

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

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

121 Surprisingly, *C. difficile* was not immediately detectable in the stools of the PEG-treated mice
122 that were allowed to recover for 10 days prior to challenge. We decided to examine what other
123 bacteria were changing during the post-infection period when *C. difficile* changed from undetectable
124 to detectable. Interestingly, we found ____ bacteria were shifting during the time period when *C.*
125 *difficile* was becoming detectable in the stools (Fig. S1)

126 **5-day laxative treatment does not promote more severe CDIs despite altering the mucosal**
127 **microbiota.** Given that previous work had demonstrated that PEG treatment disrupts the mucus
128 layer and alters the immune response in mice (16), we decided to examine the impact of PEG
129 treatment on the mucosal microbiota and CDI severity. We examined CDI severity by evaluating
130 cecum and colon histopathology [].

131 ***C. difficile* challenge does not have a synergistic disruptive effect on the microbiota of**
132 **PEG-treated mice** Because *C. difficile* itself can have an impact on the microbiota (ref), we
133 also sequenced the tissue and stools of mock-challenged mice. Examining the stools of the
134 mock-infected PEG- and clindamycin-treated mice revealed similar bacterial disruptions as the *C.*
135 *difficile* challenged mice (Fig. S2A). Similarly, there was no difference between the tissues of mock
136 and *C. difficile* challenged mice (Fig. S2B). Thus, most of the microbiota alterations we observed in
137 the PEG-treated mice were a result of the laxative and not an interaction between the laxative and
138 *C. difficile*.

139 **1-day laxative treatment results in transient *C. difficile* colonization and minor microbiota**
140 **disruption** + Explain motivation for experiment, set-up of experiment (Fig. 4A). + Transient *C.*
141 *difficile* colonization in 1-day PEG treated mice with all PEG mice clearing by 7dpi (Fig. 4B) + PCoA
142 results (Day has larger R2 than treatment group). PEG mice are closer to baseline communities

143 after 7-day period (Fig. 4C) + Shannon results show alpha diversity is only transiently disrupted
144 (Fig. 4D) + Highlight bacteria that are disrupted by PEG or clindamycin treatment but recover within
145 7 days (Fig. 4E)

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

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

- 158 • Figure 6. Specific microbiota features associated with prolonged *C. difficile* colonization in
159 PEG treated mice.
- 160 • Figure S2. Specific OTUs associated with clearance that are mostly absent in mice with
161 prolonged *C. difficile* colonization.
 - 162 – Ex. *Muribaculum intestinale*.

163 **Discussion**

- 164 • Summary of major findings (Fig. 7A)
- 165 • Discussion of prolonged persistence. *C. difficile* sequences detected in tissue samples.
166 Association with mucin-degrading bacteria suggested by recent papers.
- 167 • Discuss why we might not have observed more severe histology in PEG mice relative to

- 168 clindamycin-treated mice
- 169 – Antibiotics may also impact mucus layer
- 170 – Strain of bacteria used
- 171 • Protective bacteria missing in PEG-treated mice
- 172 • Discuss what these findings might mean for human patients (Fig. 7B)
- 173 – What's known regarding laxatives and susceptibility to CDIs
- 174 – Relevance to human FMTs? Unclear what the best administration route is because there
- 175 have been no studies designed to evaluate the best administration route for FMTs.

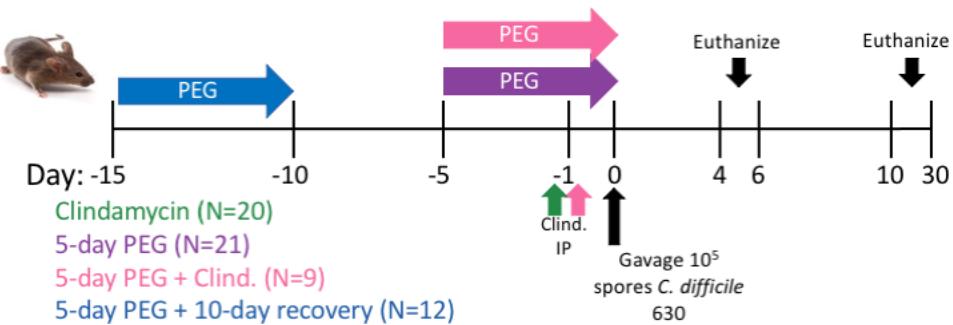
176 **Conclusions**

177 **Acknowledgements**

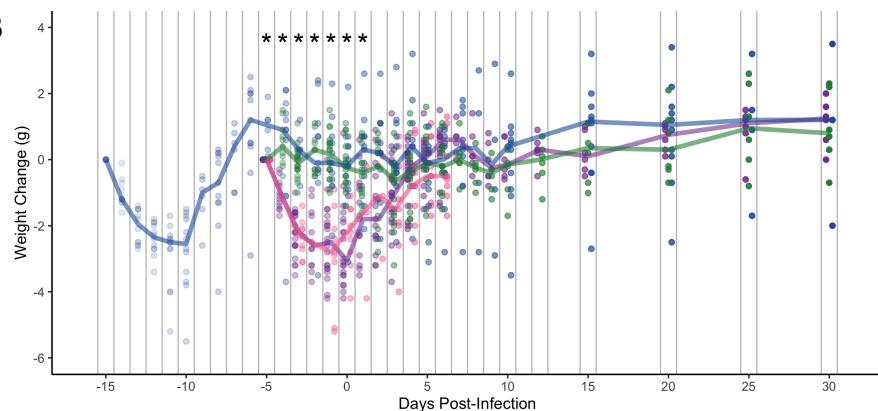
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188 **Materials and Methods**

