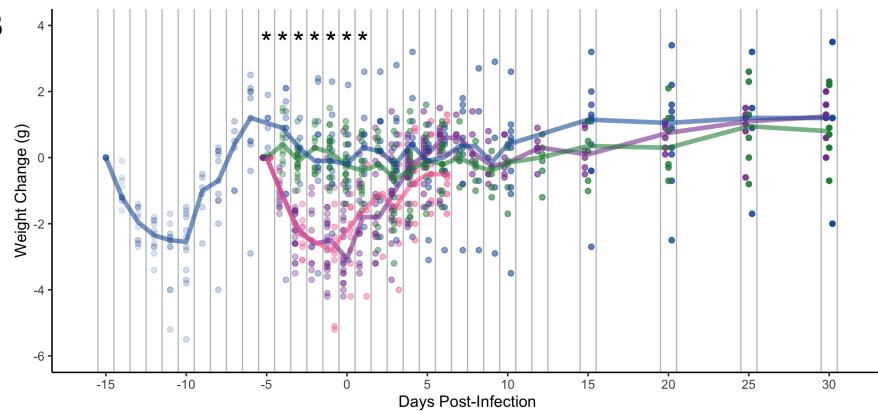
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215 **Materials and Methods**

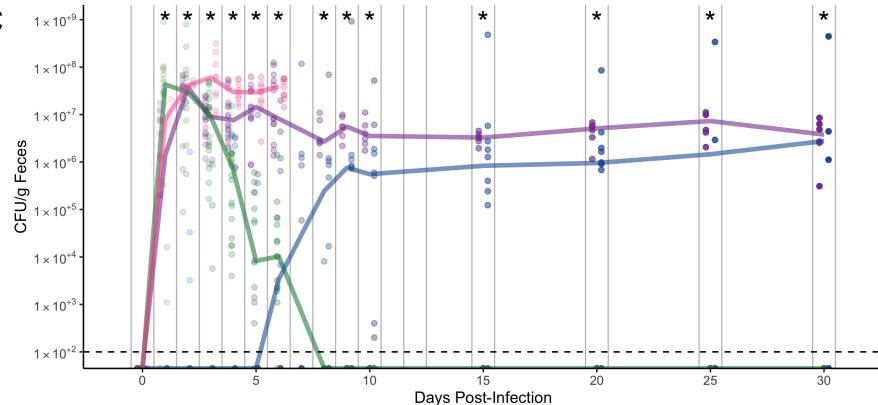
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B



