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ORIGINAL PAPER

Fermentation of Polygonati Rhizoma aqueous extract using *Lactiplantibacillus plantarum* under the condition of eutrophication

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Abstract

In this experiment, the eutrophication system was established by adding sucrose and yeast powder, and the pH and dissolved oxygen were measured in a bioreactor in real time to study the effect of aerobic environment on the fermentation process of Polygonati Rhizoma extract by *Lactiplantibacillus plantarum*. To further analyze metabolic changes, UPLC-Q-Exactive MS was used for metabolomic analysis and metabolic profiling. Multivariate analysis was performed using principal component analysis and Orthogonal projections to latent structures discriminant analysis. Finally, 313 differential metabolites were selected, 196 of which were annotated through database matching. After fermentation, the content of short-chain fatty acids, lactic acid, and their derivatives increased significantly, and there were 13 kinds and 4 kinds, respectively. Both compounds and their derivatives are beneficial to the intestinal flora. Consequently, incorporating *L. plantarum* into the aerobic fermentation process of Polygonati Rhizoma extract within the eutrophic system is potentially advantageous in enhancing the impact of its fermentation solution on the gut microbiota and its effects on human health. Our findings for this kind of edible and medicinal material research and development offer useful insights.

Highlights

- First report of polygonati Rhizoma fermentation in eutrophic bioreactor system.
- Fermentative components were characterized by UHPLC-QE-MS/MS and multi-data analysis.
- The different metabolites were obtained by comparing before and after fermentation.
- Short-chain fatty acids, lactic acid and their derivatives contents increased.

Keywords Fermentation · Polygonati Rhizoma · *Lactiplantibacillus plantarum* · Eutrophication system

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Introduction

Polygonati Rhizoma is one of the first 87 edible medicinal materials published by the former Chinese Ministry of Health in 2002, and it is an important Chinese traditional medicine resource. With strong adaptability to climate and soil, Polygonati Rhizoma can be grown in various areas (Gong et al. 2019) and has great potential as a food source. For example, Polygonatum sibiricum polysaccharide has blood-enriching effects (Wang et al. 2022a), anti-obesity effects (Liang et al. 2021), and antidepressant effects (Shen et al. 2022), Polygonati Rhizoma saponin is hypoglycemic (Chai et al. 2021), protects the pancreas (Luo et al. 2023), etc. Both as a food source and in terms of its pharmacological activity, Polygonati Rhizoma has great research and development value. However, raw Polygonati Rhizoma is generally not eaten directly and needs to be processed by steaming and drying to remove its unpleasant taste (Yao et al. 2022). In recent years, Polygonati Rhizoma is also processed by fermentation; probiotic fermentation of Polygonati Rhizoma has gradually become the focus of the food field (Wang et al. 2019; Hu et al. 2021).

Probiotics are a kind of active microorganisms that are beneficial to the host by colonizing in the human body and changing the composition of the flora in a certain part of the host. Lactobacillus plantarum is a type of probiotic bacteria (Leroy and Vuyst 2004). When it is fermented together with traditional Chinese medicine, its ferments often have special physiological functions. For instance, *Lactiplantibacillus paraplantarum* CRL2051 and *L. plantarum* CRL2030 ferment pomegranate juice, and this functional drink may protect against weight gain, liver damage, and dyslipidemia induced by a high-fat diet consumption (Isas et al. 2023). *Petasites japonicus* fermented with lactic acid bacteria has an anti-allergic effect (Bae et al. 2009), and kiwi juice fermented with *Bifidobacterium bifidum* 6169 and *L. plantarum* 21,805 can regulate cholesterol levels in mice with hyperlipidemia (Wang et al. 2022b).

Lactic acid bacteria fermentation process is influenced by many factors, and changing one factor even a little can make a big difference (César et al. 2023). In *L. plantarum* aeration and anaerobic culture catabolism of glucose was observed in the end product of substantive differences. Oxygen leads to acetic acid growth in aerobic culture and lactic acid growth in anaerobic culture (Murphy and Condon 1984).For instance, the production of γ-aminobutyric acid by *L. plantarum* N5 was affected by initial pH of 5.5, glutamic acid concentration, nitrogen source, glucose as carbon source, and incubation temperature and time (Harnentis et al. 2019). The results showed that organic nitrogen sources were more favorable for the production of bacteriocin-like inhibitory substances compared to inorganic sources during fermentation by lactic acid bacteria (Jawan et al. 2020).

