Research Article

Exploring the Effects of Solid-State Fermentation on Polyphenols in *Acanthopanax senticosus* Based on Response Surface Methodology and Nontargeted Metabolomics Techniques

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In order to optimize the process of solid-state fermentation and understand the changes of active ingredients during fermentation of *A. senticosus*, we used response surface methodology to optimize the solid-state fermentation process of *A. senticosus* and analyzed the effect of fermentation on the active components of *A. senticosus* by nontargeted metabolomics techniques. The effects of fermentation conditions on polyphenol content were studied in terms of fermentation time, fermentation temperature, water content, and the inoculum level through Plackett–Burman design and response surface experiments. The optimized fermentation conditions were a fermentation time of 60 h, a fermentation temperature of 37°C, a water content of 55%, and an inoculum level of 12.5%, and the polyphenol content reached 10.25 ± 0.05 mg·g⁻¹, which was 40% higher than that before fermentation. Furthermore, the metabolites and metabolic pathways of *A. senticosus* were analyzed before and after fermentation using nontargeted metabolomics techniques, and differences in metabolites were investigated based on the analysis of the principal components. The results showed that *A. senticosus* contained 57 phenolics, and a total of 12 polyphenolic metabolites were significantly different before and after fermentation (including phenols and isoflavonoids), among which the content of caffeic acid and its derivatives with antioxidant capacity was significantly increased, which may improve the antioxidant capacity of fermented *A. senticosus*. Therefore, solid-state fermentation opens new avenues for the clinical development and application of *A. senticosus*.

1. Introduction

*Acanthopanax senticosus* Harms is a genus of *Acanthopanax* in the *Acanthopanax* family, and its dried root, rhizome, or stem were used as treatment medicine [1, 2]. It can work to strengthen the body and mind and has the efficacy of nourishing Qi, strengthening the spleen, nourishing the kidney, and soothing the nerves [3]. The primary chemical constituents of *A. senticosus* include polyphenols [4], polysaccharides [5], glycosides [6], and lignans, which have good pharmacological activities to regulating immune function [7], antifatigue effect [8], antioxidant activity [9], and anti-inflammatory properties [10]. Polyphenols are secondary plant metabolites found in traditional Chinese medicine with potential effects on health and are also the general term for plant components with several phenolic hydroxyl groups in the molecular structure, including polyphenols, tannins, phenolic acids, and anthocyanins [11].

As beneficial microorganisms, probiotics play a unique role in physical health, such as improving immunity [12], producing beneficial metabolites [13], and regulating intestinal flora balance [14]. Traditional Chinese medicine uses high-purity single high-purity strain or hybrid strain [15] and can improve active component content and efficacy [16]. Most of the active ingredients in traditional Chinese medicine are found in the cell wall. The cell wall is rich in lignin, cellulose, pectin, and other dense structural components, which increases the hardness of the cell wall and is not easy to decompose, hindering the release of the active ingredients of traditional Chinese medicine from the cell [17]. It has been demonstrated that the traditional
Chinese medicine cell wall can be degraded by various enzymes produced by microorganisms, releasing more active ingredients [14]. Therefore, the content of dynamic components [18] and efficacy of alkaloids [19], saponins, flavonoids, polysaccharides, and organic acids in traditional Chinese medicine will be improved after microbial fermentation and are more suitable as feed additives [20]. With the continuous innovation of microbial fermentation technology and the deepening of the modernization of traditional Chinese medicine, microbial fermentation and transformation of traditional Chinese medicine have aroused great interest and gradually become a new way to produce new active compounds with potential medicinal value.

Nontargeted metabolomics focuses on detecting hundreds of individual compounds rather than identifying or quantifying specific compounds [21]. Metabolomics is widely used to monitor the quality, processing, safety, and microbiology of fermented products. In this study, the previously optimized process parameters were used for the solid-state fermentation of *A. senticosus* (FAS), and the changes in the chemical components of the substrate in the fermentation process were analyzed. Using unfermented *A. senticosus* (AS) as a control, the metabolites of the compound of *A. senticosus* were identified by ultra-performance liquid chromatography and mass spectrometry [22]. The study established chromatographic and mass spectrometric conditions, used partial least squares discriminant analysis to screen compounds, matched the secondary mass spectrometry information with the database, identified related compounds, and obtained the metabolic material spectrum during the fermentation process of *A. senticosus*. Bioinformatic tools and software were used to classify and analyze the identified metabolites, systematically identify the primary metabolites and critical metabolic pathways in the fermentation process of *A. senticosus*, and provide a reference for the application of *A. senticosus*.

