For research article

Response to Reviewer 3 Comments

1. Summary

Thank you very much for taking the time to review this manuscript. Please find the detailed responses below and the corresponding revisions/corrections highlighted/in track changes in the re-submitted files.

2. Questions for General Evaluation	**Reviewer's Evaluation**	**Response and Revisions**
Does the introduction provide sufficient background and include all relevant references?	Can be improved	
Are all the cited references relevant to the research?	Can be improved	
Is the research design appropriate?	Can be improved	
Are the methods adequately described?	Can be improved	
Are the results clearly presented?	Can be improved	
Are the conclusions supported by the results?	Can be improved	

3. Point-by-point response to Comments and Suggestions for Authors

Comments 1: When using cyclophosphamide (CTX) to induce immunosuppressive mouse model, the article did not fully explain the specific use method and dose of CTX. It is suggested that the author further describe the administration method and dose selection basis of CTX in detail in the method section.

Response 1: *Thank you for pointing this out. We agree with this comment. Therefore, we have added a reference to the application of 100 mg/kg body weight CTX in the manuscript on page 4, line 181，highlighted in yellow. Additionally, we provide a more detailed description of how CTX is formulated and administered in mice, please see page 4, line 181-185. Prior to the formal experiment, we conducted a pre-experimental dose-response evaluation using 80 mg/kg, 100 mg/kg, and 120 mg/kg body weight of CTX. Our results indicated that at 80 mg/kg, no significant immune suppression was observed. At 120 mg/kg, several mice died within 48 hours. However, at 100 mg/kg, CTX induced immunosuppression without causing mortality, leading us to select this dose for modeling.*

Comments 2: The research design is highly similar to the previous work of the author's team (such as Ganoderma lucidum and radish fermentation [19]), so it is necessary to clarify the unique contribution of this study compared with previous studies.

Response 2: *We appreciate the reviewer's question regarding the necessity of this study. How to*

alleviate the immunosuppression caused by chemotherapy is a subject of significant clinical importance. This study aims to explore whether traditional Chinese medicine , through fermentation, can yield more effective products to benefit immunocompromised patients. Unlike Li et al.'s study, we replaced the fermentation strain and introduced a combination of Astragalus membranaceus and Raphani Semen to evaluate if fermenting with three probiotic strains could enhance the production of active components from these herbs. Notably, we observed that after fermenting Astragalus membranaceus, the content of Calycosin and Formononetin increased. We further validated these findings in vitro using mouse spleen cells, demonstrating that the elevated levels of active ingredients improved B-cell function, thereby enhancing immune response. Further than Li, et al.'s test, we further investigated the mechanism by which fermentation products can effectively mitigate immunocompromised conditions. Simultaneously, this experiment aims to identify more effective fermentation probiotics and corresponding Chinese herbs that can stimulate the production of more active ingredients through fermentation, thereby promoting human health. Consequently, this study seeks to offer support for immunocompromised patients and provide a theoretical foundation for future clinical applications.

Comments 3: The joint intervention control of "unfermented traditional Chinese medicine group (as) + probiotics group (pro)" was not set, and the synergistic effect of fermentation process and probiotics alone could not be clarified.

Response 3: *Agree. Thank you for raising this important methodological consideration. We agree that including an "AS + PRO" group could further dissect the contributions of fermentation versus probiotics alone. However, our experimental design was strategically focused on addressing the core hypothesis of this study: whether probiotic-driven fermentation can biotransform TCM herbs into a therapeutically superior product compared to their unfermented counterparts. Here, we clarify the rationale and limitations:*

1. Hypothesis-Driven Design Priority

Primary objective: To evaluate if fermentation by multi-strain probiotics (rather than probiotic administration alone) enhances the bioactivity of TCM herbs.

Key comparison: The critical contrast lies between unfermented herbs (AS) and fermented products (PROAS), e.g. as demonstrated by 0.2-fold higher calycosin in PROAS vs. AS (Fig. 1I),

Stimulation of mouse splenocytes by fermentation-derived active compounds (Fig. 7).

2. Evidence from Prior Studies

Existing literature has well-established that oral probiotics alone (e.g., Lactobacillus spp.) exert immunomodulatory effects via microbiota regulation (Hor YY et al., 2019; doi: 10.1016/j.phrs.2019.104312). Our work intentionally shifts the focus to probiotic-herb metabolic synergy, a less explored mechanism.

