Peer Review File

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Reviewer A

1)The annotations in Figures 4 and 5 are different from their legends. Please carefully check and make corrections.

Authors' response: We have changed the original Figures 4 and 5 into the correct version. The correct figures will be uploaded with our revision. We apologize for our mistake.

2)There are too few behavioral experiments in this study, and it is recommended to add other detection methods and results.

Authors' response: Unfortunately, only the Y-maze test and the tail suspension test were conducted during the experiments, and it is impossible for us to add any other behavioral experiment since the rats are already euthanized. We have added this limitation to the conclusion portion of the abstract, and thank you for pointing this out.

3)Suggest comparing the differences in intestinal flora among different dietary groups of cerebral palsy rats and examining whether these differences are related to gastrointestinal dysfunction.

Authors' response: Unfortunately, dietary difference wasn't considered as an experimental variable in this study. All the rats from all groups were given the same diet. Thus, we can't assess the relation of differences in diets and gastrointestinal dysfunction in this study. However, we have conducted other studies that can be referenced, for example, "Dietary fiber and probiotics based on gut microbiota targeting for functional constipation in children with cerebral palsy, Frontiers in Pediatrics, PMID: 36313885", which include two different diet groups, general diet and liquid diet. We have added this limitation to the conclusion portion of the abstract, and thank you for pointing this out.

4)What are the effects of different gut microbiota on cytokine expression in patients with cerebral palsy? It is recommended to add relevant content.

Authors' response: We have incorporated the effects of different gut microbiota on cytokine expression in patients with cerebral palsy in the Introduction of article. The details are as follows:

Different gut microbiomes have different effects on general health, as well as diseases. For example, there is evidence of prebiotics being able to reduce the amount of cytokines in humans as well as animals; however, only a few studies suggest that prebiotics can enhance immune system functions (3). As for patients with cerebral palsy, studies suggest that excessive Prevotella may be a biomarker for CP (4), and Prevotella is related to inflammation (5), suggesting that distinctive gut microbiota of CP patients contribute to their high levels of cytokines.

5) Figure 11 are not clear enough. It is recommended to provide clearer figure again.

Authors' response: A clearer version of Figure 11 has been uploaded and incorporated into the revised version of our article. We apologize for this mistake.

6)The introduction part of this paper is not comprehensive enough, and the similar papers have not been cited, such as “Gut-liver axis-mediated mechanism of liver cancer: A special focus on the role of gut microbiota, Cancer Sci, PMID: 34533882”. It is recommended to quote this article.

Authors’ response: We have cited this article in the introduction part of our paper as the ninth cited article. The details are as follows:

Gut microbiota has important effects in the general health of human, and it plays a role in the development of many kinds of diseases, for example, cancer (9) and neurological disorders (10).

7)It is recommended to add research progress on the relationship between intestinal flora and cerebral palsy in the discussion.

Authors’ response: Research progress on the relationship between intestinal flora and cerebral palsy has been added to the discussion section. The details are as follows:

We further found that though other studies suggesting that interventions with dietary fiber, lactic acid-producing bacteria, and butyric acid-producing bacteria can significantly alter gut microbiota diversity for children with CP (8), intervention with L. rhamnosus OF44 changed the gut microbial composition of CP rats to some extent, but the overall composition and diversity of intestinal flora were not significantly changed.

Thank you very much for your attention and time. Looking forward to hearing from you.

Reviewer B

1) First, the title needs to indicate the comparisons across L. rhamnosus, S. boulardii, and placebo, the research design such as a RCT, and the outcomes of this study such as inflammation biomarkers and depression-like behaviors.

We have changed the title of our article to “Randomized Controlled Trial Comparing the Impacts of Saccharomyces boulardii and Lactobacillus rhamnosus OF44 on Intestinal Flora in Cerebral Palsy Rats: Insights into Inflammation Biomarkers and Depression-like Behaviors.”

2) Second, the abstract needs some revisions. The background did not indicate the potential clinical significance of this research focus and why there is a clinical need to validate the efficacy of L. rhamnosus OF44. The methods need to specify the number of rates, how the three interventions were assigned, and efficacy outcome measures. The results need to quantify the findings by reporting the outcome measures and accurate P values. All outcomes should be reported, as well their comparative results. The conclusion needs comments for the clinical implications of the findings and the limitations of this study.