### 2. Materials and Methods

#### 2.1. Reagents and Strains

The *A. senticosus* was purchased from Liaocheng Limin Pharmacy and dried at 60°C in an oven with constant weight. After drying, the plant was crushed with a pulverizer and sieved with 200 mesh. About 1500 g powder of *A. senticosus* was used in the experiment. The powder was stored in a refrigerator at 4°C for subsequent testing. Formic acid was purchased from CNW; 2-Propanol was purchased from Merck; 2-Chloro-L-Phenylalanine was purchased from Adamas-beta. *Lactobacillus Plantarum*, *Saccharomyces cerevisiae*, and *Bacillus subtilis* were isolated from the intestine of healthy chickens in the laboratory. These strains were identified by routine methods using 16S rRNA and physiological and biochemical tests. These were sourced from laboratory-preserved strains. The concentration of all strains used for fermentation is 1 × 10⁸ CFU/ml, and the addition ratio is 1 : 1 : 1.

#### 2.2. Fermentation of *A. senticosus*

The *A. senticosus* powder (10 g) was accurately weighed and placed in a conical flask; 10 ml of distilled water and 0.1 g of glucose were added and sealed separately; the sealed conical flask was placed in an autoclave for 30 minutes, taken out, and placed on an ultraclean bench, and it was cooled to room temperature. *Lactobacillus Plantarum*, *Saccharomyces cerevisiae*, and *Bacillus subtilis* were added to their enrichment medium, placed on a shaker, and incubated and treated at 37°C for 24 h. The concentration of bacteria was adjusted to 1 × 10⁷ CFU/mL. The bacteria and *Saccharomyces cerevisiae* were sterilized and mixed on the ultraclean bench and placed in a constant temperature incubator for fermentation. After the fermentation was finished, they were baked in a blast dryer until they reached a constant weight and then used for the later experiments.

#### 2.3. Extraction of Polyphenol from *A. senticosus* and Calculation of Its Content

Dried FAS powder (1.0 g) was weighed. Polyphenols were extracted using the ultrasonic-assisted method (time = 40 min and power = 600 W) with 10 ml of 60% ethanol as the extraction solvent. In this paper, the extraction temperature was 60°C and the solid-liquid ratio was 1 : 30 (g : mL). After that, it was cooled, filtered, and centrifuged at 10,000 rpm for 10 minutes to determine the polyphenol content of the supernatant. The content of polyphenol was determined by the Folin–Ciocalteu method described by Pham [23], with gallic acid as the standard. The polyphenol yield of *A. senticosus* (*Y*) was calculated according to the following equation:

\[
Y = \frac{(C \times N \times V)}{M},
\]

where *C* is the polyphenol content in *A. senticosus*, *V* is the volume after the extraction, *N* is the dilution ratio of the sample, and *M* is the mass of the raw material.

#### 2.4. Optimization by the Response Surface Method

The effects of fermentation time, fermentation temperature, the inoculum level, water content, glucose addition, and urea addition on polyphenol content were first investigated by single-factor tests. Then, a Plackett–Burman design (PBD) was performed, and PBD was usually applied to conduct a minimum number of experiments to assess the relative importance of various parameters. Six factors affecting polyphenol content were selected, including fermentation time, fermentation temperature, the inoculum, water content, glucose addition, and urea addition, with polyphenol content (*Y*) as the response value. A 6-factor 2-level PBD was designed using Design Expert 10 software, with two levels of high (+1) and low (−1) set for each variable (Table 1), and the effect of each variable on polyphenol content was calculated by the difference between the high and low levels of the difference between the mean values. Finally, a Box–Behnken experiment with four factors and three levels was conducted for four screened factors, including water content (A), fermentation time (B), inoculum (C), and fermentation temperature (D), based on the Plackett–Burman results (Table 2). Both Plackett–Burman and BBD experimental designs were generated using Design Expert 10 software.
TABLE 1: Factors and levels of the Plackett–Burman design.

<table>
<thead>
<tr>
<th>Trial no.</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fermentation time (h)</td>
<td>36 60</td>
</tr>
<tr>
<td>Fermentation temperature (°C)</td>
<td>33 37</td>
</tr>
<tr>
<td>Inoculation amount (%)</td>
<td>7.5 12.5</td>
</tr>
<tr>
<td>Water content (%)</td>
<td>45 55</td>
</tr>
<tr>
<td>Glucose addition (g/kg)</td>
<td>5 10</td>
</tr>
<tr>
<td>Urea addition (g/kg)</td>
<td>5 10</td>
</tr>
</tbody>
</table>

2.5. Determination of Antioxidant Capacity. In this study, we investigated the changes in the antioxidant capacity of A. senticosus before and after fermentation by measuring the scavenging ability of ABTS [24] and DPPH [25] radicals in A. senticosus and their total antioxidant capacity.