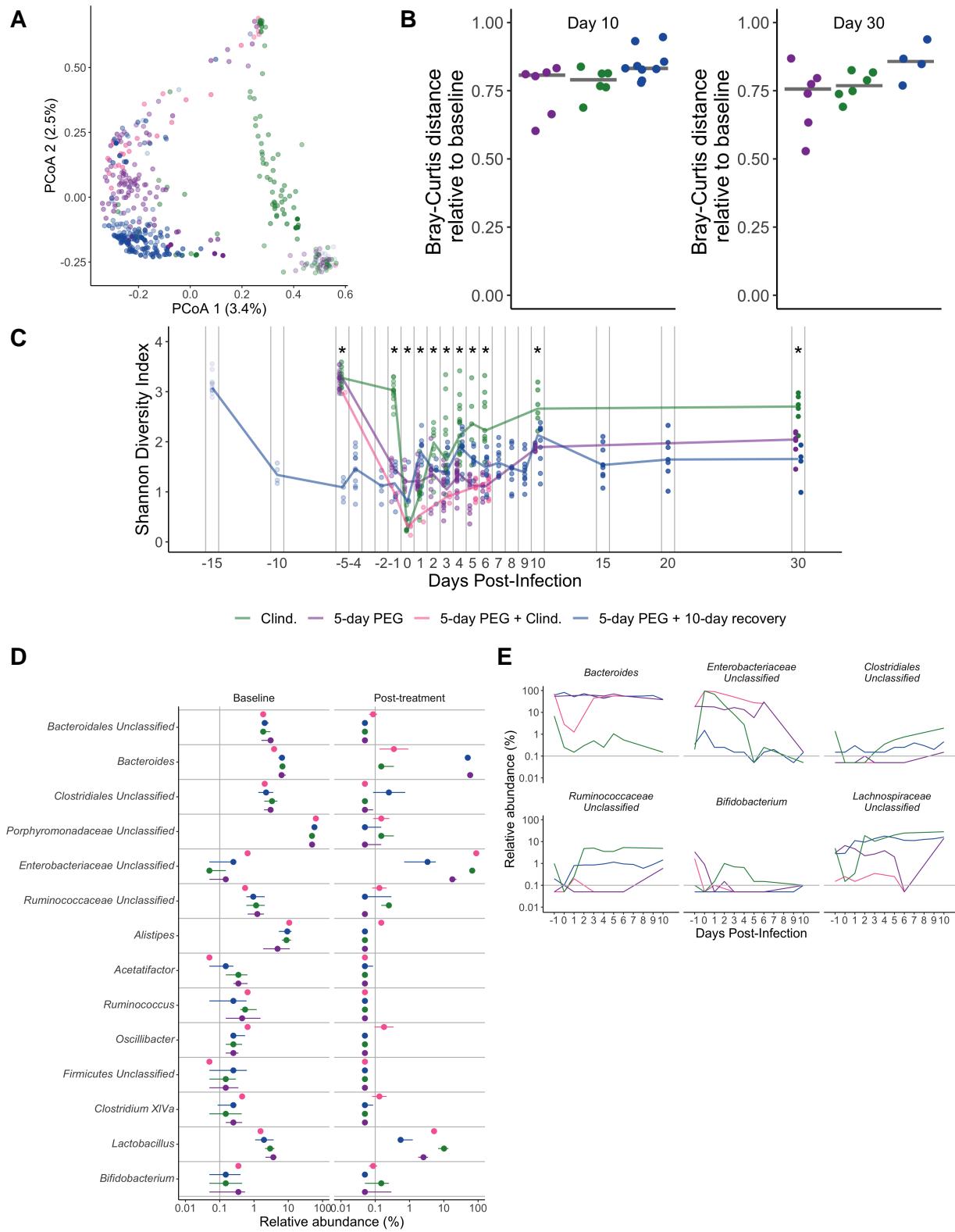
C



216

217 **Figure 1. 5-day PEG treatment prolongs susceptibility and mice become persistently**
218 **colonized with *C. difficile*.** A. Setup of the experimental timeline for experiments with 5-day PEG
219 treated mice. Clindamycin was administered at 10 mg/kg by intraperitoneal injection. 15% PEG
220 3350 was administered in the drinking water for five days. B. Weight change from baseline weight
221 in groups after treatment with PEG and/or clindamycin, followed by *C. difficile* challenge. C. *C.*
222 *difficile* CFU/gram stool measured over time (N = 16-59 mice per timepoint) via serial dilutions. The
