

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 (PERMANOVA
98 combined $R^2 = 0.50$, $P < 0.001$) with the remaining variation explained by interactions between
99 treatment group and other experimental variables including time, cage effects, and sequencing
100 preparation (Data Set S1, Sheet X). Cage effects refer to the well-documented phenomenon that
101 mice housed in the same cages have similar microbial communities due to coprophagy, we tried to
102 minimize the impact of cage effects on our experiment by breaking up cagemates when assigning
103 mice to treatment groups and primarily housing only two mice per cage. Importantly, although
104 we conducted a total of 5 separate experiments, the experiment number and its interaction with
105 treatment group was not one of the variables that significantly explained the observed variation in
106 fecal communities (Data Set S1, Sheet X). When we examine alpha diversity by looking at Shannon
107 diversity index over time, we see that although both clindamycin and PEG treatments decrease
108 diversity, the diversity index is less in the groups of mice that received PEG treatment compared to
109 those that received clindamycin through thirty days post-infection.

110 **5-day laxative treatment does not promote more severe CDIs despite altering the mucosal**
111 **microbiota.** Given previous work has shown that PEG treatment disrupts the mucus layer, we
112 decided to examine the impact of the laxative on the mucosal microbiota and infection severity by
113 evaluating histopathology.

- 114 • Figure 2. 5-day PEG treatment disrupts the stool microbiota for a longer amount of time
115 compared to clindamycin-treated mice.
- 116 • Figure S1. 5-day PEG treatment plus 10-day recovery mice microbiota dynamics
117 post-infection.
- 118 • Figure 3. 5-day PEG treatment does not result in more severe CDIs, although mucosal
119 microbiota is altered.
- 120 • Figure 4. 1-day PEG treatment renders mice susceptible to transient *C. difficile* colonization.
- 121 • Figure 5. 1-day PEG treatment post *C. difficile* challenge prolongs colonization regardless of
122 whether an FMT is also administered.
- 123 • Figure 6. Specific microbiota features associated with prolonged *C. difficile* colonization in
124 PEG treated mice.
- 125 • Figure S2. Specific OTUs associated with clearance that are mostly absent in mice with
126 prolonged *C. difficile* colonization.
127 – Ex. *Muribaculum intestinale*.
- 128 • Figure 7. Schematic summarizing findings.

129 **Discussion**

- 130 • Summary of major findings
- 131 • Discussion of prolonged persistence. *C. difficile* sequences detected in tissue samples.
132 Association with mucin-degrading bacteria suggested by recent papers.
- 133 • Discuss why we might not have observed more severe histology in PEG mice relative to
134 clindamycin-treated mice
135 – Antibiotics may also impact mucus layer
136 – Strain of bacteria used

- 137 • Protective bacteria missing in PEG-treated mice
- 138 • Discuss what these findings might mean for human patients
- 139 – What's known regarding laxatives and susceptibility to CDIs
- 140 – Relevance to human FMTs? Unclear what the best administration route is because there
- 141 have been no studies designed to evaluate the best administration route for FMTs.

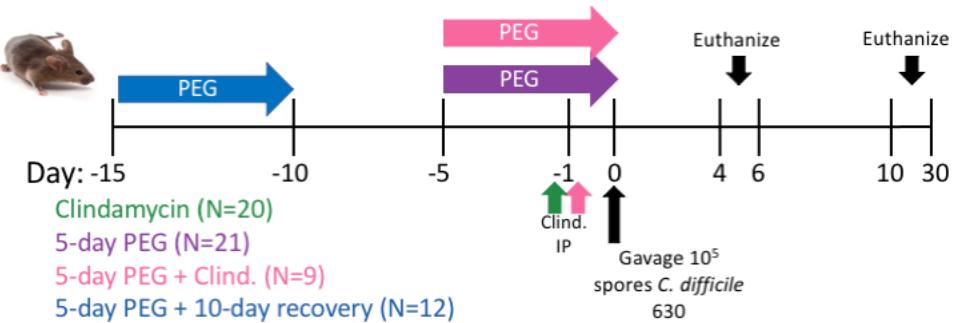
142 **Conclusions**

143 **Acknowledgements**

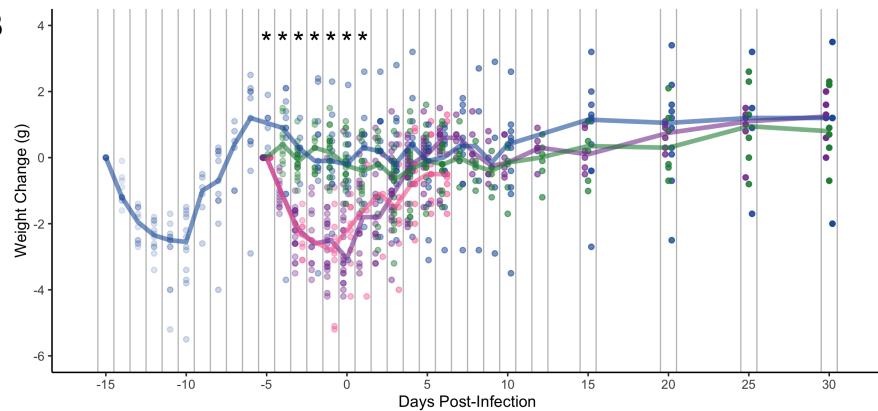
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154 **Materials and Methods**