2.6. Preparation of Nontargeted Metabonomics Samples. The samples were slowly thawed at a low temperature of 4°C. 50 mg of sample was accurately weighed into a 2 ml centrifuge tube, a 6 mm diameter grinding bead was added, and 400 µl of extraction solution (methanol: acetonitrile = 1:1 (v:v)) containing 0.02 mg/mL of the internal standard (L-2-chlorophenyl alanine) was added. The samples were ground for 6 min at −10°C at 50 Hz using a frozen tissue mill, extracted by ultrasonication for 30 min at 5°C at 40 kHz, left to stand at −20°C for 30 min, and centrifuged for 15 min at 13,000 g at 4°C, and the supernatant was removed to an injection vial with an internal cannula for analysis in the machine. Quality control (QC) samples were prepared by mixing the extracts of all models in equal volumes, with each QC sample having the same volume as the sample and treated and tested in the same way as the analytical samples.

2.7. UPLC-MS/MS Assay. The instrumental platform for this LC-MS analysis was an ultrahigh performance liquid chromatography-tandem Fourier transform mass spectrometry UHPLC-Q Exactive system from Thermo Fisher. Chromatographic conditions: the column was an ACQUITY UPLC HSS T3, mobile phase A was 95% water +5% acetonitrile (containing 0.1% formic acid), and mobile phase B was 47.5% acetonitrile +47.5% isopropanol +5% water (containing 0.1% formic acid); the flow rate was 0.40 mL/min, the injection volume was 2 µL, and the column temperature was 40°C.

2.8. Statistical Analysis. Box–Behnken experiments were designed using Design-Expert. The 8.0.6 software and Graphpad 8.0 software packages were used to analyze variance (ANOVA) and plot data. Principal component analysis (PCA), cluster analysis, partial least squares discriminant analysis (PLS-DA), and orthogonal partial least squares discriminant analysis (OPLS-DA) were used to predict the stability and reliability of the models. Multidimensional statistical analysis (using VIP values) and one-dimensional statistical analysis (p values) were used to predict the stability and reliability of the models. Metabolites with VIP > 1, p < 0.05, FC value > 1, or C value < 1 were selected as differential metabolites. Each group of different metabolites was clustered by hierarchical clustering. Each treatment was carried out in triplicate, and experimental results were expressed as the mean ± standard deviation.

3. Results

3.1. Response Surface Analysis. Using the polyphenol content in the fermentation process produced as an indicator, many factors affecting the fermentation process of A. senticosus were screened, and the PBD in Table 1 was fitted and analyzed by ANOVA using Design Expert software. The results are shown in Table 3, and the regression model was highly significant (p < 0.01) with a coefficient of determination R2 value of 0.9335, indicating that 93.35% of the variance could be estimated by this equation. Among them, fermentation temperature, fermentation time, inoculum amount, and water content reached significant levels (p < 0.05) and were the main effect factors affecting the production of A. senticosus polyphenols. A Box–Behnken design (BBD) in response surface methodology (RSM) was selected to determine the optimal fermentation conditions for A. senticosus. Based on the experimental results, the polyphenol yield was used as an indicator and four parameters, namely, water content (A), fermentation time (B), inoculation amount (C), and fermentation temperature (D), were selected as variables for optimising fermentation technology. The practical steps, the corresponding experimental results, the experimental design, and the results of the response surface are shown in Table 4.

Herein, A, B, C, and D refer to water content, fermentation time, inoculation amount, and fermentation temperature, respectively. The experimental data were designed and processed by Design Expert 8.0.6 software, and the variance analysis of the regression model was obtained. The results are shown in Table 5. After the quadratic polynomial fitting of the nonlinear regression, the prediction model was obtained as follows:

\[
Y = 10.26 + 0.18A + 0.24B + 0.08C + 0.39D + 0.07AB + 0.01AC - 0.02AD - 0.08BC - 0.13BD + 0.01CD - 0.83A^2 - 0.72B^2 - 1.01C^2 - 1.20D^2.
\]  

As shown in Table 5, the proposed mathematical model is significant (p < 0.0001), the lack-of-fit term is little (p > 0.05), and the corrected coefficient of determination R2 is 0.9545, indicating that 95.45% of polyphenol content of A. senticosus can be determined by the model to explain; R2...
is 0.9091, demonstrating that the polyphenol content of *A. senticosus* is changed and the equation established is a proper fit for the experiment. The effects of the respective variable factors on the polyphenol content of *A. senticosus* are as follows. The primary factors B (fermentation time) and D (fermentation temperature), A2, B2, C2, and D2 had rather significant effects on the polyphenol yield of *A. senticosus* \((p < 0.01)\). The effect of A (water content) on the total polyphenol content of *A. senticosus* was significant \((p < 0.05)\). AB (water content and fermentation time), AC (water content and inoculation amount), AD (water content and fermentation temperature), BC (fermentation time and inoculation amount), BD (fermentation time and fermentation temperature), and CD (inoculation amount and fermentation temperature) had no significant effect on the polyphenol content of *A. senticosus* \((p > 0.05)\).