(1) We have added information regarding the clinical significance of calidating the efficacy of L. rhamnosus OF44 in the background of the abstract. The details are as follows:

Validation of the effects of L. rhamnosus OF44 on cerebral palsy adds to its confirmed effects in treating osteoporosis and reproductive tract microbiota disorders, increasing its potential as a probiotic.

(2) We have specified that the rats were exposed to L. rhamnosus OF44, S. boulardii, or normal saline gastric gavage daily for 28 days in the methods section of the abstract. For the efficacy outcome measures, it is specified that the efficacy of the outcome is measured by performing statistical analysis like the t-test on the data to see its significance.

(3) In the results section of the abstract, we have included all outcomes with specific P values. The details are as follows:

Before intervention, CP rats failed to exhibit depression-like behavior ($P = 0.6$). L. rhamnosus OF44 treatment significantly reduced the level of IL-6 ($P = 4.8e-05$), S. boulardii treatment significantly reduced the level of (TNF)-α ($P = 0.04$). In addition, both treatments altered the composition and complexity of the gut microbiome.

(4) The conclusion part of the abstract has been changed so that it includes comments for the clinical implications of the findings and the limitations of this study. The details are as follows:

Our results indicated that L. rhamnosus OF44 has potential in alleviating inflammation and altering the gut microbial composition in CP, and that it has the potential to clinically treat CP. There are some limitations of this study. For example, dietary differences and their effects on gastrointestinal dysfunction are not considered in this study, and only two behavioral experiments were used.

3) Third, the introduction did not explain why there is a clinical need to examine the efficacy of L. rhamnosus OF44. In the presence of known effect of boulardii, why there is a need to examine L. rhamnosus OF44. The authors need to explain why the current animal study could help answer the research question. Please also explain why the proposed outcomes can indicate the potential of L. rhamnosus OF44 for CP.

We have explained the clinical significant to examine the efficacy of L. rhamnosus OF44 in cerebral palsy. We have also explained why the current animal study can help answer this question, and we have explained the ways the proposed outcomes indicate the potential of L. rhamnosus OF44 for CP. The details are as follows:

However, despite the identified effects of S. boulardii, its unpredictable and less pronounced impact on gut microbiota of cerebral palsy mice suggests it may not be the ideal microbe for cerebral palsy treatment (19). Additionally, given the proven efficacy of L. rhamnosus OF44 in treating and preventing osteoporosis and reproductive tract disorders, which both involves inflammation and changes in microbiota (20, 21), exploring its role in cerebral palsy opens avenues for investigating other potentially more effective treatments for cerebral palsy, as well as the full clinical potential of L. rhamnosus OF44.

The current animal study is crucial in addressing the research question as it delves into the efficacy of L. rhamnosus OF44 in the context of CP by elucidating the distinct impact of L. rhamnosus OF44 on inflammation, behavior, and gut microbiota. The proposed outcomes serve as meaningful indicators of the potential effectiveness of L. rhamnosus OF44 for cerebral palsy because various factors contributing to the development of CP, for instance, gut microbiota and inflammation, are addressed.

4) Fourth, in the methodology, please describe the sample size estimation procedures, randomization method, and the underlying rationale for these outcome measures. The authors need to have a separate part to describe the statistical analysis of this study, in particular how the repeated measurement data were analyzed and how the post-hoc comparisons across groups were performed.

We have explained the methods of randomization and sample size estimation. We have also explained the rationale behind these methods. The details are as follows:

We employed a randomized parallel controlled trial design with simple randomization, using 18 CP model rats and 6 healthy rats as experimental subjects. We estimated the number of rats used with references of other studies in the same area (3, 12). Simple randomization ensures that each rat has an equal chance of being assigned to each group. This minimizes the bias of selection in the experiment. By choosing to estimate sample size with accordance to other studies on cerebral palsy and gut microbiota, our study is aligned with existing research, and the validity of our study is enhanced.