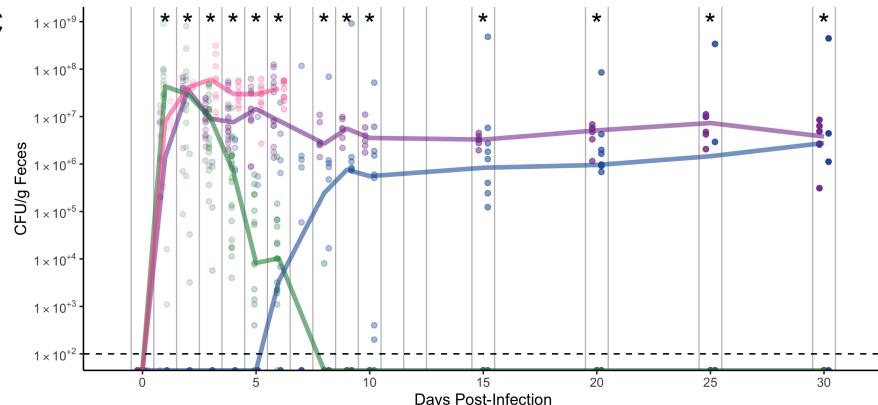
A



B



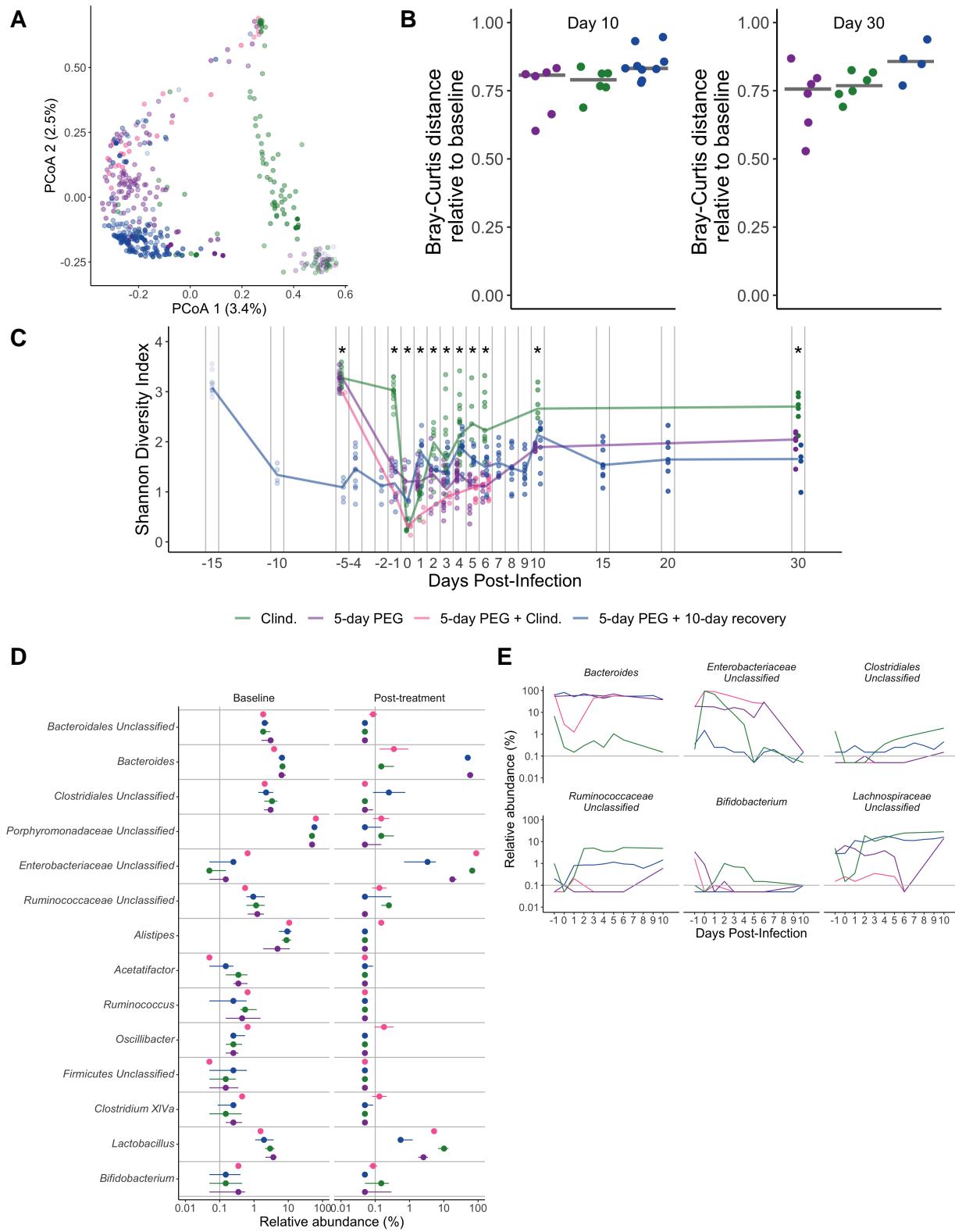
C



189

190 **Figure 1. 5-day PEG treatment prolongs susceptibility and mice become persistently**
191 **colonized with *C. difficile*.** A. Setup of the experimental timeline for experiments with 5-day PEG
192 treated mice. Clindamycin was administered at 10 mg/kg by intraperitoneal injection. 15% PEG
193 3350 was administered in the drinking water for five days. B. Weight change from baseline weight
194 in groups after treatment with PEG and/or clindamycin, followed by *C. difficile* challenge. C. *C.*
195 *difficile* CFU/gram stool measured over time (N = 16-59 mice per timepoint) via serial dilutions. The
196 black line represents the limit of detection for the first serial dilution. CFU quantification data was