223 black line represents the limit of detection for the first serial dilution. CFU quantification data was

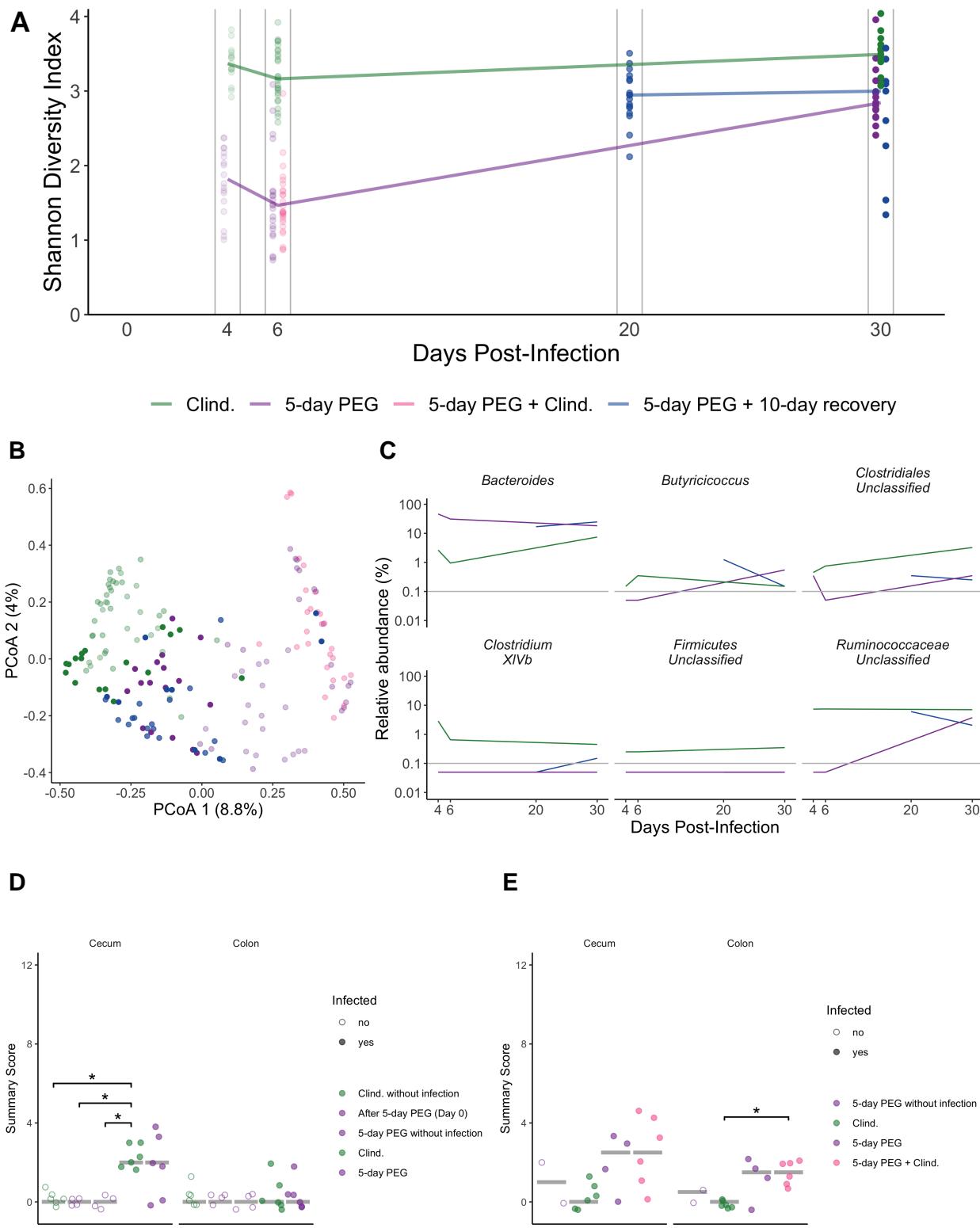
224 not available for each mouse due to stool sampling difficulties (particularly the day the mice came
225 off of the PEG treatment) or early deaths. For B-C, lines represent the median for each treatment
226 group and circles represent samples from individual mice. Asterisks indicate timepoints where the
227 weight change or CFU/g was significantly different between groups by the Kruskal-Wallis test with
228 Benjamini-Hochberg correction for testing multiple timepoints. The data presented are from a total
229 of 5 separate experiments.