At present, there are few researches on the fermentation of Polygonati Rhizoma by lactic acid bacteria. The functional yogurt produced by lactic acid bacteria has good quality and sweet and sour taste (Sun et al. 2020; Hu et al. 2021). In a previous study, we explored the fermentation process of Polygonati Rhizoma, where no extra nitrogen or carbon sources were added (Wang et al. 2023b). In that study, the fermentation lasted for 7 days, and the growth rate of lactic acid bacteria was relatively uniform and slow. This study was conducted without the addition of additional nutrients and would like to continue to investigate whether adding sufficient nutrients can speed up the fermentation process.

Based on previous studies, in this study, suitable nitrogen and carbon sources were screened for the rapid growth of lactic acid bacteria and shortened fermentation time. In this study, *L. plantarum* was added to establish the eutrophication system of fermentation. Eutrophication systems add additional nutrients so that fermentation is in a nutrient-adequate environment, rather than in a nutrient-deficient environment. Metabolic profiling and metabolomics analysis were conducted using (UHPLC-QE-MS/MS). The metabolites with significant differences obtained from metabolic analysis were classified, and one representative compound from each class was selected for verification. The significant difference in the intestinal flora were analyzed and discussed in combination, to thus provide a direction to the research and development of fermented Polygonati Rhizoma in the field of food sciences.

Materials and methods

Polygonati Rhizoma procurement and extraction

Polygonati Rhizoma (20 g; Yipuyuan Inc., China.) was added to 1 L pure water, extracted at 100℃ for 1 h, and filtered with gauze. The filtered residue was extracted again in the same way, and then the two extracts were mixed, and 40 g sucrose and 40 g yeast powder were added to prepare a eutrophication system for later fermentation (Wang et al. 2023a).

Strain and preculture

L. plantarum microbial starter was purchased from Shandong Zhongke-Jiayi Biological Engineering Co., Ltd., China. The De Man, Rogosa and Sharpe (MRS) Medium (HuanKai Microbial Inc., China.) was prepared; 1 L medium contained 10 g peptone, 10 g yeast extract, 20 g glucose, 2 g ammonium citrate, 5 g sodium acetate, 0.5 g MgSO4·7H2O,

and 80 0.5 g Twain (R). Before inoculation, the culture was grown at 37°C in a shaker for 16 h. The inoculants were cultured at 37°C on a shaker (Shanghai shenxian the rmostatic equipment factory, China) for 16 h, and the harvested strains were inoculated.

The bioreactor system

A bioreactor system with independent 2 L fermentation bottles obtained from Bipu Huarui Scientific Instruments (Beijing) Co., Ltd., China, was used for fermentation, as described in previous studies (Wang et al. 2023b). Bioreactor was monitored in real-time for pH and dissolved oxygen (DO), and the data were transmitted to the computer to record, and the data were visualized by automation.

Fermentation process

There are 6 groups in aerobic and anaerobic environment, and a total of 12 fermentation bottles. The prepared extracts were added to each fermentation bottle and sterilized at 121°C. Six groups were taken out first as extracts before fermentation, and then 12 fermentation bottles were inoculated with *L. plantarium* with a concentration of about 5 × 109 CFU/mL for fermentation. Each fermentation bottle was connected to an air pump (Yafengshuizu, China) to maintain an aerobic environment. The initial pH is 5.29 and the initial DO is 3.45. During fermentation, the daily growth of *L. plantarium* was recorded using an ultraviolet spectrophotometer to measure OD values (Omak and Yilmaz-Ersan 2022) according to the previously published method. (Omak and Yilmaz-Ersan 2022). Subsequently, the specific growth rate of *L. plantarum* was determined by applying formula (1) (Patel et al. 2022):

$$\mu = \frac{1}{X}\frac{dX}{dt} \quad (1)$$

After fermentation, one sample was taken from each bottle, and finally, there were a total of six samples, and the sample was named Aerobic fermentation supernatant (AS). Six samples of Polygonati Rhizoma were obtained before the fermentation process, named as extractives of Polygonati Rhizoma before fermentation (EB), for further analysis. The samples were promptly subjected to centrifugation (Thermo Fisher, China), resulting in the separation of cells and supernatants. These fractions were subsequently employed for UHPLC-MS/MS analysis.

Ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry analysis

Metabolomics analysis was performed by UHPLC-QE-MS/MS (Thermo Scientific, America), mainly referring to the previous methods (Xu et al. 2021). Then, 2-hydroxyisocaproic acid and 3-phenyllactic acid were validated using the Agilent 6530 UHPLC-QTOF-MS/MS system (Agilent Technologies, Wilmington, DE, USA). Chromatography was performed on InterSustain C18 column (4.6 mm × 250 mm, 5 µm, GL Sciences, Japan) at 40°C. The mobile phases were water (A) and acetonitrile (B). For verification, an elution procedure using optimized linear gradient was used: 100% A (0–2 min); 100–52% A (2 to 6 min); 52%-0% A (6–10 min); 0% A (10–12 min); 0–100% A (12–12.1 min); 100% A (12.1–15 min). The flow rate was 1.0 mL/min, the sample size was 10 µL, and the detection wavelengths were 210 nm and 245 nm, respectively. Q-TOF conditions were: scanning range 100–1000 m/z, drying gas temperature 320°C, sheath gas temperature, 320°C, drying gas (N2) flow: 8.0 L /min, capillary voltage, 3.5 kV, fragmentor, 110 v, and fragmentation was carried out with stepped normalized collision energy values of 20, 30, and 50. Verification of 2-hydroxyisocaproic acid and 3-phenyllactic acid was performed in terms of retention time and mass spectrometry.

Preprocessing and filtering of data

The techniques of data preprocessing and filtering utilized in this study were derived from previous studies (Wang et al. 2023b). Original mass spectrometry data were subjected to various processing steps, such as peak alignment, retention time correction, and peak area extraction, using MS-DIAL (ver 4.9). Subsequently, the processed data were compared with publicly available mass spectrometry databases including MassBank, NIST14, ReSpect, the Human Metabolome Database (HMDB), and others. To integrate the data from these databases, the MSP format was adopted and merged with the North American MoNA-MassBank database and the metabolite standard library developed by Shanghai Biopro file Biological Technology Co., LTD. In the extracted-ion features, only variables with more than 50% of the non-zero measurement values in at least one group were kept.

Multivariate statistical analysis

Statistical analyses were performed using RMarkdown free software (ggplot2 and MetaboAnalystR) (Balcázar-Zumaeta et al. 2023). The Pareto scale was employed to centralize the mean value of the dataset. To construct the model, principal component analysis (PCA) and orthogonal projections

to latent structures discriminant analysis (OPLS-DA) were implemented. All evaluated models were tested for overfitting using the permutation test method. The score of each variable was mathematically calculated as the weighted sum of squares of the PLS weights. The average Variable Importance for the Projection (VIP) value was 1, and a VIP value greater than 1 is generally considered significant. The metabolites were annotated using the statistical significance threshold of VIP values obtained by the OPLS-DA model, and a two-tailed Student's t-test (p-value) was performed on the normalized raw data by the univariate analysis. The importance of metabolites was assessed by conducting a one-way ANOVA test (one-way variance test), followed by identification of statistically significant metabolites based on p-value < 0.05 and OPLS-DA VIP > 1 (Wang et al. 2023b). The fold change was determined by calculating the logarithm of the average mass response ratio between two arbitrary categories.

Results

Real-time detection of pH, dissolved oxygen, and cells growth

As shown in Fig. 1A, at the beginning of fermentation, the pH was around 5.3. By day 1 of fermentation, the pH rapidly dropped to 3.9. It slowly dropped from day 2 to day 5, and by the end of day 5, the pH reached 3.75. At the beginning, the DO value remained at about 4 at the beginning, and gradually increased to about 4.5 from the second day. From day 3 to day 4, the DO level rose from 2.65 to 5.24, and overall, the DO level remained around 4.3 mg/L. Before the fifth day, the DO value fluctuated, indicating that the fermentation was still in progress, so the sample was not taken before the fifth day. In the fermentation broth of Polygonati Rhizoma, Fig. 1B displays the concentration of *L. plantarum* along with its average specific growth. Initially, the cell concentration of *L. plantarum* exhibited a fast increase, but its growth rate gradually slowed down from day 1 to day 5 of fermentation. The mean rate of growth per unit of time also exhibited a swift escalation from the inception of the fermentation process to the first day of fermentation, culminating in a peak value of 2.84 on the first day. From day 2, the specific growth rate decreased to 0.12 and then continued to decline; on day 5, the value decreased to 0.031, which was nearly zero growth. Therefore, samples were taken on day 5 of fermentation for metabolome analysis.

Principal component analysis and orthogonal projections to latent structures discriminant analysis

Figure 2 displays the PCA score plots for the before and after fermentation stages of the Polygonati Rhizoma extract, representing the positive (A) and negative (B) aspects. PCA score plots of positive mode explained more than 47.25% of the total variance (individually, PC1 and PC2 explained 29.22% and 18.03%, respectively). Simultaneously, the PCA score plots of the negative mode accounted for over 55.88% of the total variance. PC1 and PC2 individually contributed 37.29% and 18.59% to the explained variance, respectively. OPLS-DA is the discriminant analysis of another kind of supervision and statistical methods. Figure 2C and D present the OPLS-DA score plots of EB and AS data in positive and negative modes, respectively, where an apparent separation between groups can be seen. In the positive mode, Q2 = 0.923, and in the negative mode, Q2 = 0.961.