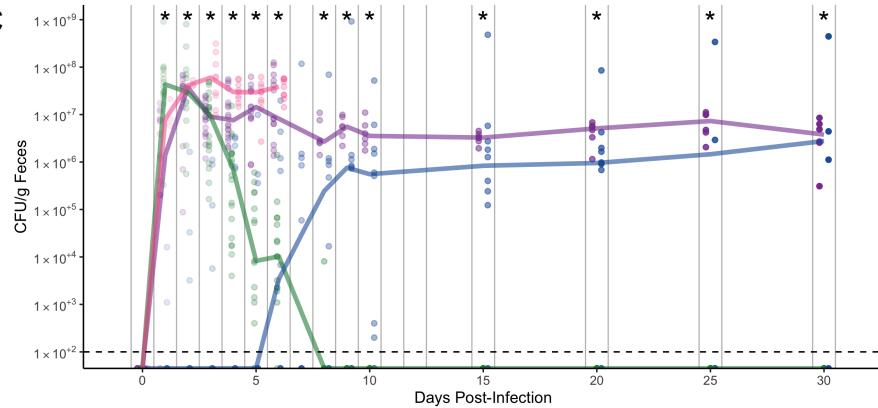
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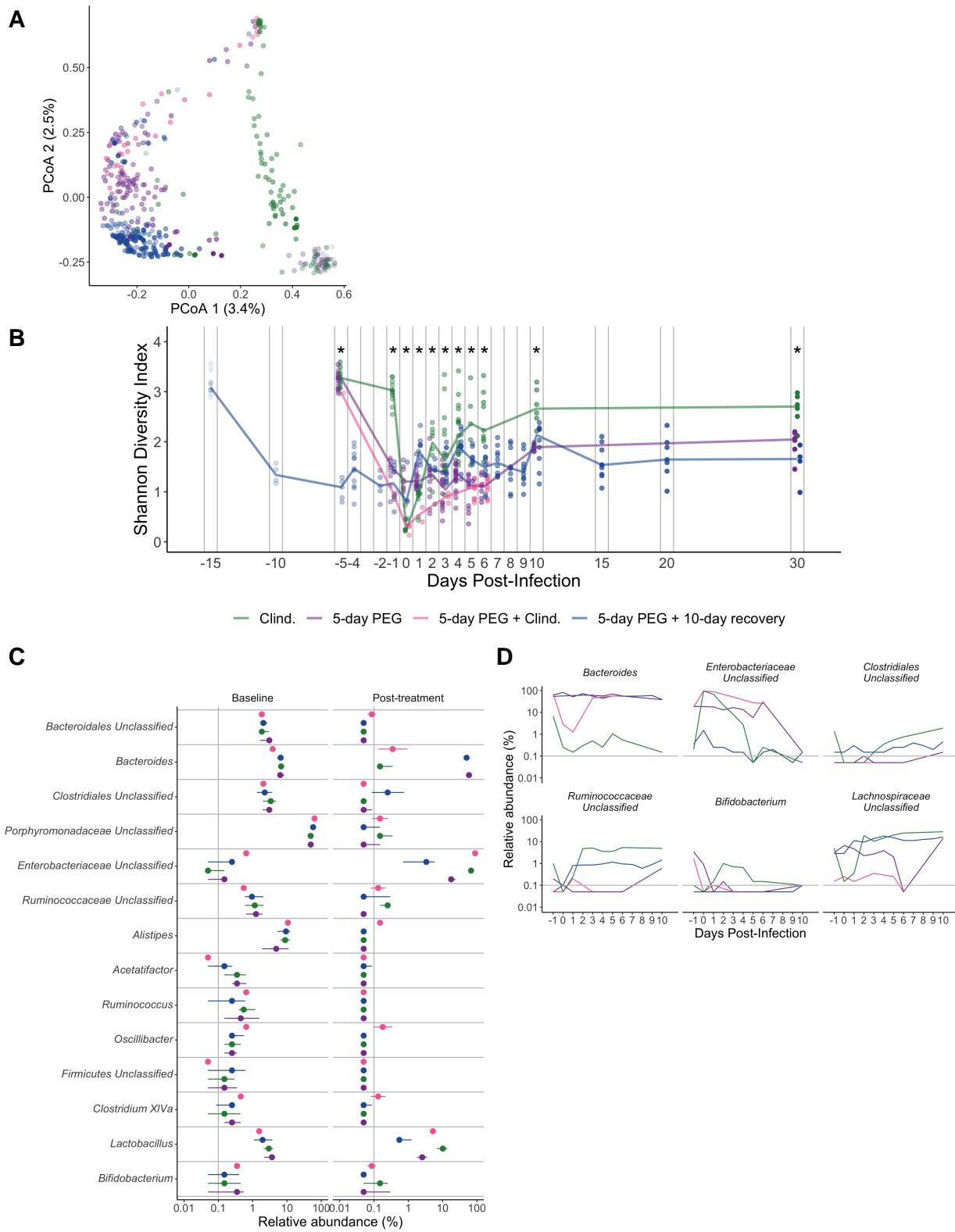
C