230

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

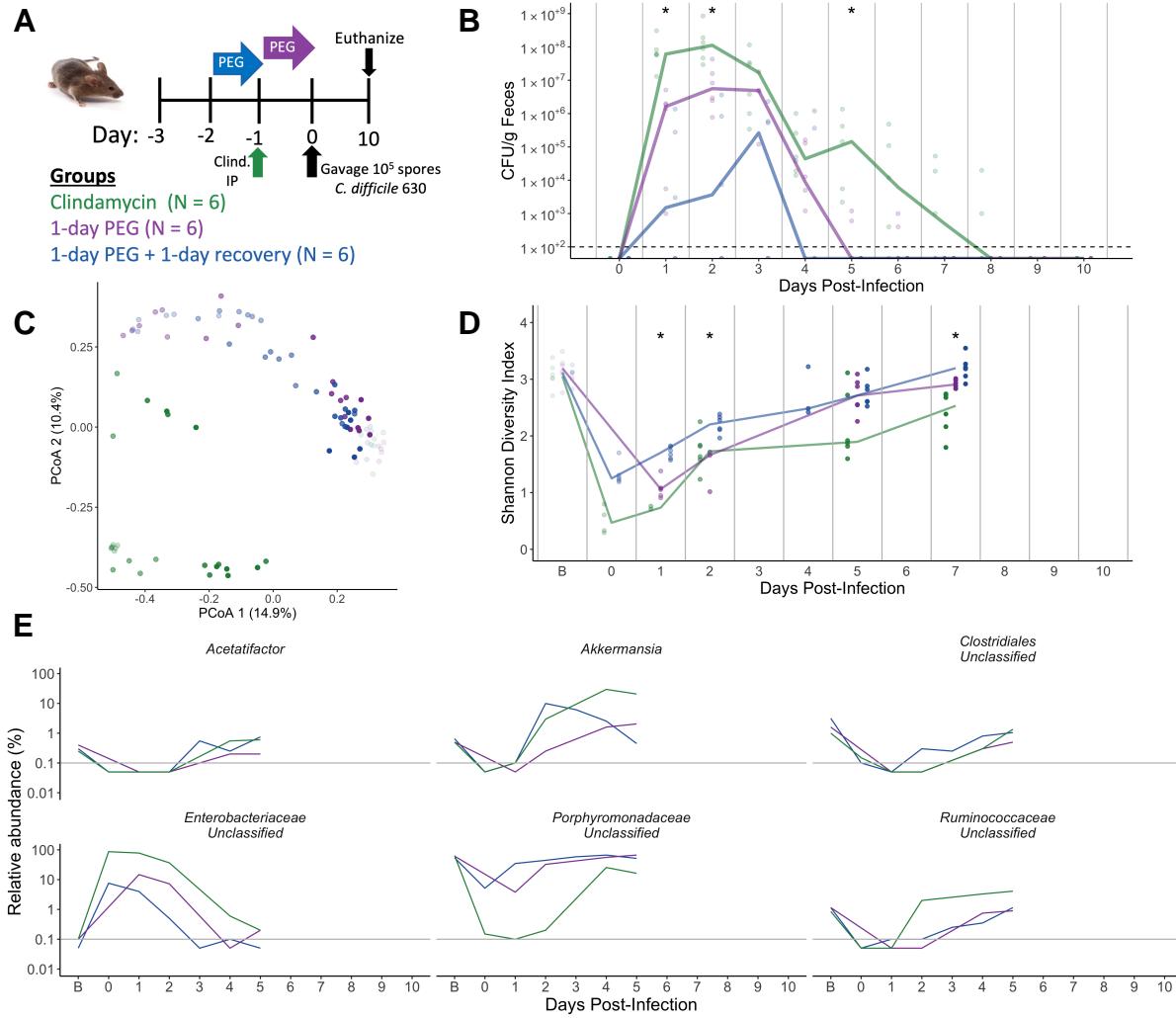
232 **compared to clindamycin-treated mice.** A. PCoA of Bray-Curtis distances from stool samples
233 collected throughout the experiment.