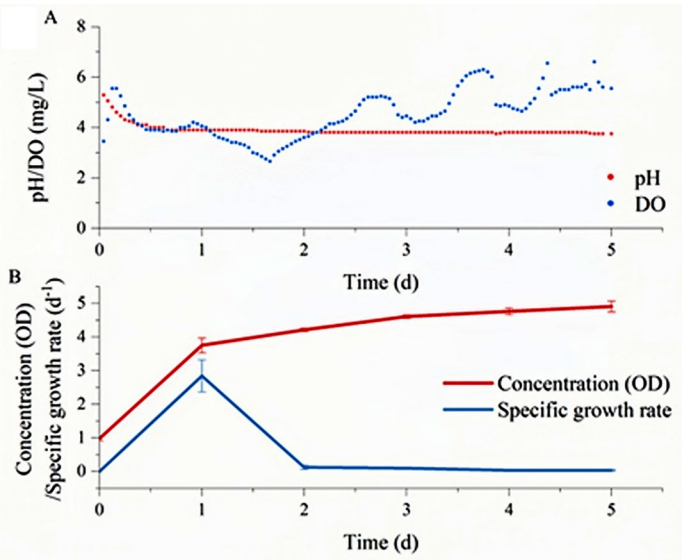

Fig. 1 Real-time pH and DO values (**A**) of *Lactiplantibacillus plantarum* fermentation broth of Polygonati Rhizoma under aerobic conditions and the cell concentration and average specific growth (**B**) of *Lactiplantibacillus plantarum* fermentation broth of Polygonati Rhizoma under aerobic conditions. DO, dissolved oxygen; OD, optical density

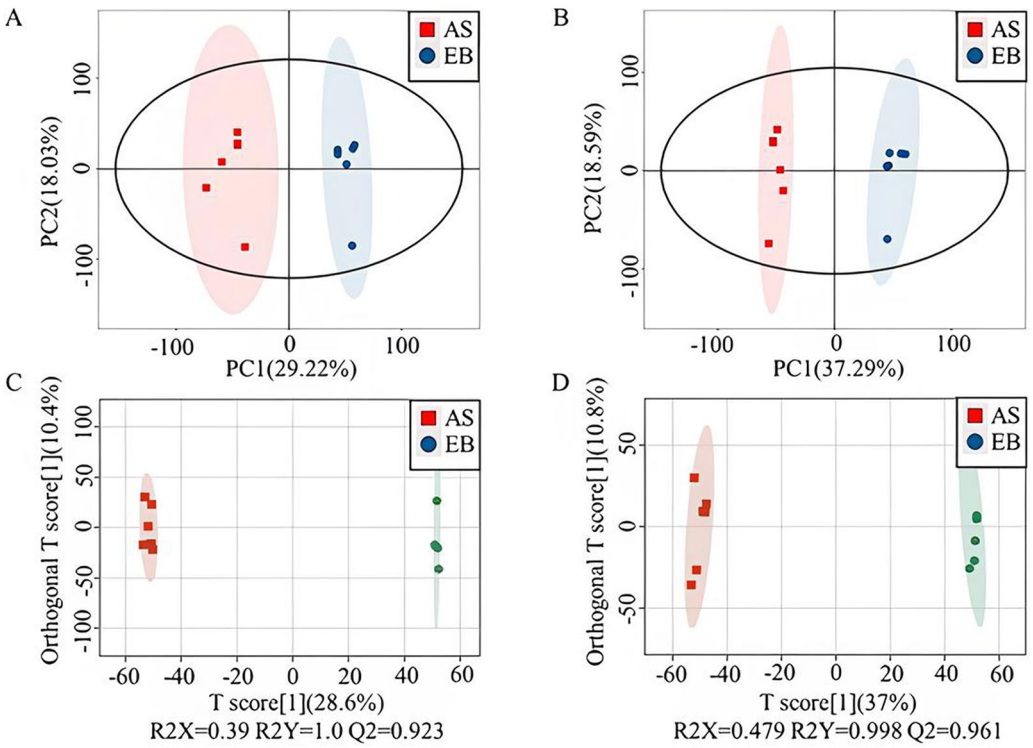

Fig. 2 PCA score plots of positive ionization (**A**) and negative ionization (**B**) metabolite profiling of Polygonati Rhizoma extract (PRE) before and after fermentation using *Lactiplantibacillus plantarum*. Orthogonal projections to latent structures discriminant analysis (OPLS-DA) analysis plots of positive ionization (**C**) and negative ionization (**D**) metabolite profiling data of PRE before and after fermentation using *L. plantarum*. Aerobic fermentation supernatants (AS); extracts of Polygonati Rhizoma before fermentation (EB)

Differential metabolite selection

To further analyze the composition changes between EB and AS groups, volcano plots were used to show the amount and significance of different metabolites between the two groups. Two-hundred-seven significant metabolites were detected in the positive mode, as shown in Fig. 3A. One-hundred and six significant metabolites were detected in the negative mode, as shown in Fig. 3B. These data also further confirmed that under the condition of eutrophication, adding *L. plantarum* to Polygonati Rhizoma, caused variations in the composition of Polygonati Rhizoma extract before and after fermentation.