The regression analysis results drew the response surface contour map and the response surface three-dimensional map. The three-dimensional map intuitively reflected the effects of the interaction of each factor on the response value, and the optimized parameters and the interaction between the parameters were found. The response surface could be used to evaluate the influence of various factors, with the impact becoming more noticeable as the surface became steeper. Figure 1 illustrates the effects of A, B, C, and D on the polyphenol content of *A. senticosus*. Through the response surface experiment, the optimized process conditions of *A. senticosus* fermentation were determined as follows.

### Table 3: Factor levels and significance analysis of Plackett–Burman.

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>Df</th>
<th>MS</th>
<th>F-value</th>
<th>(p) value</th>
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<td>1.78</td>
<td>11.7</td>
<td>0.0082</td>
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<tr>
<td>Fermentation time (h)</td>
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<td>1.97</td>
<td>12.9</td>
<td>0.0157</td>
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<tr>
<td>Fermentation temperature (°C)</td>
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<td>5.91</td>
<td>38.73</td>
<td>0.0016</td>
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<td>Inoculation amount (%)</td>
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<td>1.43</td>
<td>9.38</td>
<td>0.028</td>
</tr>
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<td>Water content (%)</td>
<td>1.31</td>
<td>1</td>
<td>1.31</td>
<td>8.59</td>
<td>0.0326</td>
</tr>
<tr>
<td>Glucose addition (g/kg)</td>
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<td>0.0143</td>
<td>0.0936</td>
<td>0.7719</td>
</tr>
<tr>
<td>Urea addition (g/kg)</td>
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<td>0.0748</td>
<td>0.4908</td>
<td>0.5148</td>
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<tr>
<td>Residual</td>
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<td>5</td>
<td>0.1525</td>
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<td>Cor total</td>
<td>11.46</td>
<td>11</td>
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</table>

*Note.* SS: sum of squares; DF: degree of freedom; MS: mean sum of squares. *Statistically significant at 95% of the probability level.

### Table 4: Response surface design and experimental results.

<table>
<thead>
<tr>
<th>Number</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>Phenol yield (mg·g(^{-1}))</th>
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<tr>
<td>1</td>
<td>55</td>
<td>36</td>
<td>12.5</td>
<td>33</td>
<td>7.56</td>
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<td>7.5</td>
<td>37</td>
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<td>37</td>
<td>8.45</td>
</tr>
<tr>
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<td>60</td>
<td>12.5</td>
<td>41</td>
<td>8.96</td>
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<td>37</td>
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<td>45</td>
<td>84</td>
<td>12.5</td>
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<td>12.5</td>
<td>37</td>
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<td>25</td>
<td>65</td>
<td>60</td>
<td>17.5</td>
<td>37</td>
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<tr>
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<td>55</td>
<td>84</td>
<td>12.5</td>
<td>33</td>
<td>8.20</td>
</tr>
</tbody>
</table>
The fermentation temperature is 37.632°C, the inoculation amount is 12.5%, the fermentation time is 63.562h, and the water content is 55%. Therefore, the total phenol content in the fermentation broth is predicted to be 10.309mg/g. Under this condition, the polyphenol content in the fermentation broth is predicted to be 10.25mg/g.

Table 5: Variance analysis and regression coefficient estimation of the response surface.

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of squares</th>
<th>Df</th>
<th>Mean square</th>
<th>F-value</th>
<th>p value</th>
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<td>Model</td>
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<td>14</td>
<td>1.36</td>
<td>20.99</td>
<td>&lt;0.0001</td>
<td>**</td>
</tr>
<tr>
<td>A</td>
<td>0.4087</td>
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<td>0.4087</td>
<td>6.3</td>
<td>0.025</td>
<td></td>
</tr>
<tr>
<td>B</td>
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<td>0.6933</td>
<td>10.69</td>
<td>0.0056</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.0847</td>
<td>1</td>
<td>0.0847</td>
<td>1.31</td>
<td>0.2723</td>
<td></td>
</tr>
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<td>D</td>
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<td>1.91</td>
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<tr>
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<td>144.36</td>
<td>&lt;0.0001</td>
<td>**</td>
</tr>
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<td>14</td>
<td>0.0649</td>
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<tr>
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<td>10</td>
<td>0.0799</td>
<td>2.94</td>
<td>0.1554</td>
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<tr>
<td>Pure error</td>
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<td>4</td>
<td>0.0272</td>
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<tr>
<td>Cor total</td>
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<td>28</td>
<td></td>
<td></td>
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</tbody>
</table>

Note: Y, A, B, C, and D are polyphenol content, water content, fermentation time, inoculation amount, and fermentation temperature, respectively; A2, B2, C2, and D2 are secondary factors of A, B, C, and D, respectively; A.B., A.C., A.D., BC, B.D., and CD are interaction factors.