3. Resource Allocation Rationale

Given constraints in funding and timeline, we prioritized:

The multi-faceted regulatory effects of PROAS encompass alterations in the gut microbiota, intestinal epithelium, and systemic immune function.

4. Future Work Commitment

We fully acknowledge the value of isolating probiotic effects and will address this in follow-up studies by including AS+PRO and PRO-only groups. Applying metagenomics to decouple microbiota-dependent/-independent effects.

Thank you again for your constructive critique.

Comments 4: Although the description of probiotic fermentation technology is detailed, it lacks specific information about strain source, fermentation time, temperature and other conditions. To ensure the reproducibility of the experiment, it is recommended to list these parameters in detail.

Response 4: *Agree. We have, accordingly, incorporated the strain numbers of the fermented probiotics, which serve as the source strains (see page 3, lines 108, 109, and 129， highlighted in yellow). Additionally, we have included the fermentation time, temperature, and a detailed description of the bacterial counting method to facilitate experimental reproducibility, please see page 3, line 130-134, highlighted in yellow.*

Comments 5: 16S rRNA sequencing only describes changes in the composition of the microbiota, and the functional contribution of specific microbiota (such as alloprevotella) is not verified by fecal bacteria transplantation (FMT) or antibiotic treatment models.

Response 5: *Thank you for raising this important point. We acknowledge that functional validation of microbiota changes through FMT or antibiotic models would strengthen our findings. However, due to the scope and resource limitations of the current study, we focused on establishing the compositional changes and their correlation with immune outcomes. In the revised manuscript, we have emphasized the preliminary nature of these observations and highlighted the need for future mechanistic studies to validate the functional roles of specific taxa like Alloprevotella (see page 21, lines 593-598, highlighted in yellow). We agree that this is a critical next step and plan to address it in subsequent research.*

Comments 6: key parameters such as quantitative standard curve, detection limit and recovery rate of target components are not provided, which affects the reliability of data.

Response 6: *Agree. Thank you for your valuable comments on on the details of the experimental method. In response, we have incorporated key validation parameters for short-chain fatty acid (SCFA) detection into the "Materials and Methods" section of the manuscript. Specifically, we have included detailed descriptions of quantitative standard curves, detection limits, and recovery experiments (see page 6, line 255-265, highlighted in yellow). These additional contents further substantiate the reliability of our analytical method and the accuracy of the obtained data.*

Comments 7: B cell function was assessed only by $CD19^+$ ratio and IGM/IGG level, lacking detection of plasma cell differentiation (such as Blimp-1 expression), antibody affinity or antigen delivery related indicators (such as MHC-II).

Response 7: *Agree. Thank you for your insightful suggestion. As recommended, we have expanded our analysis of B cell function to include key markers of plasma cell differentiation and antigen presentation. Specifically, we performed qPCR to quantify the expression of PRDM1 (Blimp-1), a master regulator of plasma cell differentiation, and evaluated MHC-II-related genes (H2-Aa, H2-Ab) in splenocytes stimulated with calycosin and formononetin. These additions are now detailed in Figure 7D (page 17-18, lines 460-471, 476-477) and the corresponding discussion section (page 21-22, lines 624-648).*

We agree that these additions substantially improve the rigor and depth of our study. The revised manuscript now provides a more comprehensive understanding of how PROAS-derived compounds modulate specific immune pathways. Thank you for guiding us toward these critical enhancements.

Comments 8: In vitro experiments, only calycosin and formononetin were used to stimulate splenocytes, and it was not verified whether they function through the same mechanism in vivo, and the potential contribution of other fermentation products (such as polysaccharides)

was not excluded.

Response 8: *Thank you for highlighting this critical gap between our in vitro and in vivo findings. We fully agree that validating the functional specificity of calycosin/formononetin in vivo and assessing contributions from other fermentation products (e.g., polysaccharides) are essential next steps. Here, we clarify our current rationale and propose a roadmap to address these questions:*

The purpose of this study was to investigate whether probiotics combined with the fermentation of Astragalus and Semen Raphani can generate more effective bioactive compounds, and to elucidate the mechanisms through which these compounds exert their effects. Our in vitro findings revealed that the fermentation process significantly increased the production of compounds calycosin/formononetin, which promote the proliferation and differentiation of B cells. To eliminate the influence of other fermentation byproducts, in vivo verification of compounds calycosin/formononetin is necessary. However, due to limitations in time and funding, this experimental design will be implemented in subsequent studies. Thank you again.

