To:

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Re:
Manuscript Ref.: 86113
Title: Novel, Non-colonizing, Single-strain Live Biotherapeutic Product, ADS024, Protects Against *Clostridioides difficile* Infection Challenge *In Vivo*

Dear Dr Jin-Lei Wang, Dr Somchai Amornyotin, Kusum Kharbandaand, Dr Tsutomu Nishida,

We are pleased to resubmit our manuscript entitled “Novel, Non-colonizing, Single-strain Live Biotherapeutic Product, ADS024, Protects Against *Clostridioides difficile* Infection Challenge *In Vivo*” for publication consideration in *World Journal of Gastrointestinal Pathophysiology.*

Thank you to you and the peer reviewers for thoroughly assessing our manuscript. Please find responses to each point below. Page numbers are referenced to the “Clean” version of the manuscript. Edited or new text can be seen as highlighted changes in the clean manuscript (Main Text file).

In addition to our response to the reviewer’s comments, we have made an edit to page 14: Among the 7 genera positively impacted by the exposure to ADS024, increases in *Bifidobacterium* (approximately 12%; $P < 0.00003$) and *Bacteroides* (approximately 2%; $P < 0.05$) were found compared with media-only

treatment (Figure 3D). The original P value was rounded to 4 decimals points. The number is 0.0000332820948665567 and we have elected to round the figure to 5 places as 0.00003.

We remain appreciative of your time and consideration as you review our submission. Should you require additional information, please feel free to reach out via the contact information listed below.

We look forward to your response.

Sincerely,

Laurent Chesnel, PhD

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Chesnel et al.
Point-by-Point Response to Editors

Reviewer #1

Overview

The manuscript by Murphy et al describes the promising Bacillus velezensis strain ADS024 as a live biotherapeutic product that protects significantly against infections caused by Clostridioides difficile in the animal model study. Overall, the study is an extension of the previous work reported by the same group where they have characterized the strain ADS024 isolated from the human fecal sample and studied its potential to inhibit the CDI in vitro assays. The current study takes into account if the strain can be used as a live biotherapeutic agent in future human trials against CDI. The study reveals promising results, specifically its antagonistic potential against C. difficile. The study has been well designed, executed, and presented well. I congratulate the authors for their nice work. As such I do not have any specific comments for the authors, but would like to get some clarifications that may be incorporated in the manuscript.

Point 1A

The fig. 1B shows the log10 CFU of ADS024 for different treatment groups for different time durations. However, in the placebo (no ADS024 addition) the CFU has also been represented for ADS024 which is difficult to understand. The authors may clarify this.

Point 1A Response

We thank the reviewer for raising this important point. In figure 1B we are showing *Bacillus*-like colonies for the placebo group. ADS024 colonies (assessed by quantitative polymerase chain reaction [qPCR] using ADS024-specific primers), were present only in the ADS024-treated groups. The placebo group, which did not receive ADS024, by growing colonies (albeit non-ADS024 ones), still has Log10 CFU/g values for *Bacillus*-like colonies. We have elaborated on this point to ensure greater clarity on page 11 and in the Figure 1 legend.

Point 1B

The single dose vs triple dose of ADS024 does not show a significant difference in the study which needs some additional clarification in the text.

Point 1B Response

Thank you for the opportunity to further clarify this point. The stress from 3x /day oral dosing in the animals likely negated any positive effect of the ADS024. Stress would likely increase assessed disease markers if there were an infected placebo arm with the same dosing regimen. We had already implicated stress in the original version's Results section on page 13: "In contrast, the ADS024 TID group did display adverse clinical features (Figure 2E and F), perhaps due to the TID oral gavage–related stress." We have now further emphasized 'stress due to oral gavage' as a plausible explanation for the lack of a significant difference in efficacy between the two aforementioned doses in the Discussion section on pages 17, which is also supported by a new reference (#32).