List of Responses

Dear Editors and Reviewers:

We are truly grateful for your letter and for the reviewers' comments concerning our manuscript entitled "Leech *Poecilobdella manillensis* Protein Extract Ameliorated Hyperuricemia through Restoring Gut Microbiota Dysregulation and Affecting Serum Metabolites" (Manuscript NO: 94050). Those comments are all valuable and very helpful for revising and improving our paper, as well as the important guiding significance to our researches. We have studied comments carefully and have made correction which we hope meet with approval. Revised portion are highlighted in yellow in the paper. The main corrections in the paper and the responds to the reviewer's comments are as following:

Responds to the reviewer's comments:

Reviewer #1:

(1) Correct the locations of the sentences. Material and Methods section includes different numbered headings which are incorrectly identified in the text. Ex: "Hepatic tissue supernatant (detailed methods in 3.8.) was employed for the assessment of xanthine oxidase (XOD) activity" (Lines 5-6 form bottom page 8).

Response: We are sorry for our negligence in the incorrect referencing of sections within our manuscript. We have corrected the locations of the sentences and highlighted these corrections in yellow within the text to ensure they are easily identifiable.

(2) How was the tissue (liver and kidney) homogenates obtained for measuring activities and the expression of transporters? Were detergents been included in the homogenization buffer?. (Material and Methods, Section 8 and 9).

Response: Thank you for your regarding the preparation of tissue homogenates used in our study for measuring the activity and expression of transporters. For the homogenization of liver, jejunal and kidney tissues, the tissues were initially rinsed with pre-cooled PBS (0.01 M, pH=7.4) to remove any residual blood. After weighing, the tissues were finely chopped on ice. The chopped tissues were then mixed with PBS at a specific ratio of 10 mg of tissue to 100 μL of PBS, effectively making 1 mL of buffer equivalent to 0.1 g of tissue. This volume was carefully adjusted to meet our experimental requirements. Protease inhibitors were added to the PBS to prevent protein degradation. The mixture was then homogenized in a tissue grinder. To further lyse the tissue cells, the homogenate was subjected to ultrasonication, as required by our experimental protocol. Finally, the homogenate was centrifuged at 5000 × g for 8 minutes at -4 ℃, and the supernatant was collected for analysis. Please note that no detergents were included in the homogenization buffer to preserve the integrity of the proteins and transporters under study. We have highlighted these corrections in yellow within the text to ensure they are easily identifiable.

(3) The eventual histological alteration of liver and kidney induced by the experimental hyperuricemia model in mice would be interesting to be shown. In particular, "signifying the ameliorative effect of LTP on renal barrier impairment in HUA mice" as well as the "the risk of intestinal inflammation and susceptibility to pathogenic invasion" indicated in the Discussion section suggest tissue injury (line 7, page 32; lines 7-8 from bottom 33).

Response: Thank you for your insightful suggestion regarding the inclusion of histological data to demonstrate the tissue alterations induced by the experimental hyperuricemia model in mice. We fully appreciate the significance of visual evidence in substantiating the biochemical results reported in our study. In response to your valuable feedback, we have incorporated histological analyses of renal tissues from the hyperuricemia model, primarily using HE staining. These images are now included in the revised manuscript to directly showcase the effects of LTP treatment on tissue architecture, supporting our discussions regarding its ameliorative effects on renal barrier impairment and the susceptibility to pathogenic invasion. This new data provides a clear visual representation of the tissue recovery facilitated by LTP treatment, particularly in renal tissues. The histological sections illustrate a reduction in tissue damage and improved structural integrity in these critical areas, thereby reinforcing our discussion on the mechanisms by which LTP protects against renal injury in hyperuricemia. Due to the limitations of experimental traditions, we could not provide direct visual evidence of intestinal tissue changes. Moving forward, we will consider addressing this aspect in future studies. We have highlighted these additions in yellow within the manuscript for ease of identification and review.

(4) Typos errors "…that posit alterations in tight junctions…" (line 8, page 32).

Response: Thank you for pointing out the issues with the phrasing in our manuscript. We have corrected "…that posit alterations in tight junctions…" as "This aligns with previous findings that suggest alterations in tight junctions play a crucial role during the repair of renal injury and participate in the pathophysiological processes involved in renal recovery." We believe that this revision more accurately reflects the intended meaning and enhances the

readability of our text.

Reviewer #2:

1. While the methods are generally well-described, more specifics on the dosages used in comparison to traditional treatments could help clarify the potential translational impact of the findings. For instance, comparing the dose response of LTP with standard treatments like allopurinol in terms of efficacy and side effects would provide valuable insights.

Response: Thank you for your comments and suggestions. In pre-experiment, we found that the dosage of 49 mg/kg exhibits the highest efficacy on lower-uric acid when compared with other dosages. Therefore, we utilized 49 mg/kg of LTP to treat the mice. Comparing with allopurinol (5 mg/kg) in the consequent research, we found that the efficacy of LTP was higher than allopurinol, and the side effects was fewer.

In future research, we plan to conduct a more comprehensive assessment of these parameters to thoroughly investigate and compare the dose-response relationships of LTP and allopurinol and their potential clinical translational impacts. This approach will enable us to provide a more complete set of safety data and efficacy comparisons, forming a solid scientific foundation for clinical applications.

2. Consider improving the resolution and labeling of the figures for better readability. Adding more detailed legends that describe the experimental conditions and outcomes can also aid in understanding the results without referring back to the text.

Response: Thank you for your constructive feedback concerning the clarity and presentation of the figures in our manuscript. We have carefully

considered your suggestions and have taken steps to improve both the resolution and the labeling of all figures to enhance readability. Additionally, we have expanded the legends to provide more detailed descriptions of the experimental conditions and outcomes, ensuring that they can be understood independently of the main text.

3. The discussion effectively connects the findings with broader implications, but could be strengthened by discussing potential mechanisms through which LTP influences gut microbiota and metabolic pathways. Speculative mechanisms should be clearly identified as such to avoid overinterpretation.

Response: Thank you for your constructive feedback regarding the discussion section of our manuscript. We have revised this section to better elucidate the potential mechanisms through which LTP influences the gut microbiota and metabolic pathways. We have clearly identified speculative mechanisms as such to avoid overinterpretation. These revisions are highlighted in yellow in the manuscript.

Special thanks to you for your good comments.

We tried our best to improve the manuscript and made some changes in the manuscript. These changes will not influence the content and framework of the paper. And here we did not list the changes marked them in yellow in the revised paper.

We appreciate for Editors/Reviewers' warm work earnestly, and hope that the correction will meet with approval.

Once again, thank you very much for your comments and suggestions.