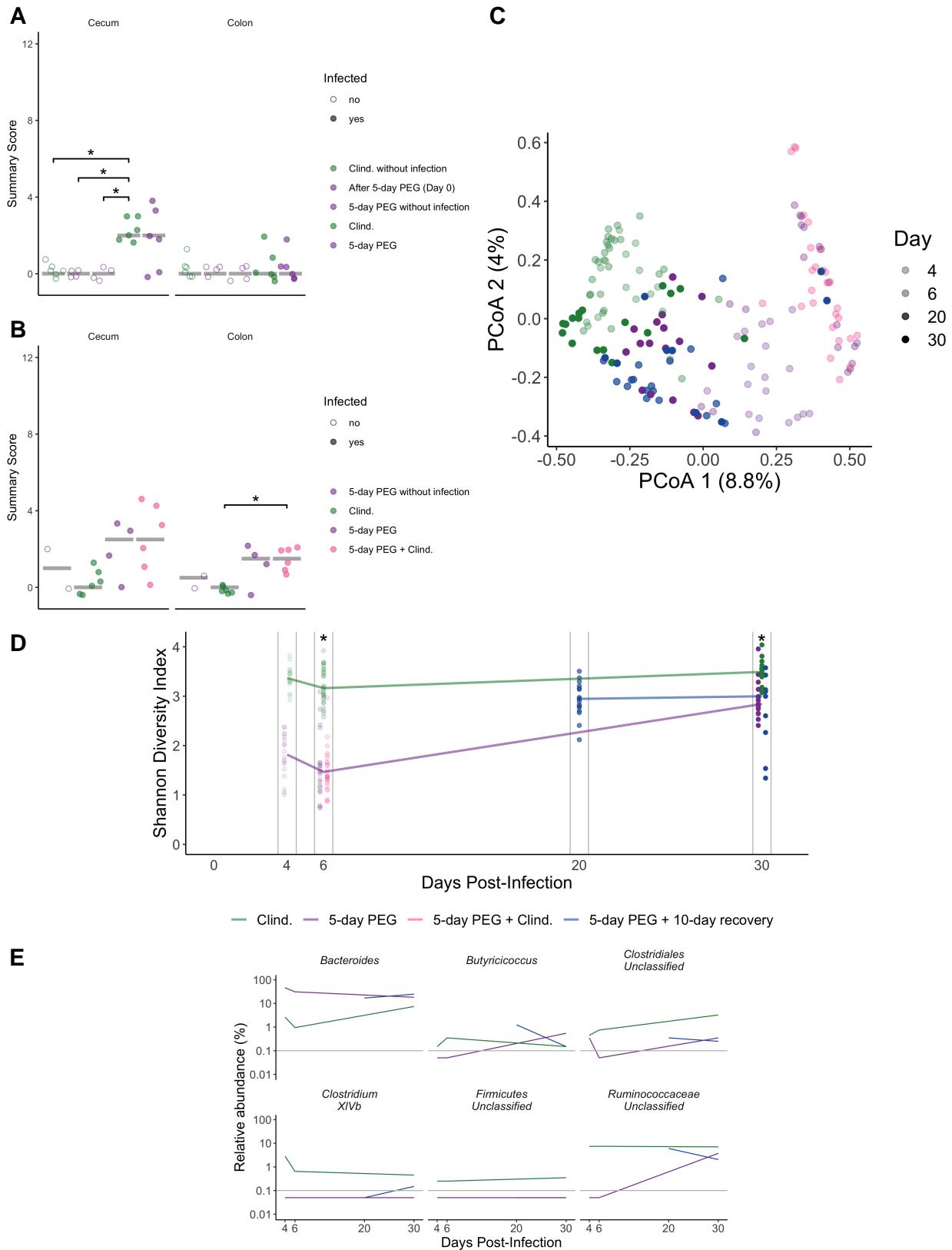
197 not available for each mouse due to stool sampling difficulties (particularly the day the mice came
198 off of the PEG treatment) or early deaths. For B-C, lines represent the median for each treatment
199 group and circles represent samples from individual mice. Asterisks indicate timepoints where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



