

For research article

Response to Reviewer 2 Comments

1. Summary

Thank you very much for taking the time to review our manuscript and offer valuable suggestions in your busy schedule. Your review is of great value to our research work. For each of your suggestions, we have provided detailed individual responses below along with their positions (lines) in the revised manuscript. Meanwhile, in the resubmitted manuscript file, we have clearly marked all the corresponding revisions, corrections and modifications made based on your opinions in a highlighted (red) manner for your review.

We believe that the revisions made in response to the reviewers' comments have substantially enhanced the manuscript. We thank the reviewers once again for their constructive feedback, which has undoubtedly improved our work. We hope that the revised manuscript now meets the high standards of Microorganisms and is suitable for publication.

Sincerely,

On behalf of all authors,

Yingchao Sun

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

Comments 1: What is the hypothesis of your work?

Response 1:

We sincerely thank the reviewer for raising this important point. The hypothesis of our study is now clearly stated in the revised Introduction (Section 1, final paragraph) as follows:

"We hypothesized that exogenous addition of 4-pentenoic acid (4PA)—an endogenous antimicrobial metabolite in nettle silage—would selectively inhibit proteolytic *Clostridium* spp., thereby reducing protein degradation and ammonia production, while malic acid (MA) would primarily enhance lactic acid bacterial activity via metabolic utilization, offering a comparative perspective on acid-mediated preservation strategies."

This hypothesis is directly supported by our previous findings that 4PA exhibits strong antibacterial activity against spoilage bacteria but also inhibits beneficial LAB, whereas MA serves as a fermentable substrate for LAB [3,11]. The current study explicitly tests these mechanistic roles in the context of whole-plant nettle silage preservation.

Comments 2: The wording of your objective is unclear.

Response 2:

We sincerely appreciate the reviewer's feedback. We have revised the manuscript to clearly state the research objectives in the final paragraph of the Introduction. The objectives are now explicitly listed to enhance clarity and alignment with the study's hypothesis and experimental design.

Comments 3: Please carefully review the entire document. Some sections are highlighted in yellow, so I believe they have already been reviewed. If not, please remove the color.

Response 3:

We sincerely thank the reviewer for their thorough review and for bringing this to our attention. The yellow highlights were indeed remnants from our internal revision process, used to track changes made in response to the previous round of reviewers' comments. These highlights were not intended to remain in the final version.

We have now carefully removed all yellow highlighting from the manuscript to ensure a clean and professional presentation. We appreciate your vigilance in helping us improve the quality of the manuscript.

Comments 4: Please review your statistical analysis because it does not describe all the analyses performed and their correlations.

Response 4:

We sincerely thank the reviewer for this critical observation. We have now thoroughly revised the Statistical Analysis section (**Section 2.4**) to provide a complete and transparent description of all statistical methods employed in the study, including the correlation analysis. The updated text explicitly details the software, methods, and significance thresholds used for each analysis.

We are grateful to the reviewer for highlighting this oversight. The revised methodology section now fully discloses all analytical approaches, ensuring the manuscript meets high statistical reporting standards.

Comments 5: I think you should consider the timeframe for your analyses and see if there are interactions by time and treatment.

Response 5:

We sincerely thank the reviewer for this insightful suggestion. We fully acknowledge that evaluating time-by-treatment interactions could provide additional insights into temporal dynamics. However, after careful consideration, we have chosen to retain the current analytical approach for the following scientifically justified reasons:

1. Alignment with Core Research Objective: The primary goal of this study was to compare the efficacy and mechanism of 4-pentenoic acid (4PA) versus malic acid (MA) in inhibiting *Clostridium* and preserving protein in nettle silage. Our hypothesis focused on the overall effect of each acid treatment against spoilage processes, rather than on how these effects change dynamically over time. The chosen methodology (**analysis within time points**) directly and clearly tests this central hypothesis by allowing a precise comparison of treatment effects at key fermentation stages (early, mid, late).

2. Biological Justification for Independent Timepoint Analysis: Silage fermentation is a process with distinct phases (e.g., aerobic phase, fermentation phase, stable phase). Microbial communities and chemical profiles undergo rapid and non-linear shifts. Analyzing each time point (7, 30, 60 days) independently is a well-established approach in silage research [21,22] because it allows for the clear identification of treatment effects within a specific fermentation context. A significant time-by-treatment interaction from a two-way ANOVA would indeed show that effects change over time, but it would not provide the specific, phase-separated comparisons that are most interpretable for assessing

preservation efficacy at critical endpoints (e.g., final protein content at day 60).

3. Clarity in Interpretation: Presenting the results within each time point offers a more straightforward and intuitive interpretation of the data, which is crucial for demonstrating the practical, preservative outcome of adding these acids. For instance, showing that **4PA resulted in the lowest ammonia-N and butyric acid levels specifically at day 60—the endpoint representing storage stability**—is a powerful and clear result that directly addresses our objective.

4. Addressing the Reviewer's Underlying Concern: We understand the reviewer's interest in temporal patterns. While we have not performed a formal statistical interaction test, we have effectively captured and presented the temporal trends requested by the reviewer through our extensive within-time-point analysis and discussion. Figures 1-4 and Table 1 are all structured to visualize changes across time for each treatment. Furthermore, in the Results and Discussion, we explicitly describe how the effects of the treatments evolved (e.g., **"4PA's inhibition of Clostridium intensified from day 30 to 60"**). This approach provides a rich, qualitative understanding of the temporal dynamics without the complexity of interpreting interaction effects, which could potentially obscure the main findings for readers.

Modification Made to the Manuscript:

To further emphasize that our analysis was designed to compare treatments at each stage and to acknowledge the temporal perspective, we have added the following sentence to the **Statistical Analysis section (2.4)**:

"The effects of acid additives on fermentation characteristics and bacterial community were assessed within each sampling time point (7, 30, and 60 days) to evaluate treatment efficacy at distinct stages of the ensiling process."

This revision reinforces that our analytical design was intentional and aligns with our research goals.

In summary, we believe our current approach of analyzing data within time points is not only statistically sound but also the most appropriate method to directly answer our primary research question regarding the comparative efficacy of 4PA and MA. We have ensured that the temporal dimension is thoroughly presented and discussed throughout the manuscript. We hope this clarification addresses the reviewer's concern satisfactorily.