234

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

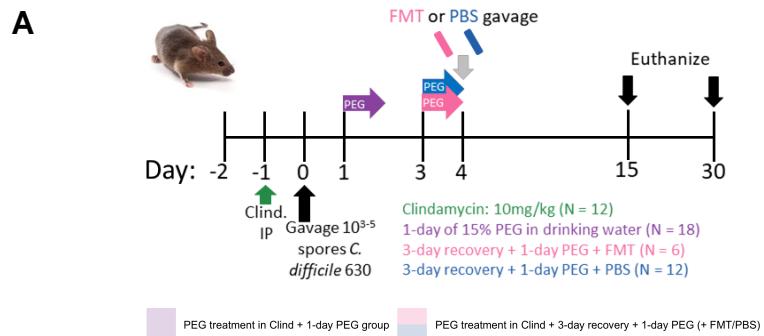
²³⁶ **microbiota is altered.** A.



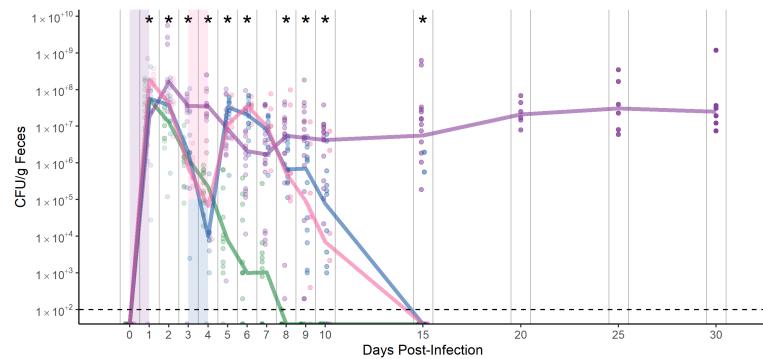
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238 **Figure 4. 1-day PEG treatment renders mice susceptible to transient *C. difficile***
 239 **colonization.** A. Setup of the experimental timeline for the 1-day PEG treated subset of
 240 mice. B. CFU/gram stool measured over time (N = 12-18 mice per timepoint) via several dilutions.
 241 The black dotted line represents the limit of detection for the first serial dilution. Asterisks indicate
 242 timepoints where the CFU/gram was significantly different between groups using the Kruskall-Wallis
 243 test with a Benjamini-Hochberg correction for multiple timepoints. C. Principle Coordinate Analysis
 244 plot of the groups over time with the alpha representing the same time scale as in panel D (day:
 245 $R^2 = 0.43$; group: $R^2 = 0.19$). D. Shannon diversity Index of the groups over time. Only days with
 246 samples from all groups are shown. E. Line plots of relative percent abundance of selected genera
 247 over time. Only days with samples from all groups shown. The gray line represents the limit of
 248 detection.

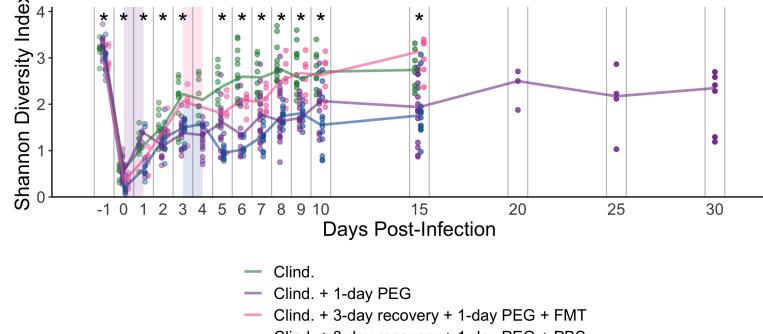
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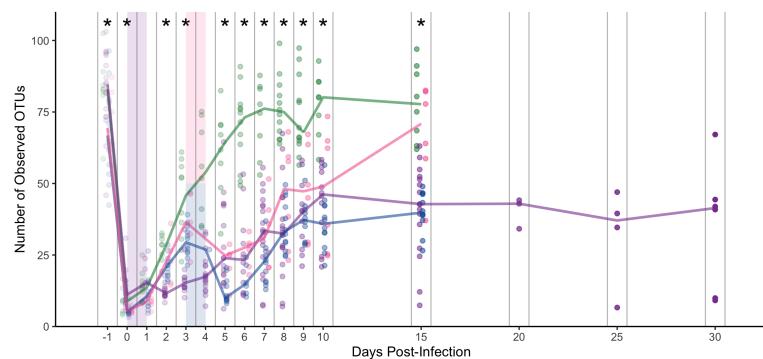
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C