203

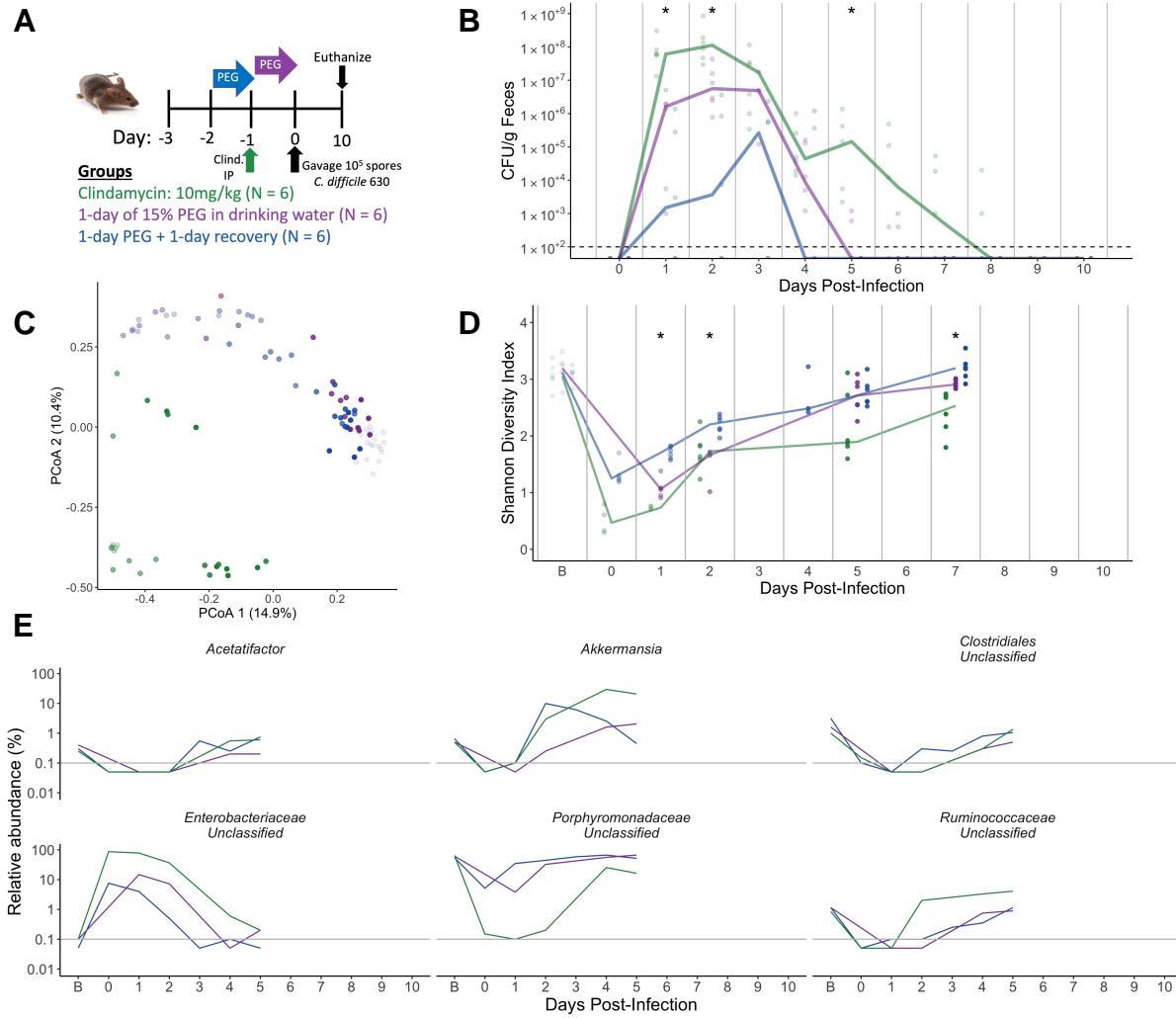
204 **Figure 2. 5-day PEG treatment disrupts the stool microbiota for a longer amount of time**

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



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

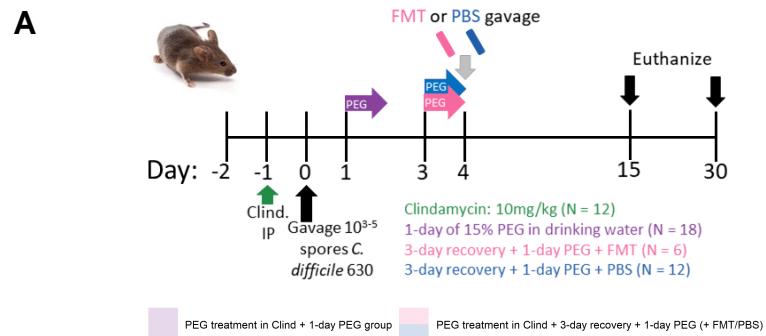
209 **microbiota is altered. A.**



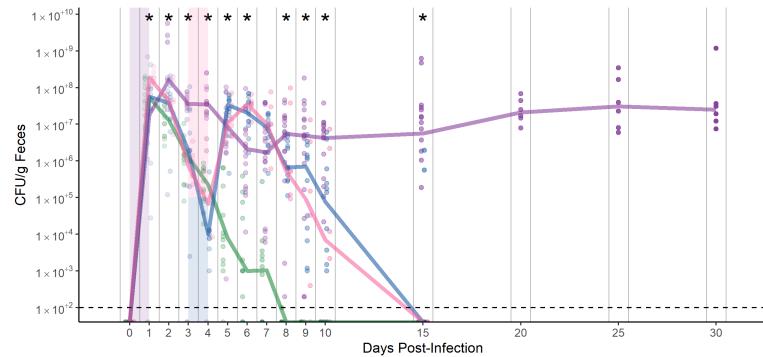
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211 **Figure 4. 1-day PEG treatment renders mice susceptible to transient *C. difficile***
 212 **colonization.** A. Setup of the experimental timeline for the 1-day PEG treated subset of
 213 mice. B. CFU/gram stool measured over time (N = 12-18 mice per timepoint) via several dilutions.
 214 The black dotted line represents the limit of detection for the first serial dilution. Asterisks indicate
 215 timepoints where the CFU/gram was significantly different between groups using the Kruskall-Wallis
 216 test with a Benjamini-Hochberg correction for multiple timepoints. C. Principle Coordinate Analysis
 217 plot of the groups over time with the alpha representing the same time scale as in panel D (day:
 218 $R^2 = 0.43$; group: $R^2 = 0.19$). D. Shannon diversity Index of the groups over time. Only days with
 219 samples from all groups are shown. E. Line plots of relative percent abundance of selected genera
 220 over time. Only days with samples from all groups shown. The gray line represents the limit of
 221 detection.

