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Research article

Analysis of metabolic differences between Jiaosu fermented from dendrobium flowers and stems based on untargeted metabolomics

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ABSTRACT

Dendrobium officinale is an important traditional Chinese medicinal herb containing bioactive polysaccharides and alkaloids. This study characterized metabolite differences between jiaosu (fermented plant product) from Dendrobium flowers versus stems using untargeted metabolomics. The jiaosu was fermented by mixed fermentation of *Saccharomyces cerevisiae*, Lactobacillus bulgaricus and Streptococcus thermophilus. Liquid chromatography-mass spectrometry analysis identified 476 differentially expressed metabolites between the two Jiaosu products. Key results showed downregulation of flavonoid metabolism in Dendrobium Stems Edible Plant Jiaosu (SEP) but increased flavonoid synthesis in Dendrobium Flowers Edible Plant Jiaosu (FEP), likely an antioxidant response. SEP displayed upregulation of lignin metabolites with potential antioxidant properties. The findings demonstrate significant metabolite profile differences between SEP and FEP, providing the basis for developing functional jiaosu products targeting specific health benefits.

1. Introduction

Dendrobium officinale Kimura et Migo, a member of the Orchidaceae family, primarily comprises polysaccharides and contains polyphenols, flavonoids, alkaloids, amino acids, and vitamins [1,2]. Recent pharmacological research has shown that Dendrobium, including its polysaccharide component, provides various health benefits, including protection against cancer and liver damage and reducing blood cholesterol, triglyceride levels, and blood pressure [3,4].

The edible plant Jiaosu (EPJ), or fermented juice, is produced by fermenting various vegetables, fruits, grains, seaweeds, and mushrooms with naturally occurring or added bacteria or yeast [5]. Currently, natural fermentation is the primary method used in the manufacturing of EPJ [6]. While natural fermentation is a cost-effective and straightforward process, it presents challenges such as a more complex microflora, a longer fermentation cycle, unknown changes in active chemicals, and difficulties in quality monitoring. Using artificial inoculation for strain determination can effectively address the limitations associated with the prolonged and unpredictable natural fermentation of EPJ. This approach leads to a more regulated fermentation process and improved nutrient composition. According to Lee et al. [7], the fermentation of papaya juice with a combination of *S. cerevisiae* and W. saturnus strains resulted in the generation of more complex fragrance compounds and elevated ethanol levels, in contrast to papaya juice fermented

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with a single yeast strain.

Untargeted metabolomics involves the thorough examination of both known and unknown metabolites within a biological system without any predetermined assumptions. Liquid chromatography combined with mass spectrometry (LC-MS) is widely utilized as an analytical method for untargeted metabolomic studies, enabling the comprehensive analysis of microbial metabolism on a global level [8]. There needs to be more scholarly investigation into the metabolic byproducts of EPJ in the realm of metabolomics, resulting in a lack of comprehensive knowledge regarding the specific metabolites and metabolic pathways that contribute to the advantageous effects of EPJ. Consequently, scientific interest is in characterizing the fermentation processes associated with these products.

Most of the current Dendrobium EPJ is produced through the fermentation of Dendrobium stems as the primary raw material. In a study by L. Jiang et al. [9], Dendrobium huoshanense was used as the raw material to examine the active ingredients and in vitro antioxidant capacity of Dendrobium EPJ during fermentation. Furthermore, Dendrobium flowers contain diverse bioactive compounds, such as naringenin, anthocyanins, polysaccharides, amino acids, and dendrobium alkaloids. These compounds demonstrate antioxidant, hepatoprotective, hypoglycemic, and hypotensive effects [10]. Nevertheless, there is limited research on the Dendrobium flower EPJ. Meanwhile, most research on Dendrobium EPJ has focused solely on quantifying specific metabolites and antioxidant capacity [11]. However, comprehensive information regarding metabolites and specific antioxidants in Dendrobium EPJ remains unclear.

The objective of this study was to analyze the evolving metabolomics of Dendrobium stem EPJ and Dendrobium floral EPJ and to identify significant differential metabolites, as well as potential anti-inflammatory and antioxidant compounds linked to these two distinct EPJ production pathways through artificial inoculation. To establish a foundation for the food industry to develop various Dendrobium EPJ products tailored to diverse health benefits and consumer demands. The metabolomic analysis was conducted using liquid chromatography and mass spectrometry (LC-MS).