Classification of significantly different metabolites

A collective of 313 distinct metabolites were selected; among these, 196 were annotated by matching the database. The main categories were organic acids and derivatives (Fig. 4), with a total of 43 (21.94%), and these include 2-hydroxybutyric acid, N-acetyl-L-aspartic acid, levodopa, and other compounds. Organoheterocyclic compounds, lipids and lipid-like molecules, organic oxygen compounds, nucleosides, nucleotides, and analogues, phenylpropanoids and polyketides and benzenoids, respectively 41 (20.92%), 29 (14.8%), 18 (9.18%), 17 (8.67%), 17 (8.67%), 16 (8.16%).

Effects of significantly different metabolites on intestinal flora

Under the condition of eutrophication, *L. plantarum* was added to ferment Polygonati Rhizoma, whose content changed greatly before and after fermentation. Particularly notable are the two broad categories of short-chain fatty acids and their derivatives and lactic acid and their derivatives (Fig. 5). Among short-chain fatty acids and their derivatives, the most significant was 2-hydroxyisocaproic acid, followed by 2-hydroxybutyric acid and dihydroxy-valerate. The change in lactic acid content and its derivatives in the class was the most significant for 3-(4-hydroxyphenyl)-lactate, followed 3-phenyllactic acid, and then the indole lactate. By each choice from the two kinds of compounds in a compound, the statistical analysis to verify the robustness: short-chain fatty acids and their derivatives and lactic acid and their derivatives (Fig. 6). The two compounds were compared with the standard substances, and their response time in the EB and AS groups was found to be consistent with that of the standard substances. The m/s of 2-hydroxyisocaproic acid, EB group and AS group

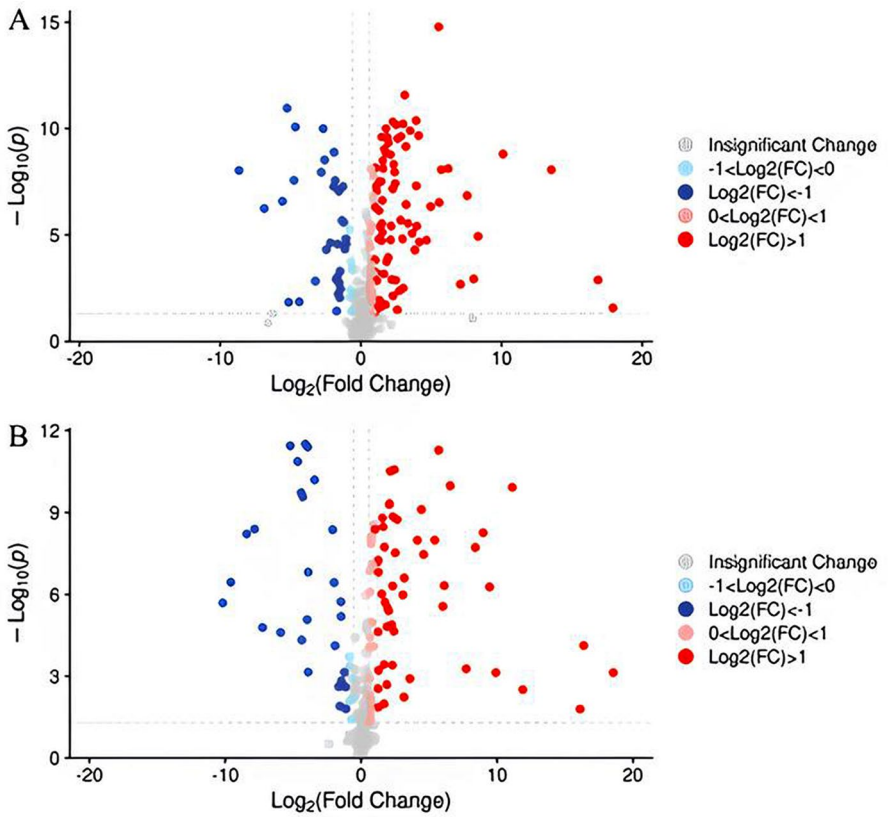

Fig. 3 The volcano plot of the comparison group extracts of Polygonati Rhizoma before fermentation (EB) vs. aerobic fermentation supernatants (AS) in positive mode (**A**) and negative mode (**B**), with FC > 1.5 or FC < 0.667 and P-value < 0.05 as the screening criteria