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156 **Figure 1. 5-day PEG treatment prolongs susceptibility and mice become persistently**
157 **colonized with *C. difficile*.** A. Setup of the experimental timeline for experiments with 5-day PEG
158 treated mice. Clindamycin was administered at 10 mg/kg by intraperitoneal injection. 15% PEG
159 3350 was administered in the drinking water for five days. B. Weight change from baseline weight
160 in groups after treatment with PEG and/or clindamycin, followed by *C. difficile* challenge. C. *C.*
161 *difficile* CFU/gram stool measured over time (N = 16-59 mice per timepoint) via serial dilutions. The
162 black line represents the limit of detection for the first serial dilution. CFU quantification data was

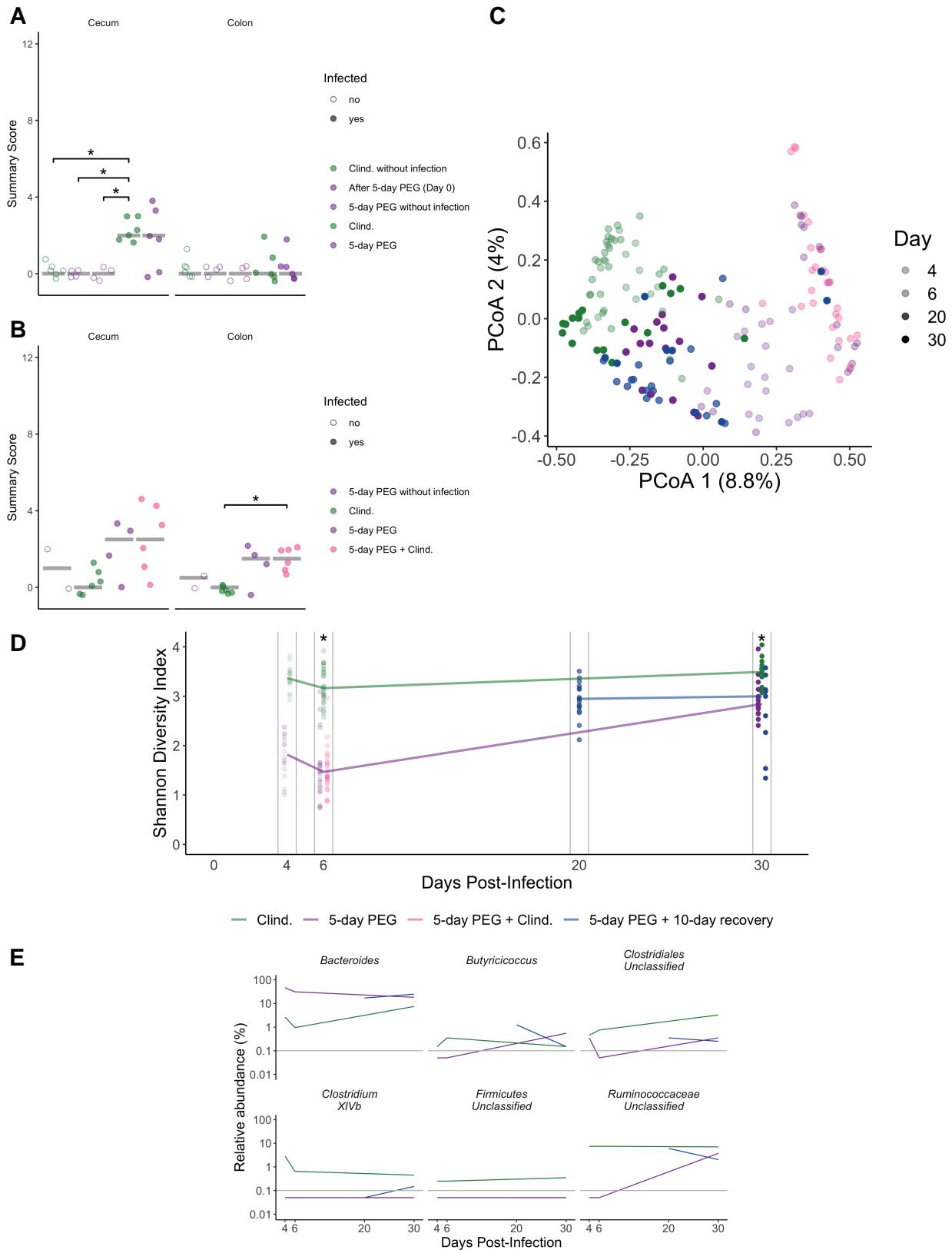
163 not available for each mouse due to stool sampling difficulties (particularly the day the mice came
164 off of the PEG treatment) or early deaths. For B-C, lines represent the median for each treatment
165 group and circles represent samples from individual mice. Asterisks indicate timepoints where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