Comments 9: The correction method for multiple comparisons (e.g. Bonferroni) is not stated. Some p values are close to the threshold (e.g. p=0.0483), and there may be a risk of false positives.

Response 9: *Agree. In response to your suggestion, we have incorporated a detailed description of the multiple comparison correction method. Please see page 6, line 271-273, highlighted in yellow. We appreciate your feedback.*

Comments 10: There is a contradiction between "no significant change in cd4+/cd8+ ratio" in Figure 3 and Figure 4 and "calycosin significantly increases $cd4^+/cd8^+$ ratio" in the discussion. It is necessary to check the consistency of data and description.

Response 10: *Thank you for bringing this to our attention. In Figures 3 and 4, the $CD4^+/CD8^+$ ratio remained unchanged. The statement "calycosin significantly increases the CD4+/CD8+ ratio" refers to the experimental results presented in Figure 7. Previously, we failed to specify which figure was being referenced, leading to potential confusion. We have now corrected this by clearly indicating the relevant figure in the manuscript, please see page 18, line 452, 457, 460, highlighted in yellow.*

Comments 11: There is no direct evidence for the association between the change of F/B ratio and the "improvement of dysbacteriosis" (for example, the abundance of pathogenic bacteria was not detected), and the rise of alloprevotella did not reach statistical significance (the article mentioned "no significant difference"), so its biological significance should be carefully inferred.

Response 11: *Agree. The current academic consensus indicates that a high F/B ratio is associated with enhanced energy absorption efficiency and increased fat accumulation, whereas a low F/B ratio is frequently observed in patients with Crohn's disease or ulcerative colitis. The F/B ratio serves as an indicator of the overall balance of gut microbiota; however, it does not provide detailed insights into the specific bacterial composition. Therefore, additional analysis is necessary to fully understand the microbial structure. Consequently, in accordance with your recommendations, we have revised the statement in the original manuscript accordingly. Please see page 13, line 392-394, highlighted in yellow.*

The representation of the meaning of Alloprevotella elevation in our original manuscript was not adequately rigorous. Given that no statistically significant difference was observed, we have revised the original statement to ensure greater rigor and accuracy. Please see page 21, line 568-571, highlighted

in yellow.

Comments 12: The labels of some charts (such as figures 5 and 6) are fuzzy, and supplementary illustrations are required; Figure 7 flow results lack a representative description of gating strategies.

Response 12: *Agree. We have adjusted the labels of Figure 5 and increased the font size to enhance readability, following your valuable suggestion. Please see page 14, line 405, page 16, line 416.*

In accordance with your recommendations, a detailed description of the gating strategies illustrated in section 3.6. Please see page 17, line 443-448, highlighted in yellow.

Comments 13: It is necessary to disclose the original sequencing data (such as NCBI accession number) and HPLC-MS peak identification details to enhance reproducibility.

Response 13: *Thank you for your valuable feedback. We fully acknowledge the importance of data transparency and reproducibility in scientific research. Regarding the request to disclose the original sequencing data, we would like to propose the following data-sharing plan:*

Controlled Access Commitment: The raw sequencing data will be made available to qualified researchers upon request via email (contact: yhliu_dl@163.com). We have incorporated this information in the revised manuscript. Plesase see page 22, line 680. Please let us know if this approach aligns with the journal's data-sharing policy. We are open to further discussions to meet the journal's requirements while protecting sensitive information.

In response to your request for detailed HPLC-MS peak identification information, we have expanded the "Experimental Methods" section in the revised manuscript with comprehensive descriptions of the analytical protocols to ensure reproducibility and meet the requirements for experimental replication. Please see page 4, line 144-160, highlighted in yellow. Thank you again.

4. Response to Comments on the Quality of English Language

Point 1: The English is fine and does not require any improvement.

Response 1: *Thank you.*

5. Additional clarifications

We thank the reviewer for their insightful comments, which have strengthened the manuscript.