D



A. Experimental timeline for

249 groups receiving PEG treatment post clindamycin gavage. B. Median and individual CFU/g of *C.*
 250 *difficile* measured over time via culture. Asterisks indicate time points with significant differences
 251 between groups using Kruskall-Wallis tests with a Benjamini-Hochberg correction. Limit of detection
 252 is $1e2$ CFU/g. Background shading indicates PEG treatment period for applicable groups. Opacity
 253

254 of points reflects time point. C. Median and individual Shannon Diversity measured over time.
255 Asterisks, background shading, and opacity of points consistent with (B). D. Median and individual
256 richness measured over time. Asterisks, background shading, and opacity of points consistent with
257 (B).

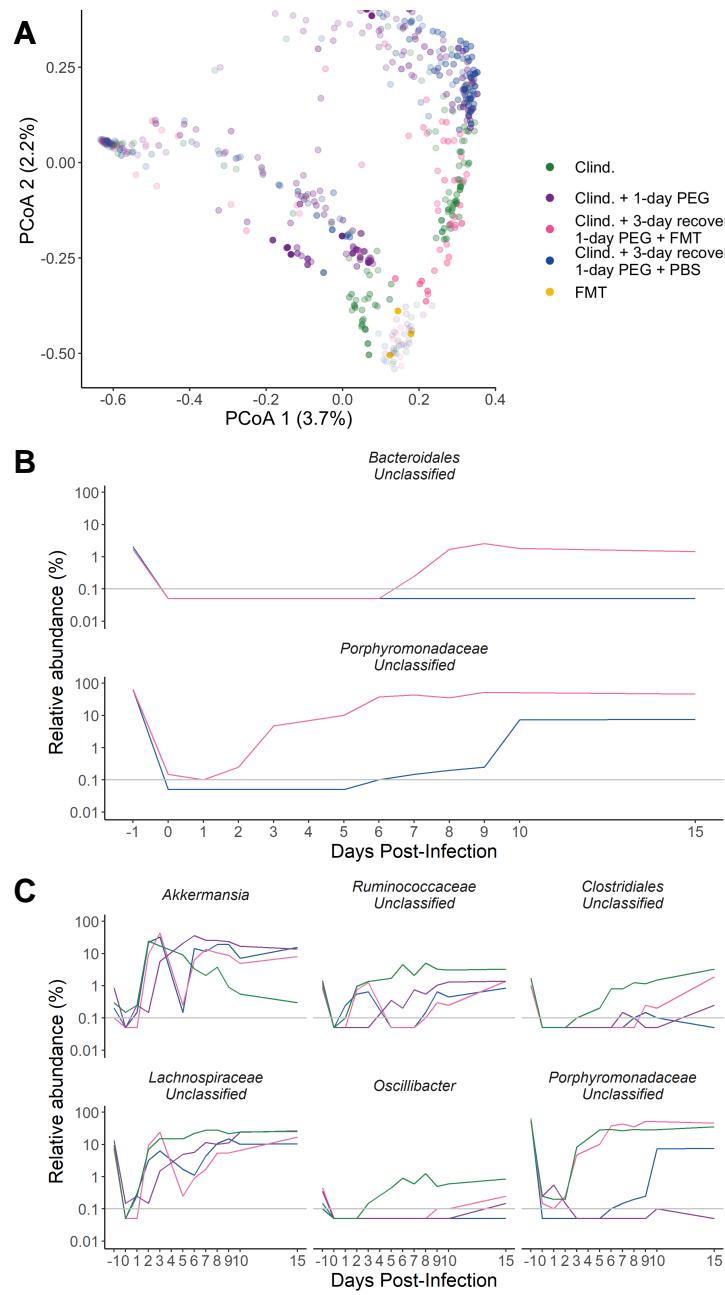
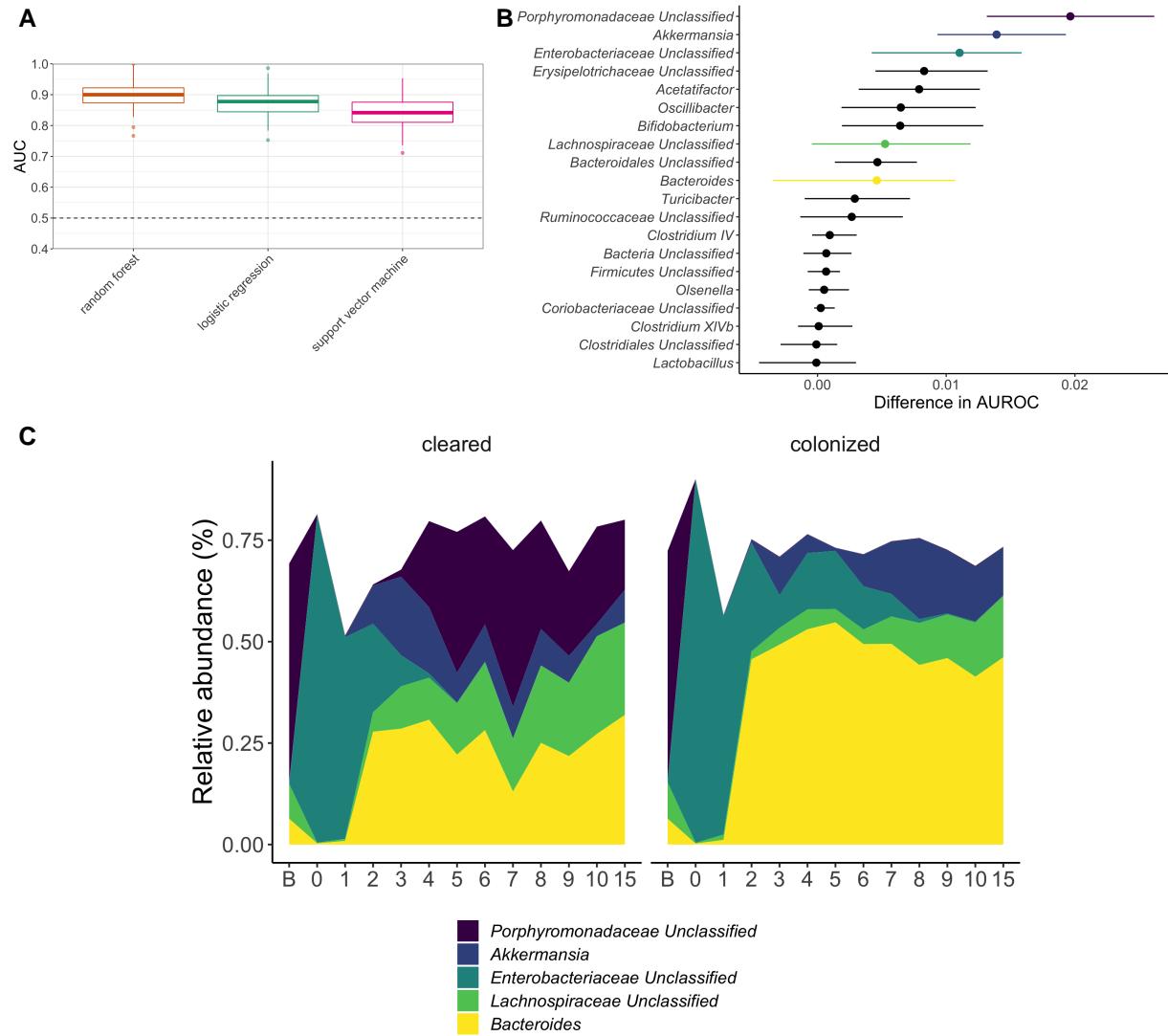


