Reviewer #1 (Comments for the Author):

In this manuscript, Tomkovich et al. examined experiments to show how PEG impacts mice susceptibility to C. difficile. They found PEG treatment alone was sufficient to render mice susceptible and 5-day PEG-treated mice remained colonized for up to 30 days post-challenge. In contrast, 1-day PEG treated mice were transiently colonized, clearing C. difficile within 7 days post-challenge. They concluded that osmotic laxatives disrupt colonization resistance to C. difficile, and prevent clearance among mice already colonized with C. difficile.

The study is very interesting and the data is strong to support their findings. I only have several minor comments following:

Line 47-49 Please clarify and make it more clear to led to your hypothesis.

> We have revised to: "These results led to the hypothesis that bacterial communities from patients experiencing diarrhea are susceptible to developing CDIs, regardless of how they developed diarrhea. For example, laxatives may disrupt colonization resistance to *C. difficile*."

Line 720 Please clear Asterisks mean. Authors noticed it as the difference between groups, it's not clear which two groups (inter-group at the same time point or same group compared to day 0) were compared and showed a significant difference, please clear it for Fig. 1B and 1C.

> We have rephrased to the following: "Asterisks indicate the specific time points on the x-axis where the weight change or CFU/g was significantly different ($P < 0.05$) between the 3-4 groups indicated on the plot by the Kruskal-Wallis test with Benjamini-Hochberg correction for testing multiple time points."

Did the author detect PEG concentration from stools along with experiments? Or any references can be referred to PEG metabolization and residence time in the gut?

> We did not measure PEG concentrations as previous studies report PEG is nonabsorbable and not metabolized by bacteria (Hammer et al. JCI 1989; Di Palma et al. The American Journal of Gastroenterology 2002).

Line 148 Did author detect the toxin concentration from fecal samples? It's interesting that more C. difficile CFU was detected and high weight loss in PEG treated group, while no significant difference in the gut.

We did not measure toxin concentration because we observed no histological differences between PEG and clindamycin treated mice. We agree it is an interesting observation, but suspect this may be partially due to the strain we used (*C. difficile* 630), and bring up this possibility in the discussion (lines 315-320).

Line 176: Is it possible to include the histopathology figures in Fig. 3D?

C. difficile 630 typically causes mild histopathology and pictures are included in Theriot et al. Gut Microbes 2011 which is referred to in the methods section (lines 441-442). Because of figure size constraints and since histopathology was mild for all groups, we decided not to include histopatholgy pictures.

Fig. 3D Label symbols of Clind. Without infection and Clind, After 5-day, 5-day PEG without infection and 5-day PEG are the same. Please correct.

The open and closed circles differentiate mice that were infected with *C. difficile* versus mice that were not. However, there were 2 sets of open circles that were the same color for the After 5-day PEG (Day 0) and 5-day PEG without infection groups, so we have added an additional shape to differentiate the Day 0 group in the Fig. 3D legend. Additionally, we have combined the scales to make the differences between groups more clear (Fig. 3D-E).

Line 276-277 Could you notice which result Figure (Maybe Fig. 3C) you are discussed to help the reader to follow?

We have added a reference to Fig. 7B in the sentence.

Line 284-285 Spores persistence in cell should be also noticed in this part as it can also contribute to C. difficile persistence. Ref: Castro-Córdova P, Mora-Uribe P, Reyes-Ramírez R, et al. Nature communications, 2021.

We have modified the sentence to the following: "One possible explanation for the prolonged *C. difficile* colonization in 5-day PEG treated mice, might be due to the bacteria's persistence in the mucosal compartment either within host cells (Castro-Córdova et al. Nature Communications 2021) or together with other bacteria."

Line 256-358 So many parts for discussion, maybe can concentrate and minimize paragraphs.

The manuscript is within mSphere's 5000 word limit (excluding the Materials and Methods, References, and figure legends) for research articles. Given all the available literature on the

microbiota, laxatives, and *C. difficile*, we wanted place our findings in context, which resulted in a longer discussion.

Line 390 Stool was collected as fresh one or obtained from cages? How did you process your stool samples? Plating every day for CFU check or stock -80 {degree sign}C and did CFU check at the end of the experiment?

We have added the following sentences to clarify our stool processing methods: "Fresh stool samples were collected from each mouse and split into two separate tubes. One tube was transferred to an anaerobic chamber the same day the sample was collected to quantify *C. difficile*, while the other tube was snap frozen in liquid nitrogen and stored in the -80°C for 16S rRNA sequencing."

Specific comments: Over-referred in this manuscript (80 references were included).

There is no limit on the number of references for mSphere research articles.

Typos Line 4 Alter-alters Line 59 Challenge-the challenge Line 181 Signifant—Significant Line 267 received Line 312 Colonization

We thank the reviewer for identifying these typos and have corrected all except for line 4. Alter was used because we are stating multiple medications alter the microbiota and just giving PEG as a specific example.

Reviewer #2 (Comments for the Author):

As expected from this group, this is a well conducted study that is timely relevant for CDI. In this work authors address an intriguing question related with how laxative treatment affects the susceptibility of the host to C. difficile infections. Authors provide a comprehensive and very detailed overview of the impact of the osmotic laxative, PEG, to shifts in fecal and mucosal microbiota, and how PEG impacted the susceptibility of mice to infection and subsequent clearance. This work is of great public relevance and complements several other studies published on this topic.

I only have minor comments for the authors.

Specific comments: Line 324: not clear please revise

We have revised the sentence to the following: "The extent to which laxatives disrupt *C. difficile* colonization resistance in human patients is unclear based on the current literature."

Figure 5: is there any reason why FMT was not used alone as a control in this experiment? There is data on how FMT impacts the microbiota composition in Figure 6, but not in the C. difficile infection experiment. Could the authors clarify this?

We did not use FMT alone in this experiment because clindamycin treated mice typically clear *C. difficile* within 10 days without any additional treatments such as an FMT.

Figure 8: Please revise the narrative of the figure legend for consistencies in language.

We have modified the legend to: "The gut microbiota of our lab's C57Bl/6 mice is resistant to *C. difficle* but treatment with either clindamycin or the osmotic laxative, PEG 3350, renders the mice susceptible to *C. difficile* colonization. Recovery of colonization resistance in clindamycin-treated mice is relatively straightforward and the mice clear *C.difficile* within 10 days post-challenge. However, recovery of colonization resistance was delayed for mice that received either a 5-day PEG pre-treatment or a 1-day PEG post-*C. difficile* challenge treatment. We found increased relative abundances of *Porphyromonadaceae* and *Lachnospiraceae* were associated with *C. difficle* clearance, while increased relative abundances of *Enterobacteriaceae* and *Bacteroides* were associated with prolonged *C. difficle* colonization."