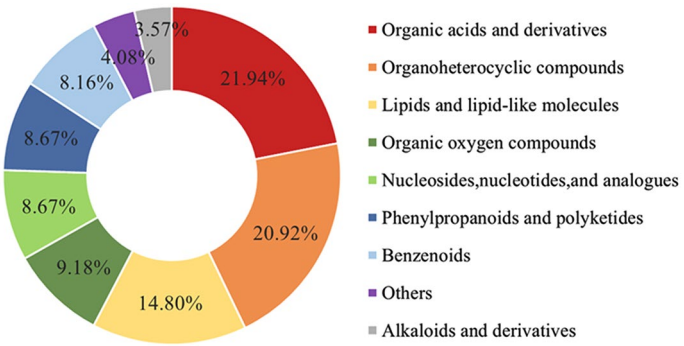

Fig. 4 The Human Metabolome Database (HMDB) classification of significantly different metabolites before and after fermentation under aerobic conditions

were 131.0734, 131.0735, and 131.0737, respectively, and that of 3-phenyllactic acid, EB group, and AS group were 165.0575, 165.0577, and 165.0581, respectively. Thus, the accuracy of the non-targeted metabolomics approach could be determined.

Discussion

In this study, pH, DO, PCA, OPLS-DA, volcano plot and Box pattern were used to study the changes of compound composition and content of *L. plantarum* fermentation extract before and after fermentation under eutrophic conditions.

The change in pH indicates that from the beginning of fermentation to day 1, a large amount of acid was produced, while from day 1 to day 5, the acid production was reduced. The prophase change of dissolved oxygen may be because the lactic acid bacteria grew rapidly in the early stage with high demand for oxygen. The fluctuation of DO value is related to the compounds generated during fermentation, which is consistent with previous studies. Later, the growth rate decreased with low oxygen demand, which led to the rise in DO value. In general, the DO value was stable at about 4.3 mg/L, indicating that during the experiment, the fermentation was always in an aerobic environment and the aerobic conditions were well maintained.

As shown in PCA, the two groups of EB and AS were well-separated along PC1, reflecting differences in their