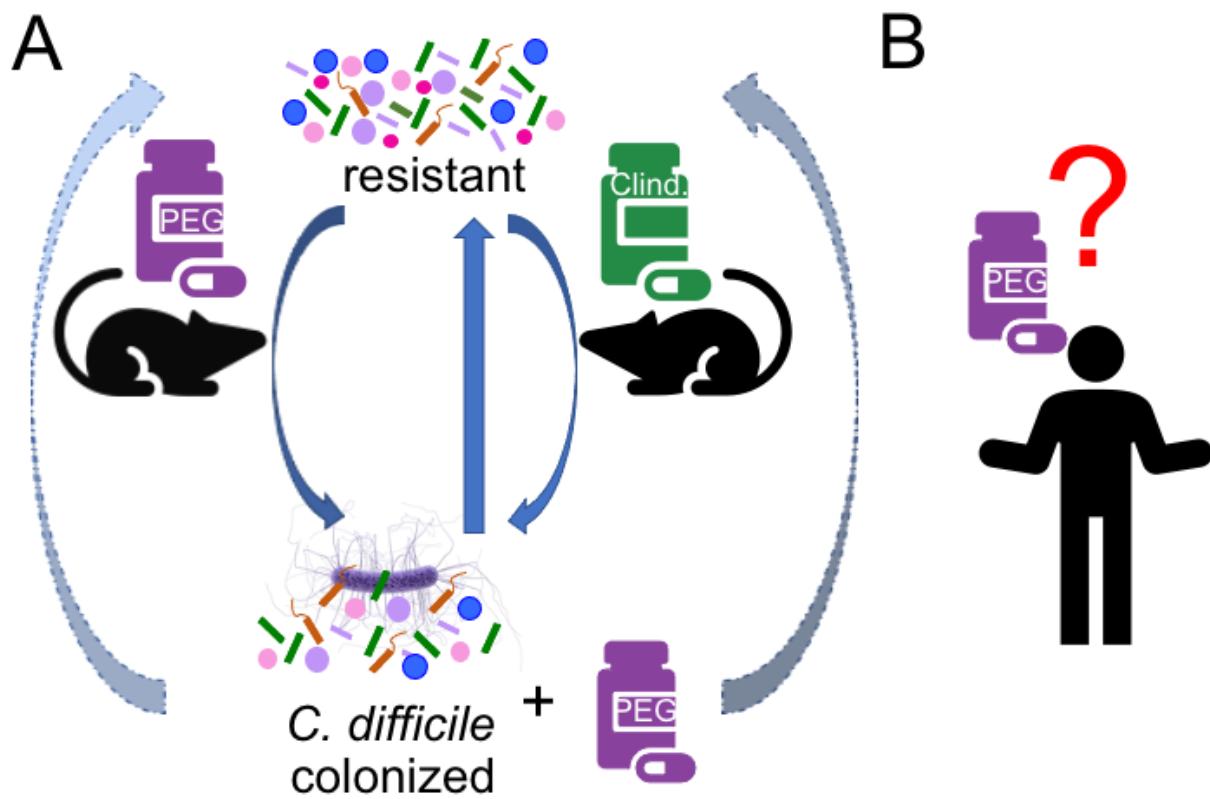
Figure 5. 1-day PEG treatment

258 **post C. difficile challenge prolongs colonization regardless of whether an FMT is also
259 administered. A. PCoA of all groups + FMT plotted over time. Opacity of points reflects time point.
260 B. Relative abundance of genera significantly different over multiple time points post FMT or PBS
261 treatment, plotted over time. Limit of detection is .1%. C. Relative abundances of top 6 significant
262 genera ranked by number of days significant, plotted over time. Limit of detection is .1%.**



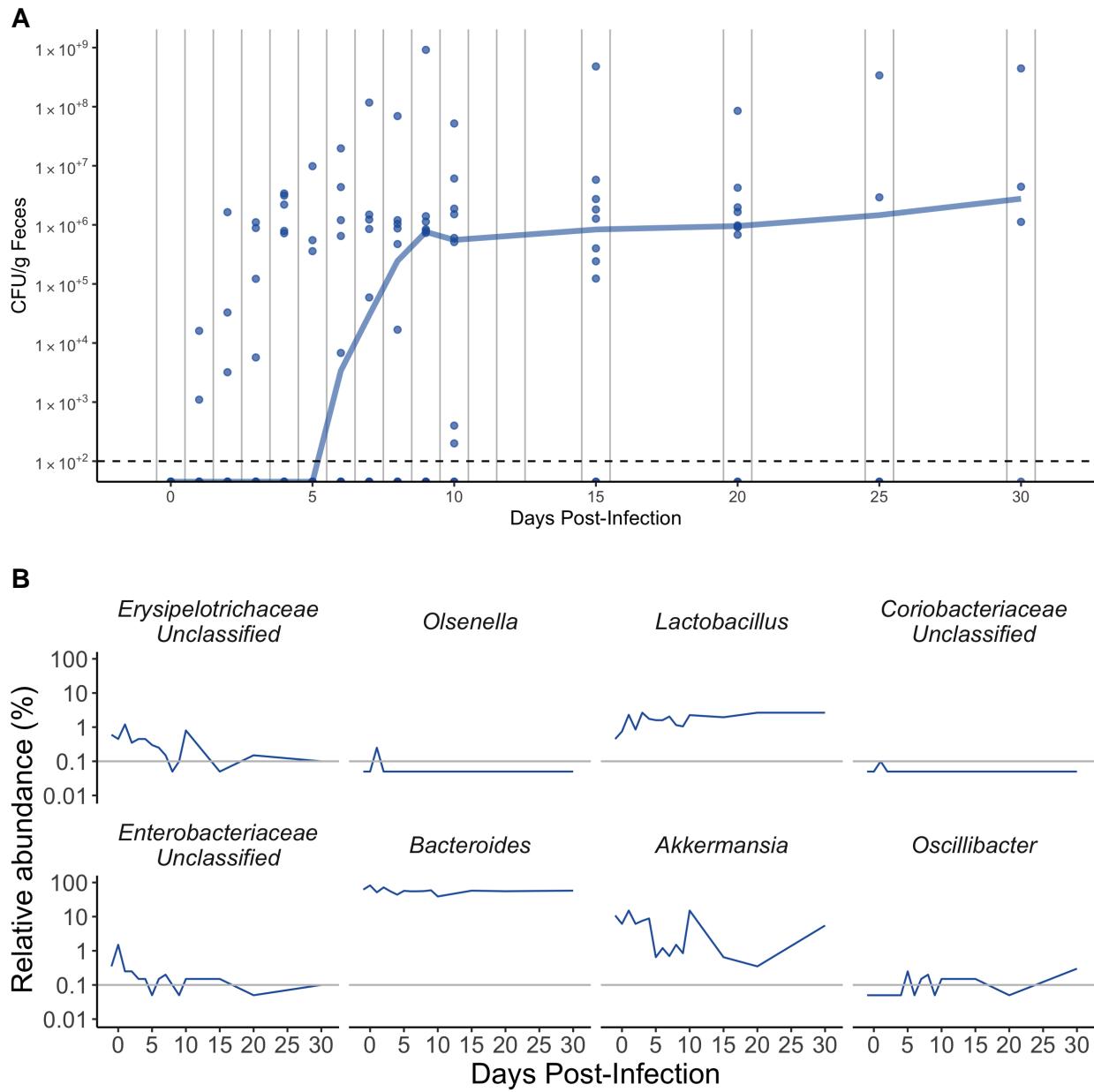
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265 **Figure 6. Specific microbiota features associated with prolonged *C. difficile* colonization**
266 **in PEG treated mice. A.**