3.2. Free Radical Scavenging Activity of Fermented A. senticosus. DPPH, as one of the few stable organic nitrogen compounds, is often used to evaluate the antioxidant activity of natural antioxidants. The DPPH radical scavenging assay has the advantages of being simple and rapid and can be widely used to determine the antioxidant activity of herbal extracts. Potassium persulfate can oxidize ABTS and produce ABTS radicals with a dark green color, and the scavenging ability of antioxidants on ABTS radicals can be assessed by detecting the decrease in absorbance, so it can be used to test the antioxidant activity of herbal extracts in various media. In this study, the total reducing capacity of A. senticosus was also examined based on the assessment of the effect of fermentation on the scavenging capacity of DPPH and ABTS free radicals in A. senticosus (Figure 2). The results showed that A. senticosus extracts had the scavenging ability for both free radicals and showed a positive correlation with increasing drug concentration. Among them, the IC50 values of fermented A. senticosus alcoholic extracts (FAS) for the elimination of DPPH and ABTS were 0.1764 mg·mL−1 and 0.6023 mg·mL−1, which were significantly higher than those of unfermented A. senticosus alcoholic extracts (AS). This shows that A. senticosus has better in vitro antioxidant ability, and the free radical scavenging ability is stronger after A. senticosus fermentation than before fermentation in the concentration range.

3.3. Nontargeted Metabonomic Analysis. Changes in the chemical composition of AS during solid-state fermentation were evaluated using nontargeted metabolomics. In the positive and negative ion modes, the total ion chromatogram is plotted with time as the horizontal coordinate and the intensity of the sum of the ion as the vertical coordinate [27]. Total ion current (TIC) overlap plots from MS analysis of mixed quality control (QC) samples were analyzed by overlap display analysis (Figure 3). The results show a high overlap of the total ion current curves for metabolite detection, indicating a reliable assessment. The metabolites in the samples were extracted and put on the mass spectrometer, and metabolites were detected using both positive and negative ion modes to obtain all of the mass spectrometry data. The metabolites are detected using both anion and cation modes. All mass spectrometry data are obtained, and the mass spectrometry results are imported into the Megi cloud platform for expression data preprocessing. Finally, 939 metabolites were identified in cationic and anionic modes (Table 6). From Figure 4, we can see that for the overall data, when the RSD is less than 0.3, the proportion of the peak is greater than 70%, so it was proven reliable. To better outline and analyze the dynamic changes of metabolites in AS during solid-state fermentation, a heatmap of sample correlations was drawn, where each...
3.4. Multivariate Analysis. To scientifically identify the differences in the accumulation of metabolites between FAS and AS, a metabolite analysis of AS was performed using LC-MS and multivariate analysis. Principal component analysis (PCA) can classify the samples and help us grasp the overall situation of metabolites in FAS and analyze the differences between FAS and AS under optimal fermentation conditions [29]. As shown in Figure 5, parallel samples of the two groups of AS samples, as well as the quality control (QC) sample group, were clustered together, indicating that all assays had good analytical stability and experimental reproducibility; meanwhile, the first principal component (PC1) and the second principal component (PC2) of cations and anions explained 37.20%, 45.50%, 23.00%, and 18.50%, respectively. The cumulative contribution of both reached 60.20% and 64.00%, respectively, which showed a more pronounced separation trend of FAS and AS, reflecting the metabolite differences among these samples. By appropriately rotating the principal components, the observed values between the groups could be effectively distinguished, and the influencing variables that led to the differences between the groups could be found. After the PLS-DA model was established, if the values of the model parameters (R2 and Q2) were high, the current PLS-DA model was successfully constructed. A substitution test was also conducted for the model parameters R2 and Q2 200 times [30]. The PLS-DA score plots and the results of the replacement test for the samples of AS before and after fermentation are shown in Figure 6. The results are similar to those of PCA. The results show that the R2 and Q2 values of
anions and cations reached above 0.95, indicating that the
PLS-DA model was successfully constructed and the re-
placement test was passed. The model was not overfitted
and had good stability and predictability, which can further
explore the fermentation process of AS. The model has good
stability and predictiveness to further explore the metabolite
differences in the fermentation process.

3.5. Identification of Metabolites. The secondary mapping
of metabolites matched the secondary mapping of standards
in the public database. The statistical results of the classifica-
tion of the substances identified at the secondary level accord-
ing to the classification information in the HMDB database are
shown in Figure 7 [31]. The results showed that 236 met-
abolites corresponded to lipids and lipid-like molecules, 103
metabolites corresponded to organic acids and derivatives,
65 metabolites corresponded to organic oxygen compounds,
57 metabolites corresponded to organoheterocyclic com-
pounds, 53 metabolites corresponded to benzenoids, and 53
metabolites corresponded to phenylpropanoids and
polyketides. The results showed that the main metabolites of
AS were 601 metabolic components after fermentation, of
which 57 were phenolic substances, including phenols,
cinnamic acids and derivatives, and flavonoids.