169

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

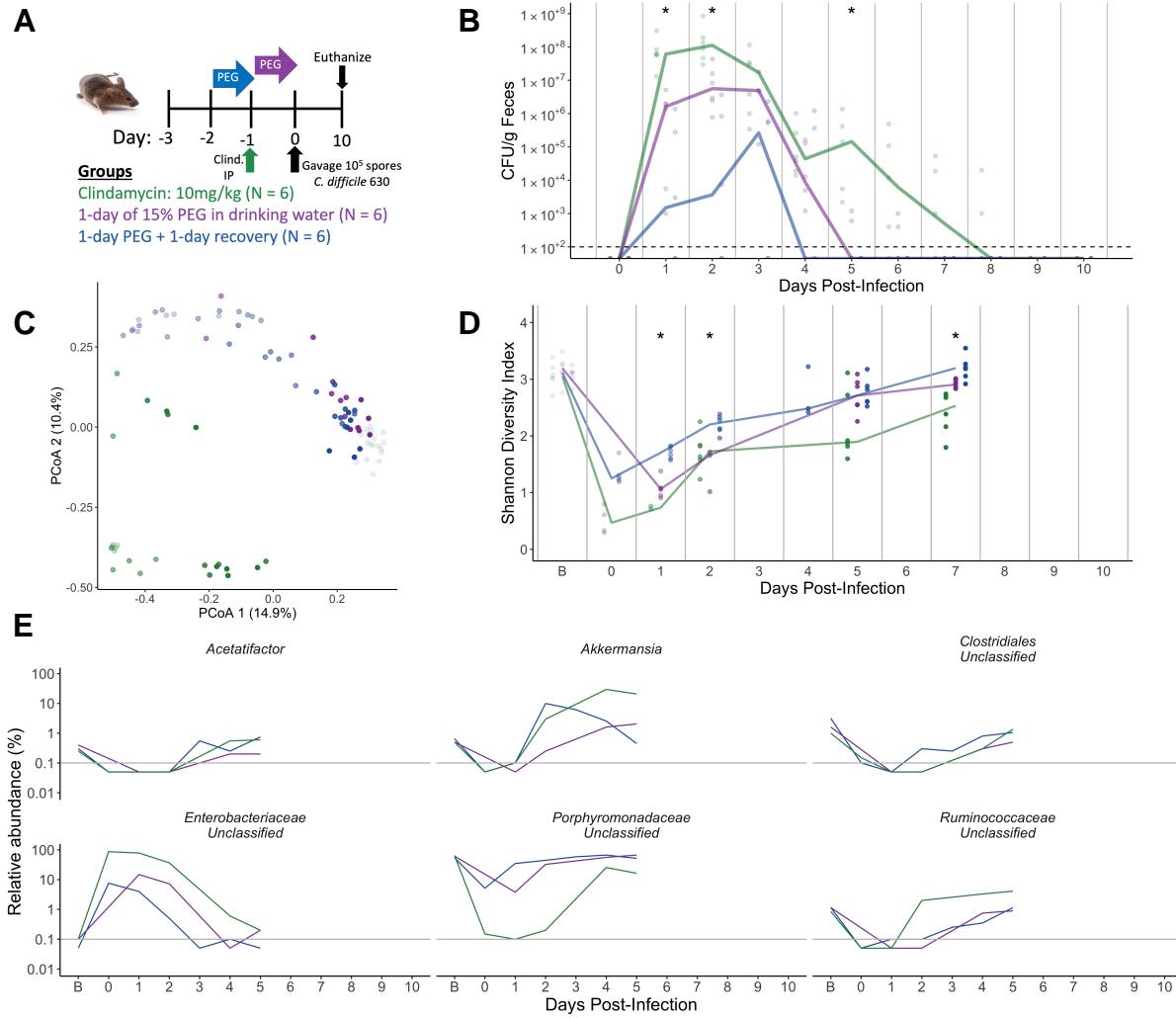
¹⁷¹ compared to clindamycin-treated mice. A.



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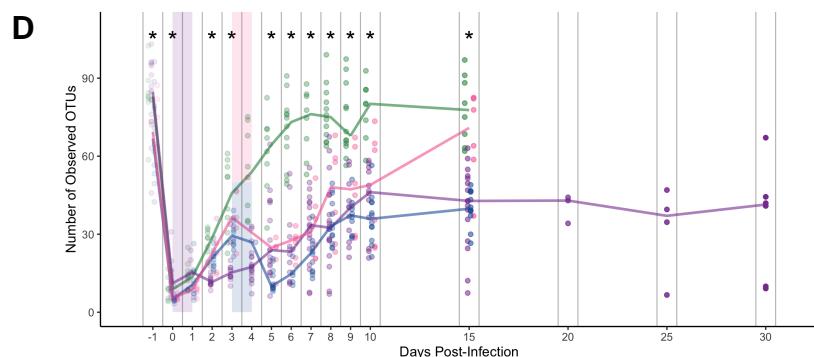
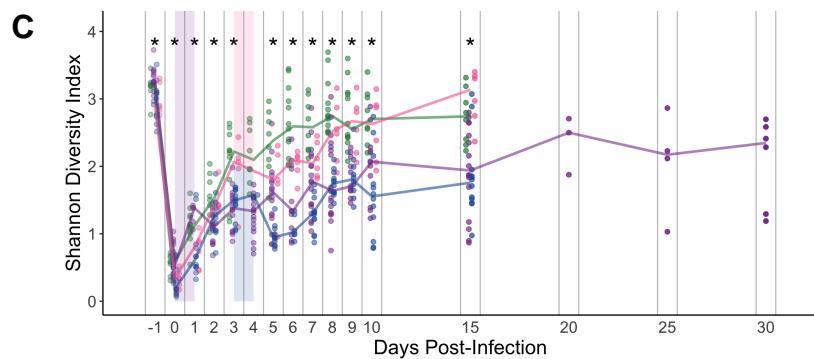
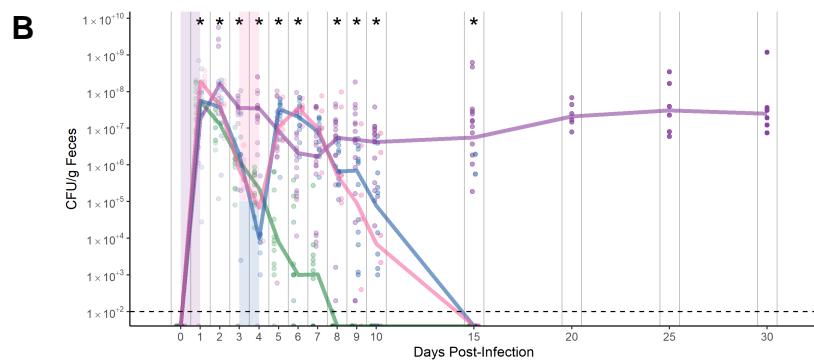
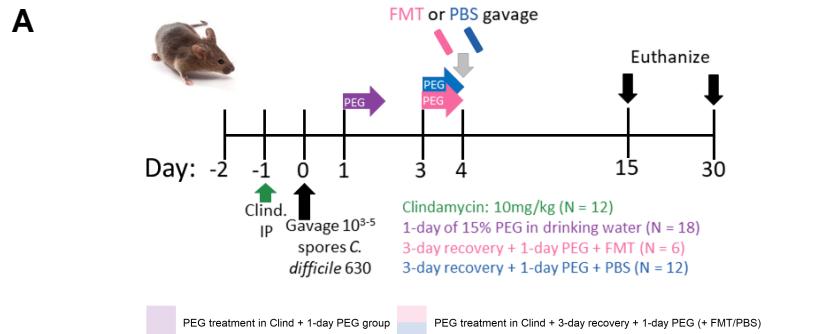
173 **Figure 3. 5-day PEG treatment does not result in more severe CDIs, although mucosal**