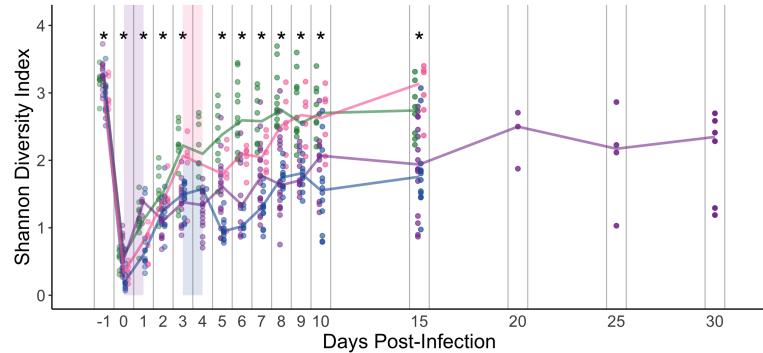
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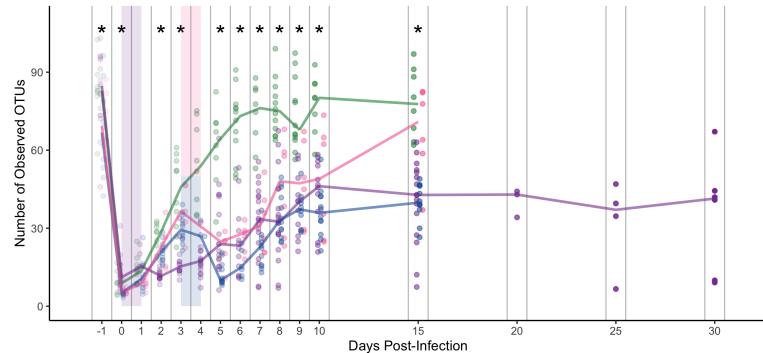
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C



D



A. Experimental timeline for

222 groups receiving PEG treatment post clindamycin gavage. B. Median and individual CFU/g of *C.*
 223 *difficile* measured over time via culture. Asterisks indicate time points with significant differences
 224 between groups using Kruskall-Wallis tests with a Benjamini-Hochberg correction. Limit of detection
 225 is $1e2$ CFU/g. Background shading indicates PEG treatment period for applicable groups. Opacity
 226

²²⁷ of points reflects time point. C. Median and individual Shannon Diversity measured over time.
²²⁸ Asterisks, background shading, and opacity of points consistent with (B). D. Median and individual
²²⁹ richness measured over time. Asterisks, background shading, and opacity of points consistent with
²³⁰ (B).