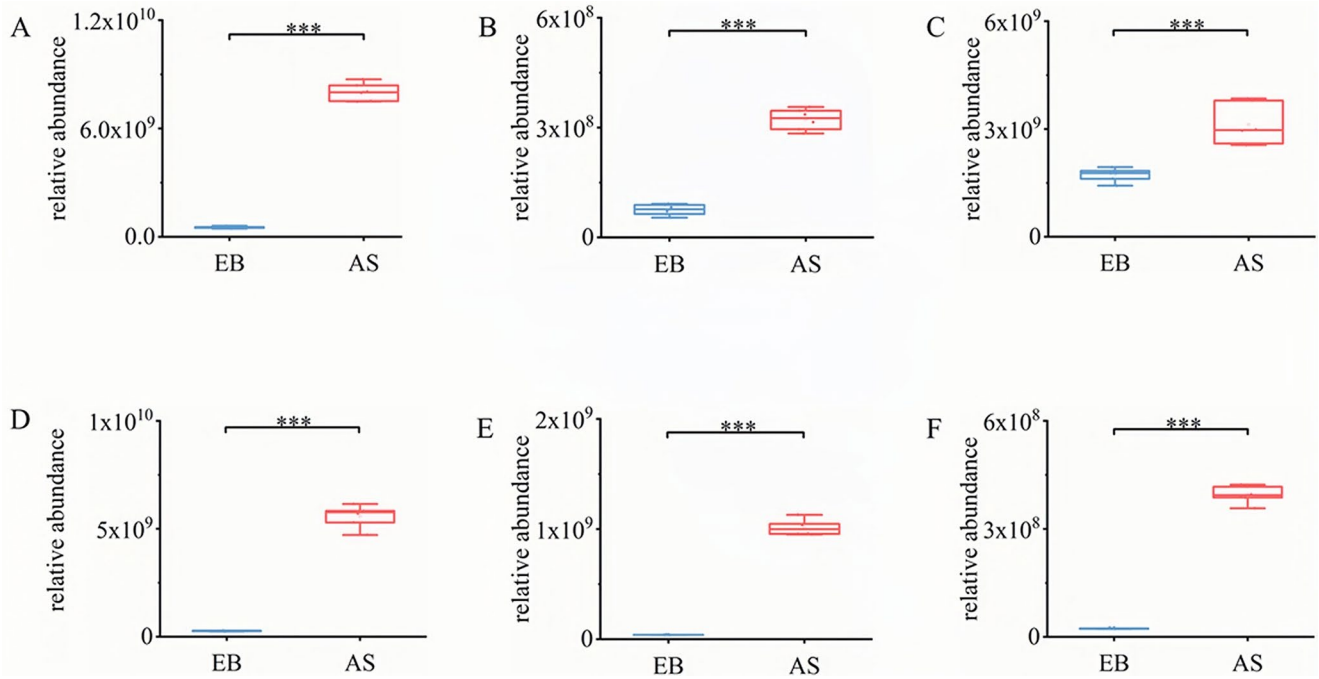

Fig. 5 Box pattern of some metabolites with significant differences in content before and after *Lactobacillus plantarum* fermentation. 2-Hydroxyisocaproic Acid (**A**); 2-Hydroxybutyric Acid (**B**); Dihydroxy-Valerate (**C**); 3-Phenyllactic Acid (**D**); 3-4-Hydroxyphenyllactate (**E**); Indolelactate (**F**); Aerobic fermentation supernatants (AS); Polygonati Rhizoma extractives before fermentation (EB). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$

metabolic profiles. The score plot for all the samples was within 95% of the Hotelling's T2 ellipse, showing good reproducibility and stability of the employed metabolomics method.

As demonstrated in the PCA analysis, the metabolic profiles of the EB and AS groups exhibited noticeable distinctions along PC1, which effectively distinguished the two groups (Zhou et al. 2018). The score plot of all the samples remained within the 95% confidence interval of the Hotelling's T2 ellipse, indicating the reliable reproducibility and stability of the utilized metabolomics technique (Qu et al. 2019). And OPLS-DA shows $Q2 > 0.5$ in all cases, indicating good predictive ability of the model. This also indicated that the composition of Polygonati Rhizoma changed before and after fermentation under the condition of eutrophication.

As the fermentation continued, it generated a large number of organic acids and derivatives, and that could be one of the main reasons for the decrease in pH during the fermentation process. These findings are consistent with the results of previous studies. Adding *L. plantarum* to ferment papaya juice reduced its pH from 5.34 to 3.55 and led to the production of a large number of organic acids such as lactic acid, oxalic acid, and tartaric acid (Chen et al. 2018).

Intestinal bacteria play a crucial role in maintaining immune and metabolic homeostasis and protecting against pathogens (Thursby and Juge 2017). In recent years, more studies have shown that Polygonati Rhizoma can regulate the body by affecting intestinal flora. Polygonatum sibiricum polysaccharide exhibits good anti-aging activity by regulating intestinal bacteria and relevant metabolites (Liu et al. 2023). The intestinal microbiota can be modulated by the polysaccharides derived from Polygonatum sibiricum Red. consequently influencing the production of short-chain fatty acids (Luo et al. 2022); probiotic-fermented products also have this effect. Likewise, *Lactobacillus*-fermented black barley exhibited a regulatory effect on the dysbiosis of high-fat diet-induced intestinal microbiota (Zhu et al. 2021).

During fermentation, the content of short-chain fatty acids and their derivatives increased continuously (Fig. 5), consistent with those mentioned in previous studies. The content of short-chain fatty acids exhibited diverse increments when different environments were subjected to various lactic acid bacteria (Hadinia et al. 2022). A protein-fermentation product of bacteria, 2-hydroxyisocaproic acid is effective in controlling the growth of some Gram-positive such as *Staphylococcus aureus* and Gram-negative bacteria, such as *Pseudomonas aeruginosa* strains (Pahalagedara et al. 2022); it can inhibit oleic acid-induced triglyceride accumulation and could also stimulate the expression of proteins in lipid degradation in hepatocytes and triglyceride lipolysis in 3T3-L1 cells (Li et al. 2022). As a class of metabolites, short-chain fatty acids play a very important role in intestinal flora. UHPLC-Q-TOF/MS analysis revealed the presence of (S)-(-)-2-hydroxyisocaproic acid in the fermented Sijunzi