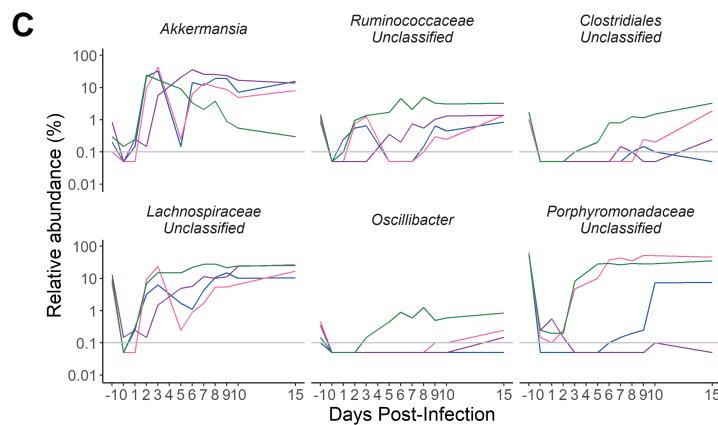
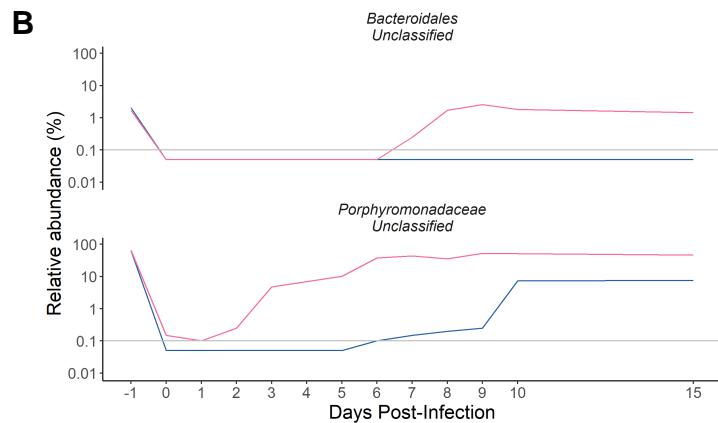
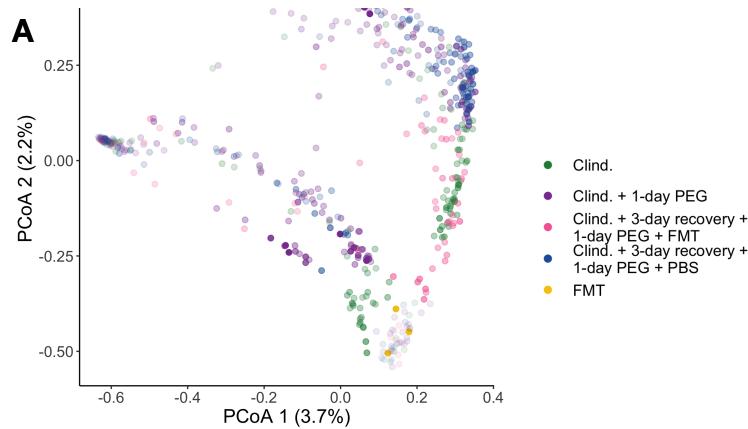
¹⁷⁴ **microbiota is altered.** A.