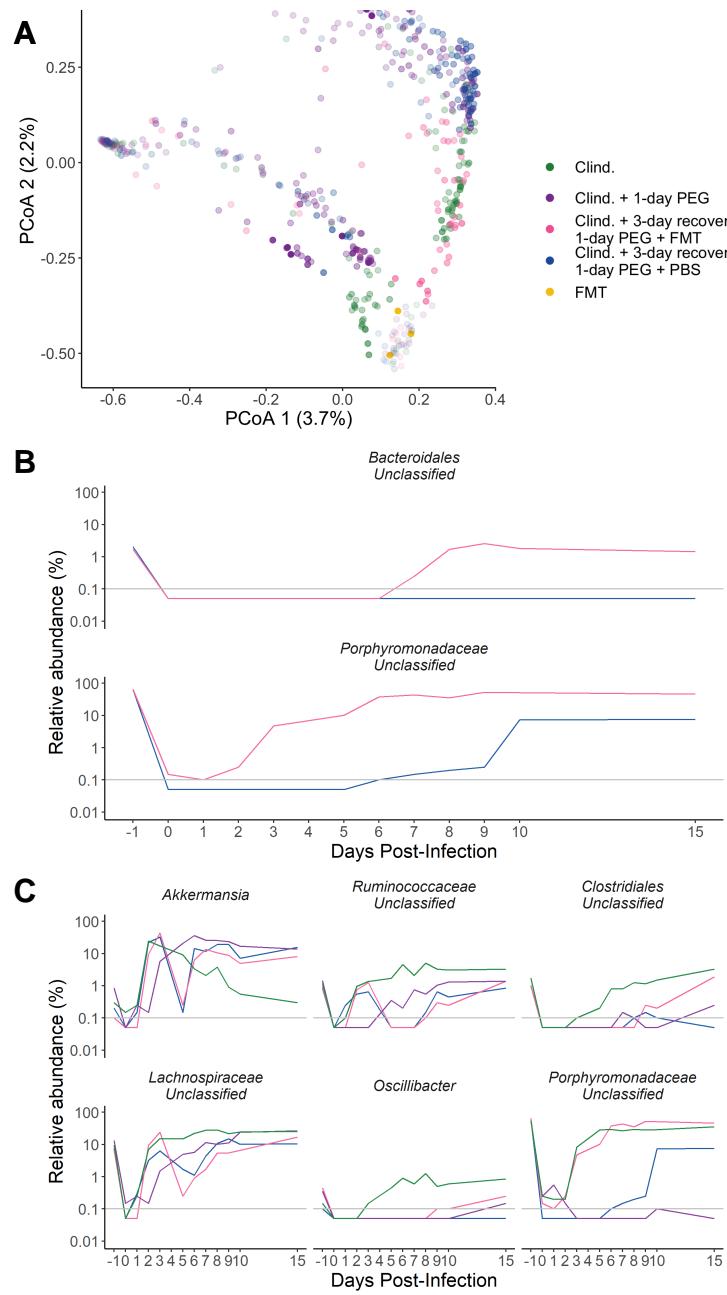
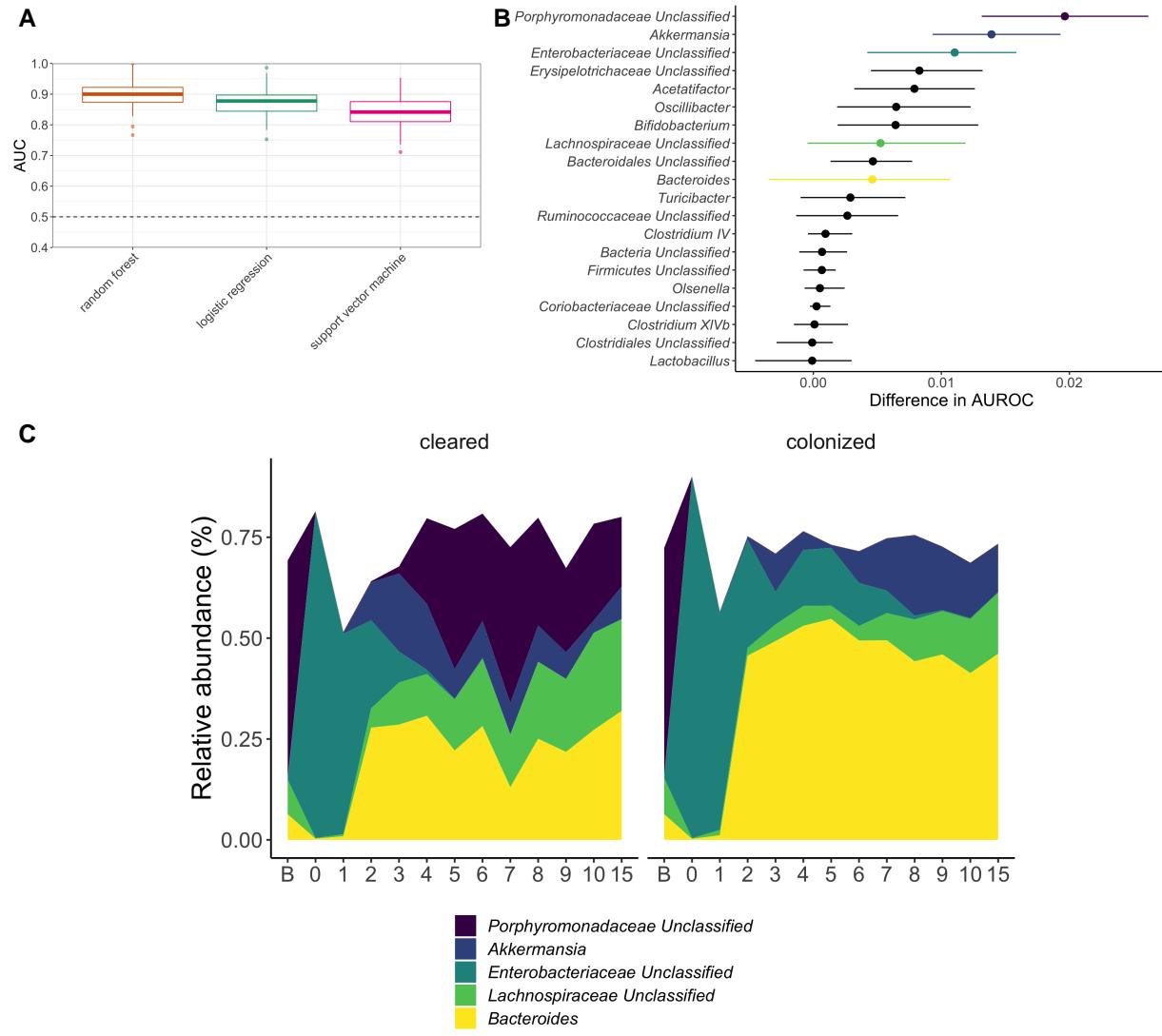