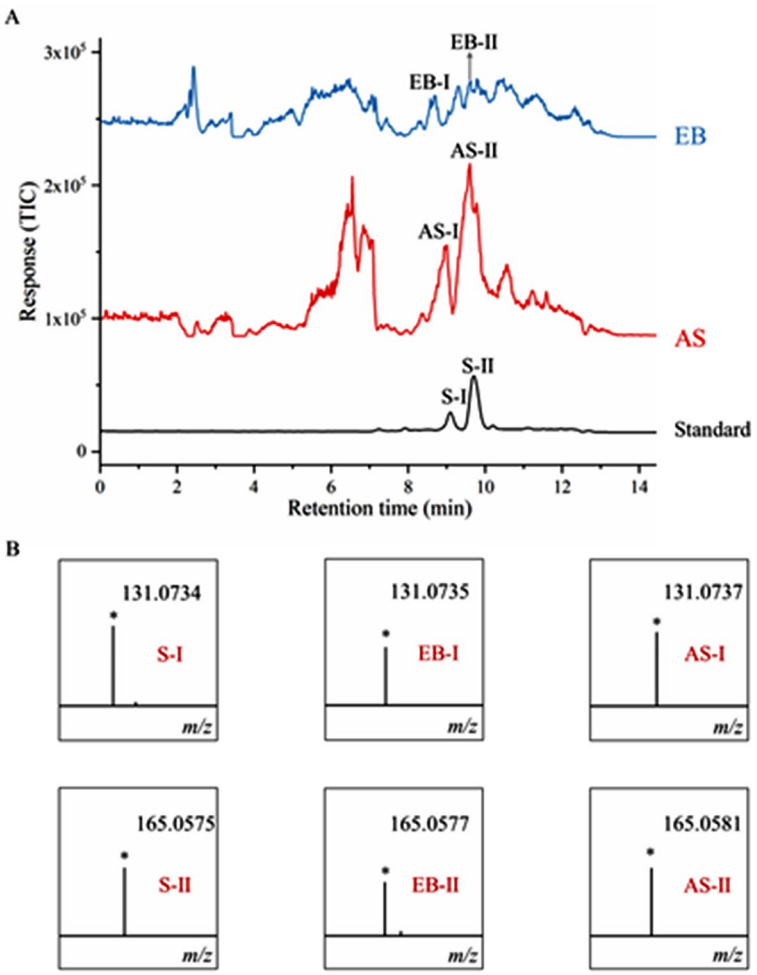

Fig. 6 Total ion chromatogram (TIC) (**A**) and mass spectrometry (MS) data of related peaks (**B**) of 2-hydroxyisocaproic acid and 3-phenyllactic acid in Polygonati Rhizoma extract before and after fermentation using *Lactiplantibacillus plantarum*. Aerobic fermentation supernatants (AS); extracts of Polygonati Rhizoma before fermentation (EB)

decoction. This compound is believed to have a potential role in regulating the composition of intestinal flora and promoting the development of villi (Guo et al. 2022). Sodium propionate can remodel the intestinal flora, in turn, significantly ameliorate vascular calcification in VDN-treated rats (Yan et al. 2022).

In addition to short-chain fatty acids, the gut microbiome is also greatly affected by lactic acid and its derivatives. Furthermore, the abundance of several species in the gut microbiome showed a notable correlation with 3-(4-hydroxyphenyl)-lactate (Caussy et al. 2018). The organic acid 3-phenyllactic acid exists widely in food fermented with lactic acid bacteria; it was found to be an ideal antimicrobial compound with broad and effective antimicrobial activity against both bacteria and fungi (Mu et al. 2012). *L. plantarum* L168 and its metabolite, indole lactate, improves intestinal inflammation, tumor growth, and gut dysbiosis (Zhang et al. 2023). The incorporation of a blend of lactic acid and glutamine into the diet has the potential to enhance growth performance in piglets by promoting the development of the small intestine, improving digestion, and maintaining a balanced microflora (Zheng et al. 2019).

The experimental results also proved that the real-time monitoring pH, DO and other functions provided by the bioreactor and other instruments were conducive to the smooth progress of the experiment, and the eutrophic system was conducive to speeding up the fermentation speed of *L. plantarum*-fermented Polygonati Rhizoma extract. However, according to the cited literature and analysis in this study, the results can only be proved to be applicable to *L. plantarum*, but it has not been proved to be applicable to Lactic acid bacteria.

Conclusion

Based on the system of eutrophication, *L. plantarum* was added to ferment Polygonati Rhizoma in an aerobic environment, and more organic acids were produced after fermentation, which may be the reason for the decrease in pH during the fermentation process. Concurrently, the levels of short-chain fatty acids, lactic acid, and their derivatives increased. These substances can function in the human body and improve the intestinal flora. The results of our study

offer potentially valuable perspectives for the exploration and advancement of this medicinal and edible substance.

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Author contributions Conceptualization, J.J.; methodology, Z.W.; data curation, C.Z.; writing—original draft preparation, Z.W. and Z.X.; writing—review and editing, J.J.; supervision, J.J. and S.Z.; project administration, J.L.; funding acquisition, W.H. All authors have read and agreed to the published version of the manuscript.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

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