5) Finally, please cite several related papers: 1. Chen D, Huang M, Yin Y, Gui D, Gu Y, Zhuang T, Chen C, Huo K. Risk factors of cerebral palsy in children: a systematic review and meta-analysis. Transl Pediatr 2022;11(4):556-564. doi: 10.21037/tp-22-78. 2. Kamal S, Hamzaid NH, Kamaralzaman S, Sharma S, Jaafar NH, Chern PM, Hassan NI, Toran H, Ismail NAS, Yusri G. Nutritional status as predictors for quality of life among caregivers of children with severe cerebral palsy. Transl Pediatr 2023;12(9):1601-1618. doi: 10.21037/tp-23-195. 3. Xue Y, Shi S, Zheng S, Yang Z, Xu J, Gong F. Therapeutic effect of scalp-based acupuncture and moxibustion as an adjunctive treatment on children with cerebral palsy comparing to conventional rehabilitation therapy: a systematic review and meta-analysis of randomized controlled trials. Transl Pediatr 2022;11(5):631-641. doi: 10.21037/tp-22-85.

These three papers are cited as the second, sixth, and seventh cited papers in our article, respectively in the Introduction section. The details are as follows:

CP is commonly triggered via brain injuries or malformation before, during, or after birth (2). Though CP has negative effects on both the patients and their caregivers (6), there currently exists no curative treatment for CP; however, therapy and treatment have been shown to alleviate the symptoms, including acceptance and commitment therapy, action observations, and bimanual training, among about 20 other treatments (4, 7).

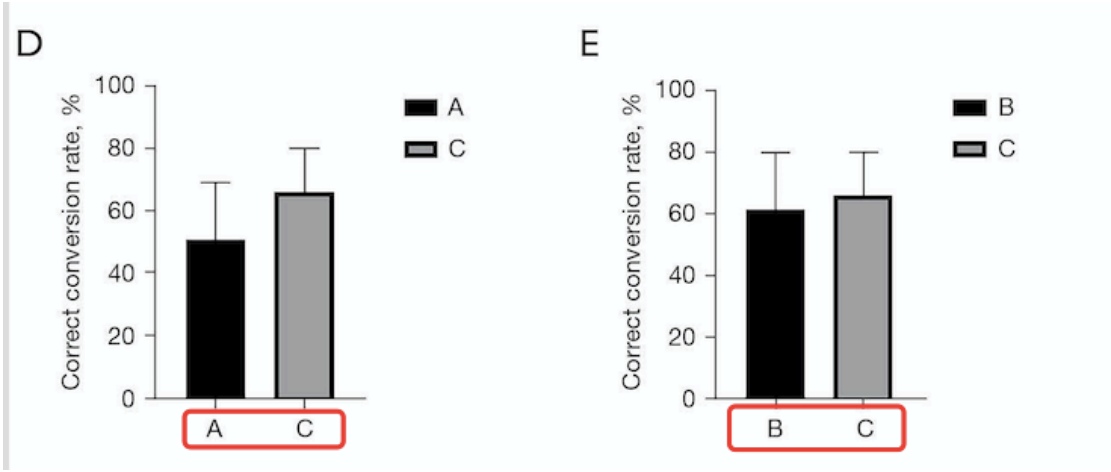
Thank you very much for your attention and time. Looking forward to hearing from you.

Reviewer C

1. Figure 2

It seems that figure legends 2D and 2E do not match the figures 2D and 2E. Please check and revise.

848 correct conversion rate between the control group and group C; (D) comparison of the
849 correct conversion rate between group B and group C; (E) comparison of the correct
850 conversion rate among the 4 groups; (F) comparison of all 4 groups. Group A, *L.*

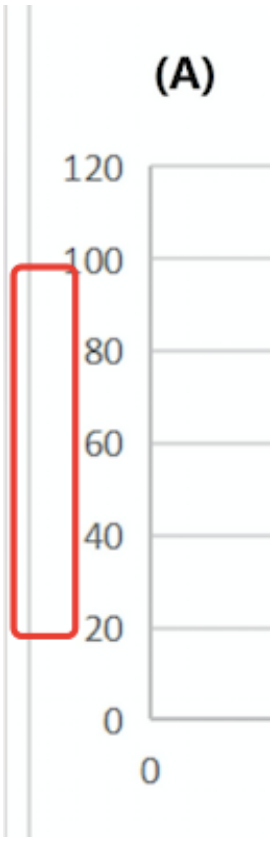


Reply: The legend of Figure 2 is revised, the details are as follows:

Figure 2 Y-maze test conversion rates of the different groups. (A) Comparison of the correct conversion rate between the control group and group A; (B) comparison of the correct conversion rate between the control group and group B; (C) comparison of the correct conversion rate between the control group and group C; (D) comparison of the correct conversion rate between group A and group C; (E) comparison of the correct conversion rate between group B and group C; (F) comparison of the correct conversion rate among the 4 groups. Group A, *L. rhamnosus* OF44 treatment group; group B, *S. boulardii* treatment group; group C, physiological saline treatment group; control, healthy control group. CP, cerebral palsy.