Figure 5. 1-day PEG treatment

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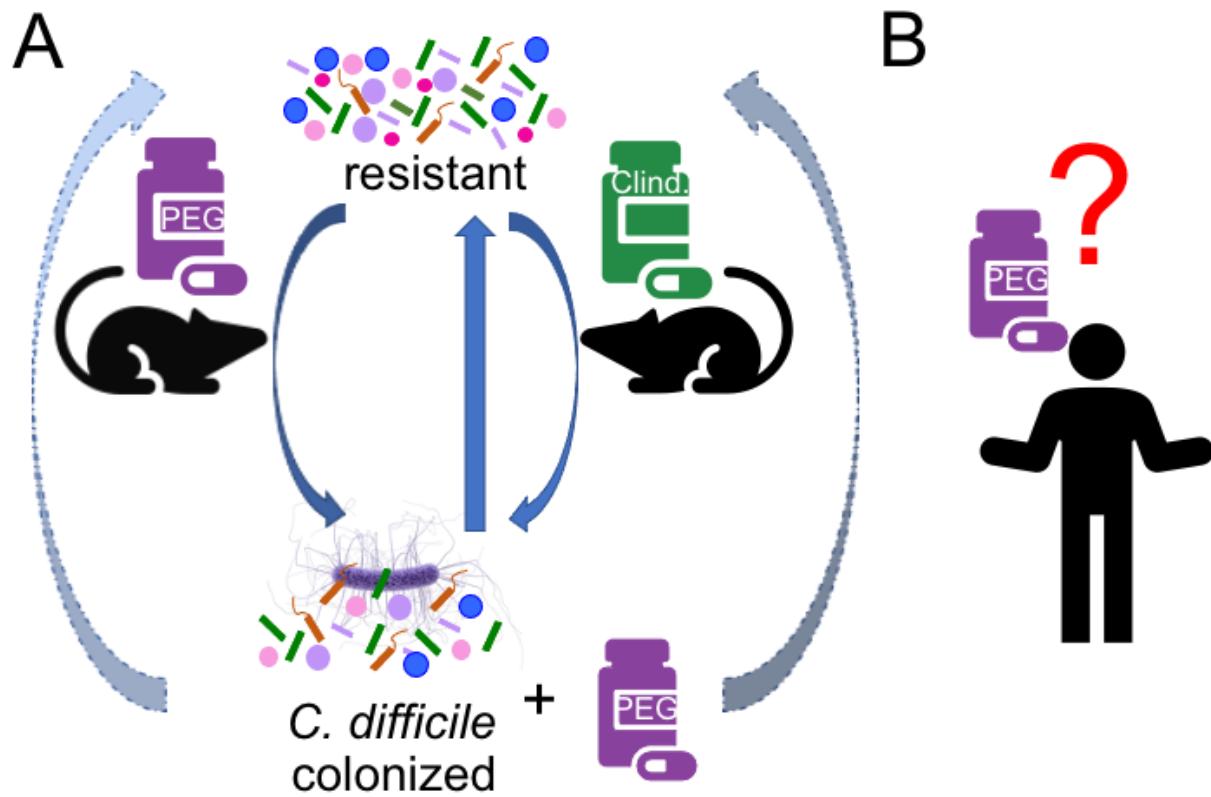
232 **post C. difficile challenge prolongs colonization regardless of whether an FMT is also**
 233 **administered.** A. PCoA of all groups + FMT plotted over time. Opacity of points reflects time point.
 234 B. Relative abundance of genera significantly different over multiple time points post FMT or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



237

238 **Figure 6. Specific microbiota features associated with prolonged *C. difficile* colonization**