175

176 **Figure 4. 1-day PEG treatment renders mice susceptible to transient *C. difficile***
 177 **colonization.** A. Setup of the experimental timeline for the 1-day PEG treated subset of
 178 mice. B. CFU/gram stool measured over time (N = 12-18 mice per timepoint) via several dilutions.
 179 The black dotted line represents the limit of detection for the first serial dilution. Asterisks indicate
 180 timepoints where the CFU/gram was significantly different between groups using the Kruskall-Wallis
 181 test with a Benjamini-Hochberg correction for multiple timepoints. C. Principle Coordinate Analysis
 182 plot of the groups over time with the alpha representing the same time scale as in panel D (day:
 183 $R^2 = 0.43$; group: $R^2 = 0.19$). D. Shannon diversity Index of the groups over time. Only days with
 184 samples from all groups are shown. E. Line plots of relative percent abundance of selected genera
 185 over time. Only days with samples from all groups shown. The gray line represents the limit of
 186 detection.

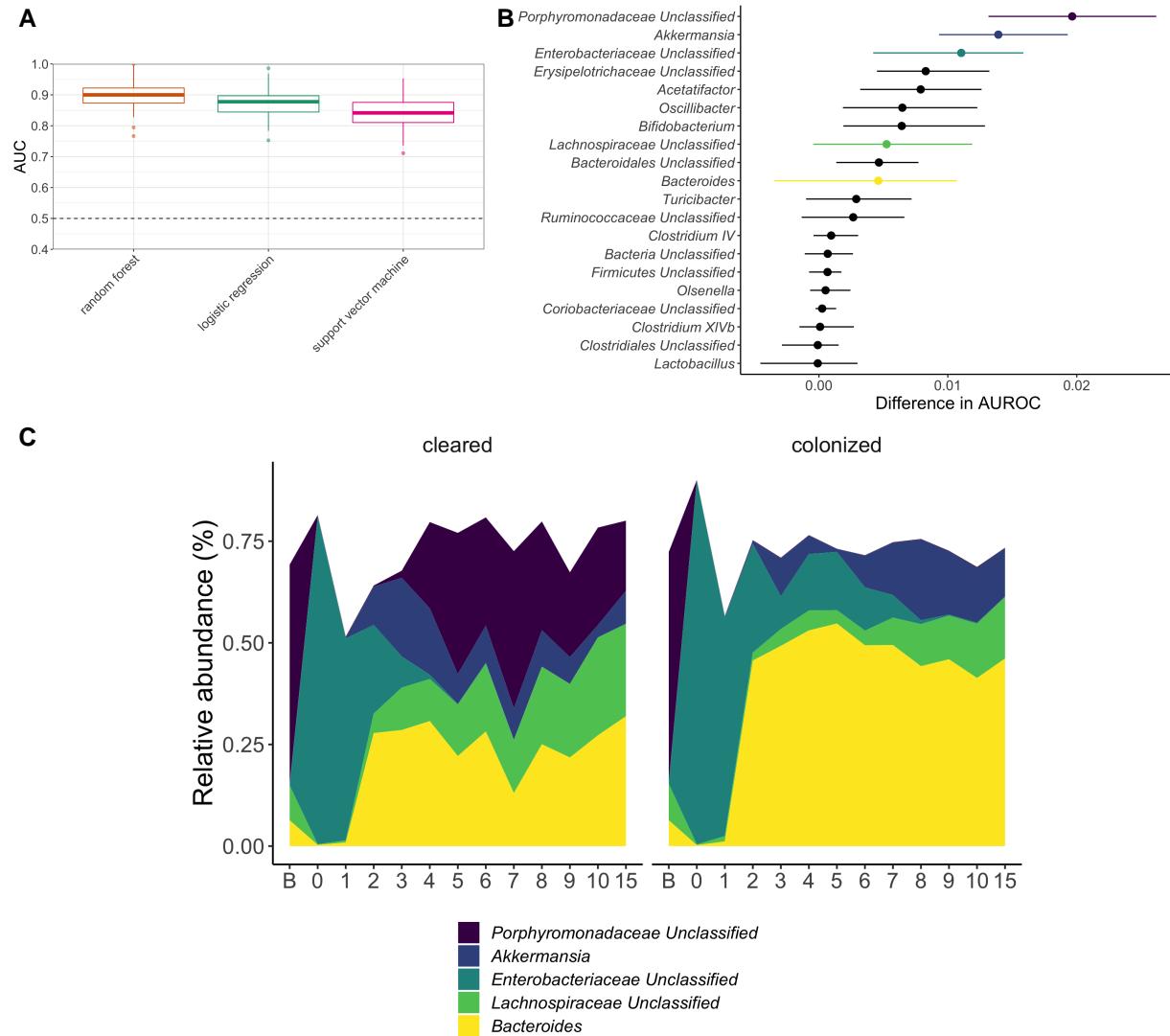




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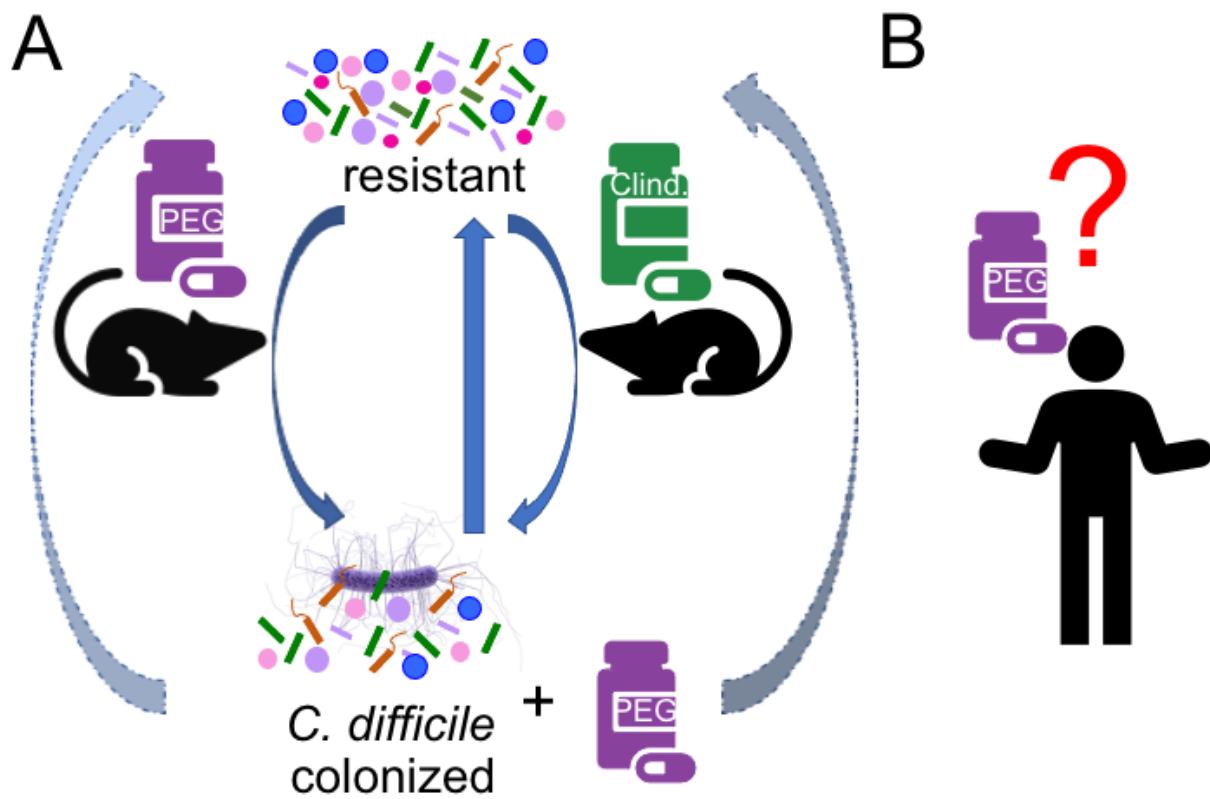
Figure 5. 1-day PEG treatment

189 **post C. difficile challenge prolongs colonization regardless of whether an FMT is also
190 administered. A.**



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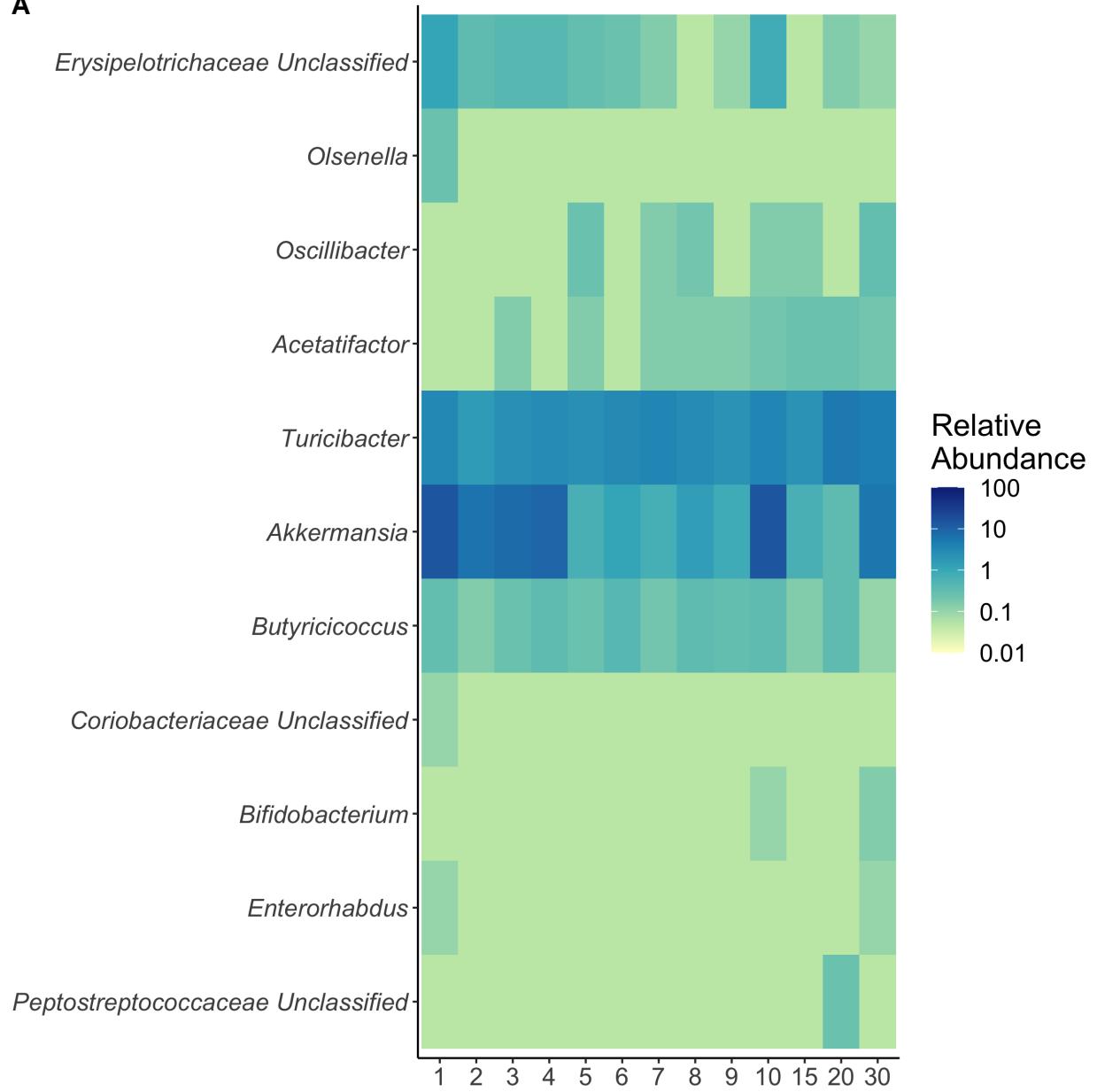
192 **Figure 6. Specific microbiota features associated with prolonged *C. difficile* colonization**193 **in PEG treated mice. A.**