2. Figure 4

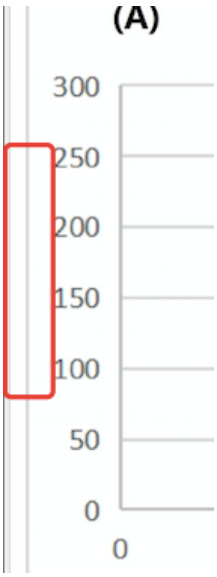
Please add the description of the Y-axis in figure 4(A-D).



Reply: We have added the description of the Y-axis in figure 4 and inserted the revised graph into the manuscript. We have also sent you a separate revised graph via email.

3. Figure 5

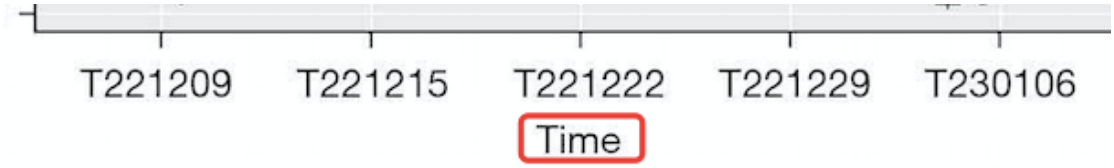
Please add the description of the Y-axis in figure 5(A-D).



Reply: We have added the description of the Y-axis in figure 5 and inserted the revised graph into the manuscript.

4. Figure 7A-C

Please add the unit of time in the X-axis.



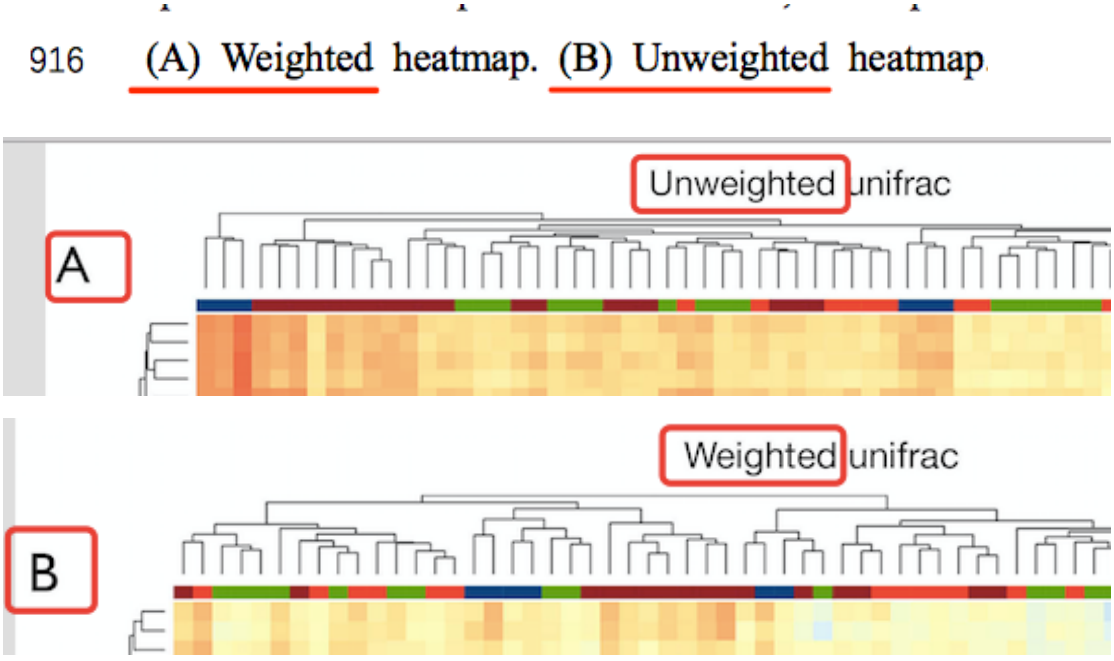
Reply: The "time" here is actually the date on which we performed the experiments. For example, T221209 means Dec 9th of 2022. We have included the explanation in the legends of Figure 7 and Figure 12. The details are as follows:

Figure 7 Changes in the gut microbiota in the 4 groups through time. The horizontal coordinate represents time points. Each time point contains the groups of different intervention methods. The vertical coordinate refers to value of the chao1 diversity indicator of the different groups. The box plot shows 5 statistics (minimal, first quartile, median, third quartile and maximal; i.e., 5 lines from bottom to top), with the outliers labeled with "o". (A) Chao1 index. (B) Shannon index. (C) Simpson index. Group A, *L. rhamnosus* OF44 treatment group; group B, *S. boulardii* treatment group; group C, physiological saline treatment group; Blank, healthy control group. CP, cerebral palsy. T221209 means Dec. 9th, 2022, or Day 0, and so forth.

Figure 12 Differential metabolic pathways in the group A rats at different intervention times. PWY, prefix for pathways; E, Entner; D, Doudoroff; Glycolysis ED pathway refers to Entner-Doudoroff pathway; LDA, Linear Discriminant Analysis. T221215 stands for Dec. 15th, 2022, or Day 7 of the experiment. T230106 stands for Jan. 6th, 2023, or Day 28 of the experiment.

5. Figure 8

The figure legends 8A and 8B do not match the figures 8A and 8B. Please check and revise.



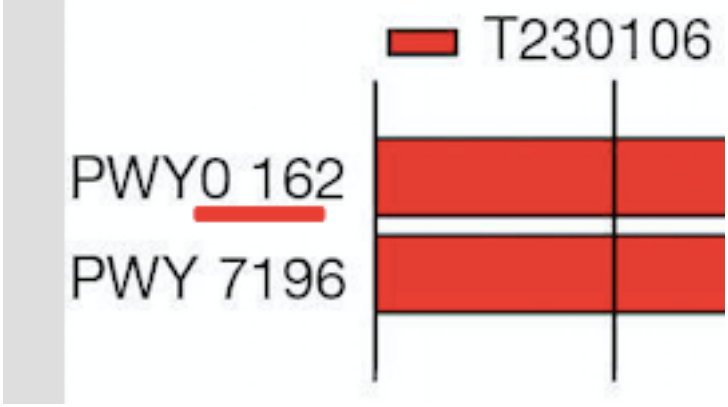
Reply: We have corrected the legend of figure 8. The details are as follows:

Figure 8 Changes in gut microbiota of the rats are shown by the beta diversity heatmap. Based on the statistical results of the differences of each specimen, cluster dissection on samples was implemented. Additionally, the difference between samples was calculated to estimate the similarity of species composition in the different specimens. If the specimens are closer, their species compositions are more similar. (A) Unweighted heatmap. (B) Weighted heatmap. Group A, *L. rhamnosus* OF44 treatment group; group B, *S. boulardii* treatment

group; group C, physiological saline treatment group; Blank, healthy control group. CP, cerebral palsy.

6. Figure 14

Should “PWY0 162” be “PWY 0162”? Please check and revise.



PWY0 162 is the correct name of the pathway instead of PWY 0162.

7. Reference

The authors mentioned “studies...”, while only one reference was cited. Change “Studies” to “A study” or add more citations. Please revise. Please number references consecutively in the order in which they are first mentioned in the text.

Previous studies have suggested that neurological diseases such as CP lead to increased inflammation levels (27).

Despite other studies suggesting that CP affects the emotional states and tail suspension test results of rats (19),

We further found that though other studies suggesting that interventions with dietary fiber, lactic acid-producing bacteria, and butyric acid-producing bacteria can significantly alter gut microbiota diversity for children with CP (8),

Reply: We have revised all sentences mentioning “studies”. The details are as follows:
A previous study has suggested that neurological diseases such as CP lead to increased inflammation levels (27).

Despite another study suggesting that CP affects the emotional states and tail suspension test results of rats (19),

We further found that though another study suggests that interventions with dietary fiber, lactic acid-producing bacteria, and butyric acid-producing bacteria can significantly alter gut microbiota diversity for children with CP (8),