The KEGG pathway to which the metabolites were
matched was classified, with the vertical coordinate being the
secondary classification of the KEGG metabolic pathway,
and the horizontal coordinate being the number of com-
pounds annotated to the pathway. The results showed that
522 metabolites were involved in the metabolic pathway,
among which 225 metabolites were categorized into
metabolism-related pathways, accounting for 43%, as shown
in Figure 8(a). Finally, pathway analysis was performed on
the metabolites identified at the secondary level, and the
identified metabolites were submitted to the KEGG website
for metabolic pathway analysis [32]. The results are shown
in Figure 8(b). The horizontal coordinates are the top 20
pathways listed, and the vertical coordinates are the number
of metabolites matched to the KEGG database. The results
showed that 17 metabolites were enriched in the bio-
synthesis of phenylpropanoid-related pathways and 16 were

Figure 2: The antioxidant test of Acanthopanax before and after fermentation. (a) Scavenging activity of ABTS radicals, (b) scavenging
activity of DPPH radicals, and (c) total reduction capacity. Note: Values are the mean ± standard deviation; * significant differences are at the
5% level.
Table 6: Total ion count and identification statistics.

<table>
<thead>
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<th>Ion mode</th>
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<th>Identified</th>
<th>Metabolites</th>
<th>Metabolites</th>
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</thead>
<tbody>
<tr>
<td>Pos</td>
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<td>564</td>
<td>406</td>
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<td>8831</td>
<td>375</td>
<td>258</td>
<td>106</td>
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</tbody>
</table>
enriched in the biosynthesis of plant secondary metabolite-related pathways. In addition to these metabolites, various amino acid-related metabolic pathways such as alanine, aspartate, and glutamate were involved.

3.6. Identification and Analysis of Differential Metabolites

3.6.1. Differential Metabolite Statistics and OPLS-DA Analysis. The S-plot of the PLS-DA model for the FAS and AS groups in the anion model is shown in Figure 9, where the points further from the origin indicate a more significant contribution to the difference between the groups and their VIP values [33]. In this experiment, metabolites that satisfy both VIP > 1 and t-test p < 0.05 were screened as differential metabolites. Two hundred thirty-eight differential metabolites were screened in the metabolites of the FAS and AS groups. Among them, the down-regulated metabolites in the FAS and AS groups compared with each other are indicated by blue dots, red dots indicate the up-regulated metabolites, and the unchanged metabolites are indicated by gray dots (Figure 10).

3.6.2. Cluster Analysis of Differential Metabolites. The metabolite distribution can be visually divided into up-regulation and down-regulation. To directly observe the trend of the concentration of the different metabolites before and after fermentation, a heatmap was made based on the relative content of each different metabolite [34], as shown in Figure 4: Correlation among samples. (a) Sample evaluation. (b) The sample correlation heatmap in the cationic mode (positive ion). (c) The sample correlation heatmap in the anionic mode (negative ion).
in Figure 11. The tree diagram of the clustering is shown on the left side, and the names of metabolites are shown on the right side. The expression profiles of metabolites in each sample in the FAS and AS groups were the same, the aggregation effect was good, and the high and low expression metabolites could be distinguished. The figure shows that the high content of differential metabolites in the fermentation group (FAS) was more than 75%, which was significantly more than that in the control group (AS), where the content of organic acids and their substituted derivatives and amino acids and their derivatives in the fermentation group was higher than that in the unfermented group. Phenolic substances are essential for plant extracts with vigorous antioxidant activity.

Phenols are a large group of secondary metabolites with diverse structures that are widely distributed in plants. Some polyphenols have a variety of structures, some of which have important pharmacological activities such as antioxidants and anti-inflammatory substances. They can also modify the palatability of fermented herbal medicines and broaden the avenues of application. To better visualize the differential phenolic metabolites, violin plots were used to compare the
3.7. Metabolic Pathway Analysis of Differential Metabolites. The plant growth process is a very complex metabolic process that must be regulated by a variety of substances and reactions together. The overall judgment cannot be made only from the content of a certain substance, so further analysis of its metabolic pathways is needed. By comparing with the KEGG database, the metabolic pathways involved in the metabolites can be known to evaluate their effects on the biological metabolic process [36]. Forty-one metabolic pathways in 7 categories were retrieved before and after the fermentation of AS. The secondary classification names of KEGG, as shown in Figure 13, were amino acid metabolism, polysaccharide biosynthesis, metabolism, biosynthesis of other secondary metabolites, nucleotide metabolism, carbohydrate metabolism, xenobiotic biodegradation and metabolism, cofactor, vitamin metabolism, another amino acid metabolism, lipid metabolism, terpenoid, polyketide metabolism, exogenous biodegradation and metabolism, carbon metabolism, and various systemic pathways.