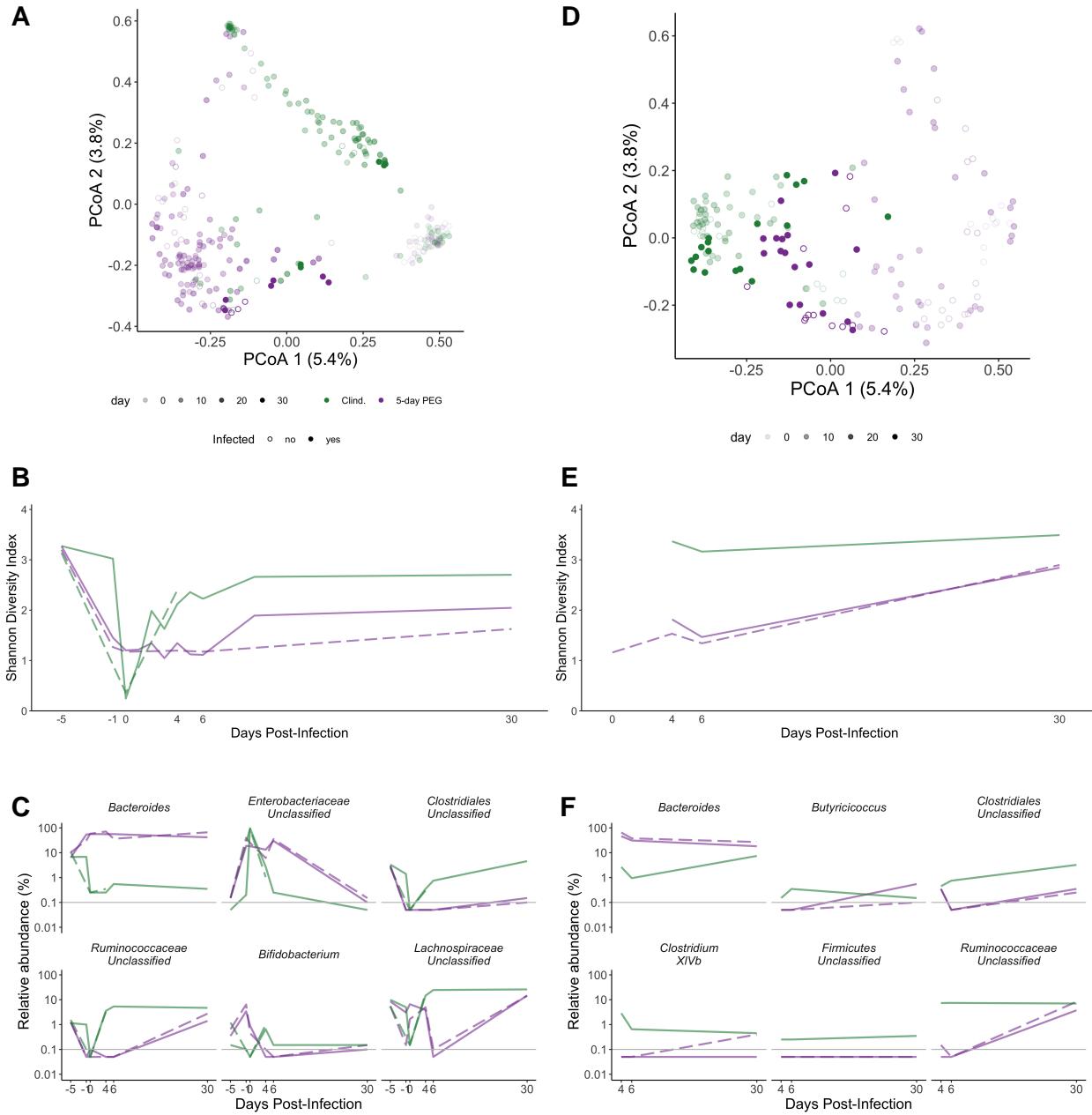
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268 **Figure 7. Schematic summarizing findings. A.**



269

270 **Figure S1.** 5-day PEG treatment plus 10-day recovery mice microbiota dynamics
271 post-infection. A.



272

273 **Figure S2. Specific OTUs associated with clearance that are mostly absent in mice with**
 274 **prolonged *C. difficile* colonization. Ex. *Muribaculum intestinalis*. A.**

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