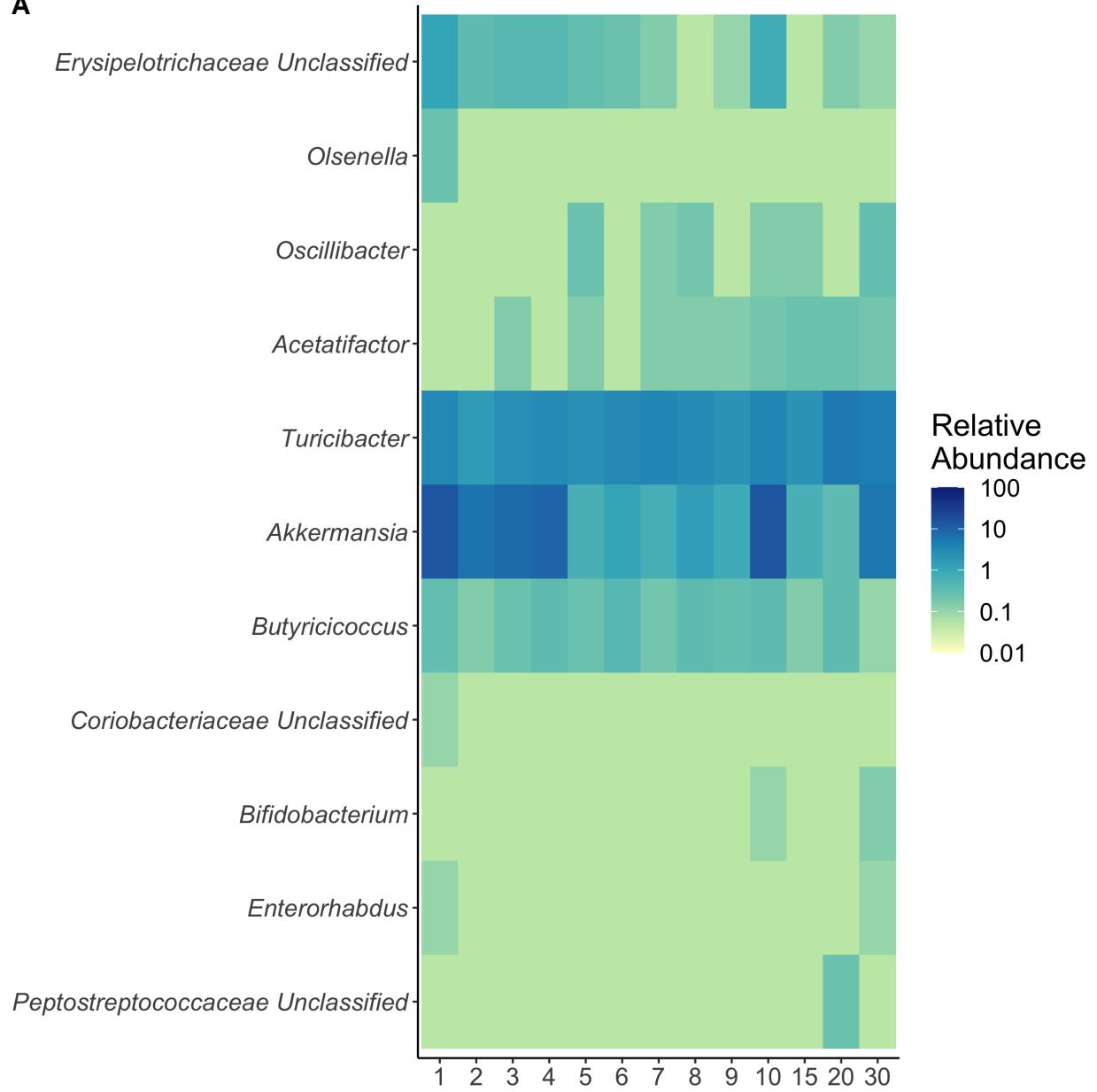
239 **in PEG treated mice. A.**



240

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

A



242

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

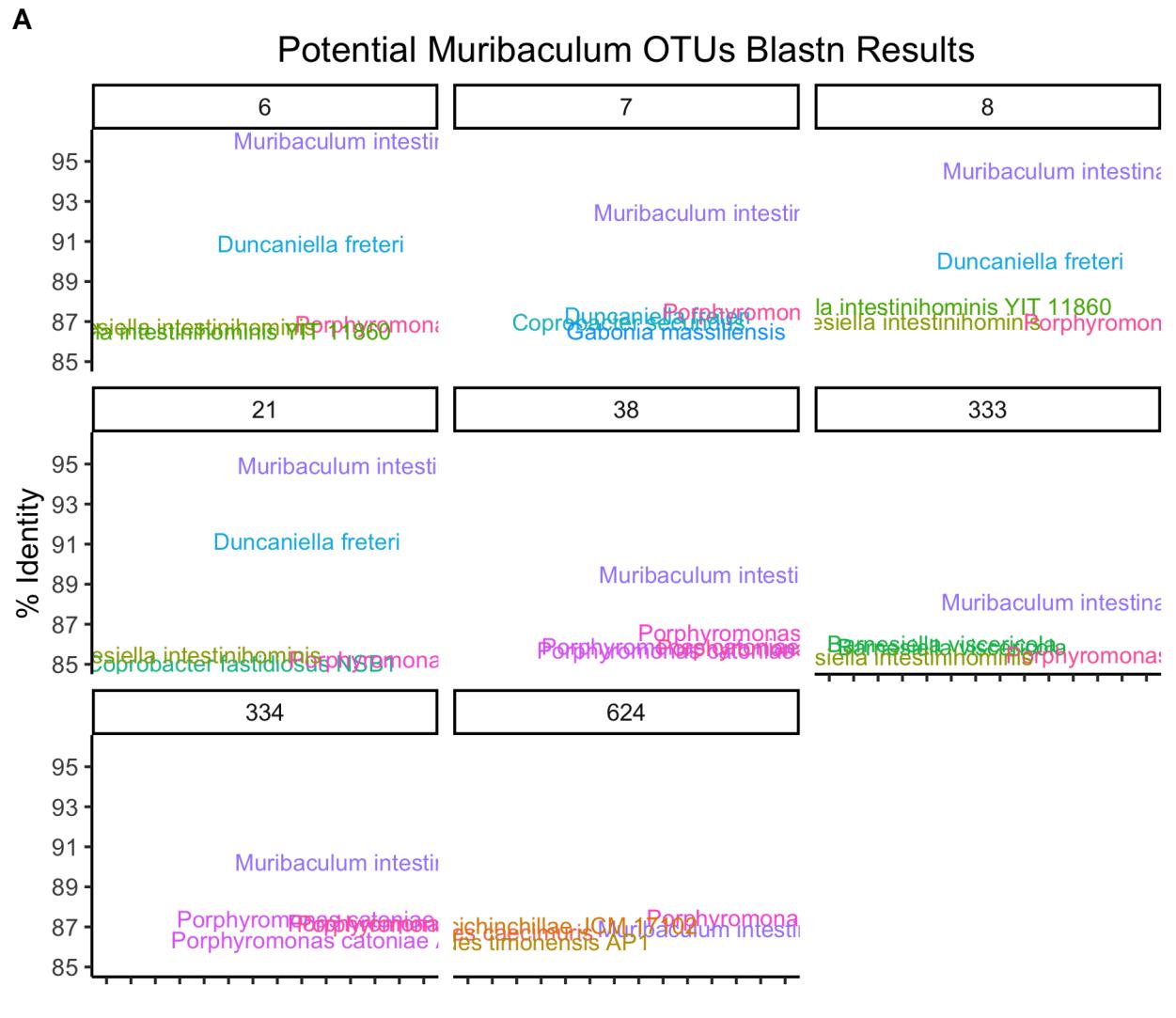


Figure S2. Specific OTUs associated with clearance that are mostly absent in mice with prolonged *C. difficile* colonization. Ex. *Muribaculum intestinalis*. A.

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