Differential metabolites identified during the fermentation of *A. senticosus* were analyzed, and enrichment analysis and topology analysis of metabolic pathways were performed based on the data from the pathway. First, differential metabolites were present in 202 metabolic pathways based on the analysis, among which 99 pathways were significantly aggregated (*p* < 0.05). The most significant key metabolic pathways were selected based on the *p* value and impact values (Table 7), which were as follows: (1) alanine, aspartate, and glutamate metabolism, including L-asparagine; (2) pyrimidine metabolism, including uracil; (3) arginine and proline metabolism, including L-arginine; (4) tyrosine metabolism, including gentisic acid; (5) citrate cycle (TCA cycle), including malic acid; (6) phenylpropanoid biosynthesis, including caffeic acid; (7) arginine biosynthesis, including L-arginine; (8) valine, leucine, and isoleucine biosynthesis, including 2-isopropylmalic acid; and (9) pyruvate metabolism, including 2-isopropylmalic acid. Figure 14 shows the pathway enrichment diagram, and the experiment was conducted to investigate the effect of fermentation on the analysis of metabolic pathways in *A. senticosus* by analyzing the differential metabolites of *A. senticosus* before and after fermentation. As shown in Figure 14(b), the synthesis of secondary metabolites and phenylpropanoid biosynthesis were dark and significant, which confirmed the significant effect of fermentation on phenolics (e.g., caffeic acid and 2-hydroxycinnamic acid) in *A. senticosus*.
Figure 8: Continued.
metabolic pathway. The phenylpropane metabolic pathway is one of the important secondary metabolic pathways for the synthesis of lignin and flavonoid substances in plants. The phenylpropane metabolic pathway was enriched in this study, which confirmed that fermentation could promote the production of phenolic substances and provide a reference for the effect of fermentation on phenolic substances in Acer sativa.

4. Discussion

In recent years, research on Chinese medicine has become a hot spot for improving the utilisation rate of Chinese medicine and developing new avenues of application. Fermentation is a biochemical reaction process that uses the metabolic function of microorganisms and plant cells to break down organic matter [37]. After microbial fermentation, Chinese medicines can significantly enhance their efficacy, reduce toxicity, and produce new active ingredients. Various studies have shown that fermented Chinese medicines have good effects in promoting growth and development of the body and preventing diseases [38-42]. The strains used in this study were all commonly used probiotics, which produce metabolites beneficial to the host during the fermentation process [43]. Compared with the single strain, the composite strain improved the fermentation efficiency and fermentation quality due to the combined effect of bacteria and enzymes. After the microbial fermentation of A. senticosus, the extracellular enzymes such as cellulase and pectinase produced by microorganisms caused its cells to rupture, allowing the precipitation of the active ingredients of A. senticosus.

Response surface methodology is an effective method to study the interaction of different factors in the fermentation process. In this study, PBD and BBD experiments were used to determine the optimal fermentation conditions for A. senticosus, using the polyphenol content as an experimental index. Polyphenols are a class of secondary
metabolites widely found in plants. Polyphenol compounds can be covalently bonded with carbohydrates, proteins, and other biological macromolecules to form insoluble bonded polyphenols. During the fermentation process, microorganisms can produce carbohydrate hydrolases such as cellulase, which decompose carbohydrates bound with polyphenols and promote the release of insoluble bonded polyphenols, thus increasing the polyphenol content. Fermentation time affects the quality of fermentation. As the fermentation time increases, the microorganisms consume nutrients to add value, which in turn increases the active ingredient of the drug [44]. The polyphenol content in this study showed a trend of increasing and then decreasing, which may be because, at the beginning of fermentation, the carbohydrate hydrolase produced by microorganisms promoted the release of insoluble bonded polyphenols, leading to an increase in the polyphenol content; the gradual decrease in the polyphenol content at the later stage of fermentation may be because the release of insoluble bonded polyphenols was complete, while the content was reduced due to the poor stability of polyphenols. The optimal fermentation time for pharyngitis tablet residue prepared by

![Figure 9: The S-plot of FAS and AS. (a) Cationic mode (positive ion). (b) Anionic mode (negative ion).](image)

![Figure 10: FAS vs. AS different metabolite volcano map. (a) Cationic mode (positive ion). (b) Anionic mode (negative ion).](image)
Figure 11: Continued.
other researchers using Lactobacillus Plantarum and Bacillus subtilis was 72 h [45], while the optimal fermentation time in this study was 60 h, which may be due to the different species of fermentation substrate and inoculated strains.

The water content will also have an important effect on the polyphenol content. Microbial pregrowth, cellulase secretion, transfer, and action all need a certain amount of water to participate, but the increase in water content will lead to the increase in viscosity of the fermentation material and reduce the air exchange, and the waste gas from growth and metabolism will affect the normal growth of the strain. Studies have shown that, at 40–60% water content in solid-
Figure 12: The relative contents of major differential metabolites. (a) (+/-)-Enterolactone. (b) (2E)-3-[3-(sulfooxy)phenyl]prop-2-enoic acid. (c) Caffeic Acid. (d) Caffeic acid 3-sulfate. (e) Caffeic acid 4-O-glucuronide. (f) Dihydrodaidzein. (g) EUGENOL. (h) Hydroxymethoxyphenyl carboxylic acid-O-sulphate. Note: On the upper side, * represents $p < 0.05$, ** represents $p < 0.01$, and *** represents $p < 0.001$. 