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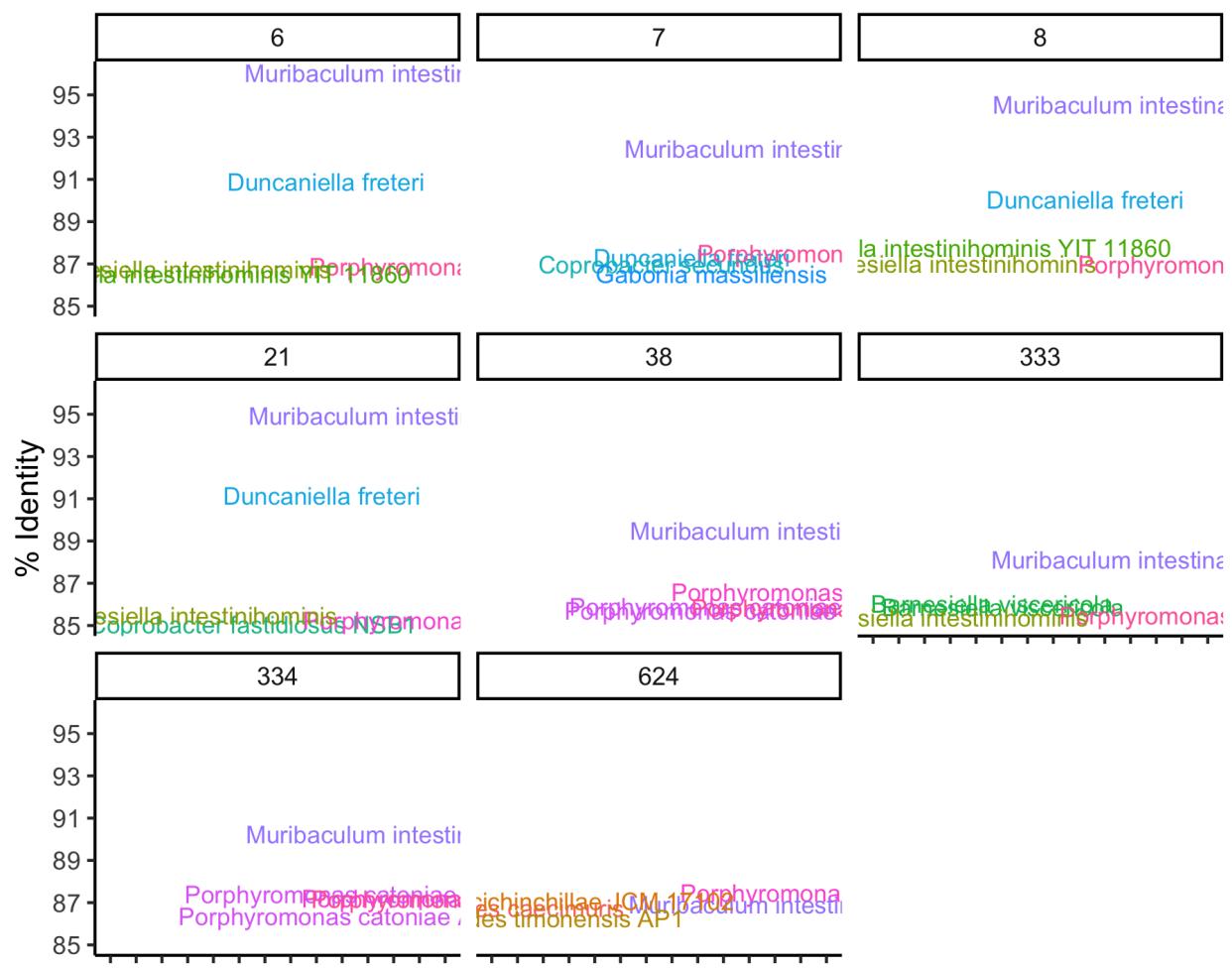
195 **Figure 7. Schematic summarizing findings. A.**

A



A

Potential Muribaculum OTUs Blastn Results



199

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

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