Figure 13: KEGG functional pathway statistics.
state fermentation, the pH of fermentable can be kept stable with a good fermentation effect [46]. However, different fermentation substrates require different water contents, and the optimized water content of 55% in this experiment was favorable for the fermentation of A. senticosus to improve the polyphenol content. The appropriate fermentation temperature is an important factor affecting the growth and metabolism of the strain. Each microorganism has an optimum temperature for growth, and the mixed bacteria fermentation system also has an optimum temperature, below or above which the growth of microorganisms will be inhibited and the metabolic rate will be reduced. The results of this study showed that 37°C was the optimum temperature when A. senticosus had the best fermentation effect and the highest polyphenol content. A suitable inoculum level enables the strain to make full use of the substrate to react. A higher inoculum level may accelerate the growth rate of the strain, but at the same time, it will increase the rate of nutrient consumption. A very high inoculum level prolongs the time required for the fermentation process and leads to incomplete fermentation of the substrate. The results of this experiment showed that the inoculum level of 12.5% had the best fermentation effect.

Metabolomics is an emerging technology for comprehensively analyzing the changes in metabolites of the samples to be tested. It mainly analyzes and determines metabolites by high throughput measurement methods such as ultrahigh liquid chromatography-mass spectrometry and uses relevant databases combined with appropriate statistical methods to find out the different metabolites that perform biological functions of the samples to be tested, to analyze the reasons for their high active functions and lay the theoretical foundation for better utilization of biological resources [47]. Natural drugs contain many pharmacological components, but plant fibres prevent the precipitation of active ingredients [48]. Fermentation increases the digestion of plant fibers and enhances the content of active ingredients in natural drugs [49]. In this study, we performed a comprehensive analysis of A. senticosus before and after fermentation. By using nontargeted metabolomics techniques,
we could not only quantify the known components but also identify the components by spectroscopy. The polyphenolic content and antioxidant activity of *A. senticosus* were enhanced before and after fermentation compared to those of *A. senticosus*.

Based on LC-MS untargeted metabolomics, we investigated the changes in metabolites before and after *A. senticosus* fermentation. We used LC-MS, univariate statistical analysis, and multivariate statistical analysis to investigate the distribution and patterns of metabolites before and after the fermentation of *A. senticosus*. PCA and PLS-DA analyses in the anion and cation modes of the samples before and after fermentation showed that the duplicate samples were relatively clustered within the group. The samples showed a clear separation trend among the groups. Through the construction and analysis of PCA, PLS-DA, and OPLS-DA models, a total of 601 secondary metabolites were identified in the anion and cation model and 238 different metabolites were identified before and after the fermentation of *A. senticosus*, mainly including lipids and lipid-like molecules, organic acids and derivatives, organic oxygen compounds, organoheterocyclic compounds, phenylpropanoids and polyketides, nucleosides, nucleotides, analog benzenoids, organic nitrogen compounds, alkaloids and derivatives, lignans, neolignans, and related compounds. The hierarchical clustering analysis of differential metabolites showed that the clustering of metabolites in both FAS and AS groups was divided into two major clusters, representing differential metabolites in AS before and after fermentation, with more than 60% differential metabolites with up-regulated expression compared to the unfermented group.

5. Conclusions

In this experiment, we used RSM to screen and optimize the optimal process for solid-state fermentation of *A. senticosus*: fermentation temperature of 37°C, fermentation time of 60 h, inoculum level of 12.5%, and water content of 55%. Under optimal conditions, the polyphenol content in *A. senticosus* was increased by 40% after fermentation. Subsequently, LC-MS untargeted metabolomic analysis was combined with multivariate analysis to systematically reveal the metabolic profile changes of *A. senticosus* during solid fermentation. A total of 601 major metabolic components were identified, 57 of which were phenolics. It was confirmed that probiotic fermentation had different effects on the content of secondary and primary metabolites of *A. senticosus*. This study further verified the feasibility and scientific validity of microbial fermentation of *A. senticosus*, theoretically supported that the active components and metabolites of *A. senticosus* changed significantly after fermentation, provided a new research strategy to elucidate the synergistic mechanism of microbial fermentation of *A. senticosus*, and offered new research ideas for the comprehensive development and utilization of *A. senticosus*. 
Data Availability
The data sets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Disclosure
Jianqing Su and Xiang Fu are the co-first authors.

Conflicts of Interest
The authors declare that there are no conflicts of interest.

Authors’ Contributions
Xiang Fu prepared the original draft, wrote and edited the review, visualized the study, and curated the data. Rui Zhang was involved in the methodology. Xiaoli Li investigated the study. Xiuling Chu conceptualized the study and managed the resources. All authors have read and agreed to the published version of the manuscript. Jianqing Su and Xiang Fu contributed equally to this work.

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References