2. Materials and methods

2.1. Chemicals and material

Methanol (HPLC grade) and acetonitrile (HPLC grade) were purchased from ThermoFisher Scientific, Co., Ltd. (USA). Formic acid were purchased from CNW Technologies, Co., Ltd. (China). Isopropanol (HPLC grade) was purchased from Merck & Co., Inc. (USA).

Dendrobium flowers and Dendrobium stems were purchased from Huoshan County Agricultural Market.

2.2. Preparation of dendrobium stem EPJ samples and dendrobium flower EPJ samples

Dendrobium Stems Edible Plant Jiaosu (SEP) and Dendrobium Flowers Edible Plant Jiaosu (FEP) were produced following the protocol described by Jiang et al. with modifications [5]. Cleanse 100g of both dendrobium flowers and stems with sterile water to remove surface dust, and then chop them into ~5 mm × 5 mm pieces using sterile scissors. Sterilize them using UV light for 45 min, flipping and repeating this process two to three times to ensure adequate sterilization. Combine the sterilized dendrobium flower/stem powder with the sterilized fermentation vessel, and then add the autoclaved sucrose solution in a ratio of 1:1:6 (dendrobium flower/stem powder: sucrose solution: water). Next, add 1% of the prepared fermenting agent consisting of *Saccharomyces cerevisiae*, Lactobacillus bulgaricus, and Streptococcus thermophilus in a ratio of 2:1:1 to the two groups of fermenters. Seal the fermenters and ferment at 37 °C for 21 days. After fermentation, extract six 5 mL samples of EPJ from dendrobium flowers and label them FEP 1, FEP2, …, FEP6. Similarly, obtain six 5 mL samples of EPJ from dendrobium stems and designate them SEP 1, SEP2, …, SEP6. Test using the two sets of 12 samples, and store the remaining EPJ in a refrigerator at −20 °C.

2.3. Metabolite extraction for LC-MS analysis

Extract 100 μl of EPJ samples from Dendrobium stems or flowers using a 1:1 (v/v) mixture of methanol and acetonitrile (400 μL). Subsequently, sonicate the mixture for 30 min at 40 kHz and 5 °C. Precipitate the proteins by storing the samples at −20 °C for 30 min. Transfer the supernatant to fresh microtubes and evaporate it to dryness using a gentle stream of nitrogen after centrifuging at 13000g at 4 °C for 15 min. Reconstitute the samples for UHPLC-MS/MS analysis using brief sonication in a 5 °C water bath with 100 μL of loading solution of acetonitrile and water (1:1, v/v).

2.4. Quality control sample

As a part of the system conditioning and quality control process, a pooled quality control sample (QC) was prepared by mixing equal volumes of all samples. The QC samples were disposed of and tested in the same manner as the analytic samples. It helped to represent the whole sample set, which would be injected at regular intervals (every 4 samples) to monitor the stability of the analysis.

2.5. (UHPLC-MS/MS) analysis

The instrument platform for LC-MS analysis is a Thermo Fisher Scientific UHPLC-Q Exactive system.

2.5.1. Chromatographic states

2 μL of the sample was separated using an HSS T3 column (100 mm 2.1 mm i.d., 1.8), and then mass spectrometry detection was performed. (Solvent A) and 0.1% formic acid in acetonitrile: isopropanol: water (Solvent B). For equilibrating the systems, the solvent gradient changed as follows: from 0 to 0.1 min, 0% B to 5% B; from 0.1 to 2 min, 5% B to 25% B; from 2 to 9 min, 25% B to 100% B; from 9 to 13 min, 100% B to 100% B; from 13 to 13.1 min, 100% B to 0% B; and from 13.1 to 16 min, 0% B to 0% B. The injection volume of the sample was two μL, and the flow rate was set to 0.4 mL/min. The column temperature was kept at 40 °C. During the analysis period, these samples were all stored at 4 °C.

2.5.2. MS conditions

The mass spectrometric data was gathered using a Thermo UHPLC-Q Exactive Mass Spectrometer with an electrospray ionization (ESI) source operating in either positive or negative ion mode. The ideal conditions were established as follows: 400 °C heater temperature;

Sheath gas flow rate, 40 arb; Aux gas flow rate, 10 arb; ion-spray voltage floating (ISVF), −2800V in negative mode and 3500V in positive mode, respectively. Normalized collision energy, rolling at 20-40-60V for MS/MS. The total MS resolution was 70,000, while

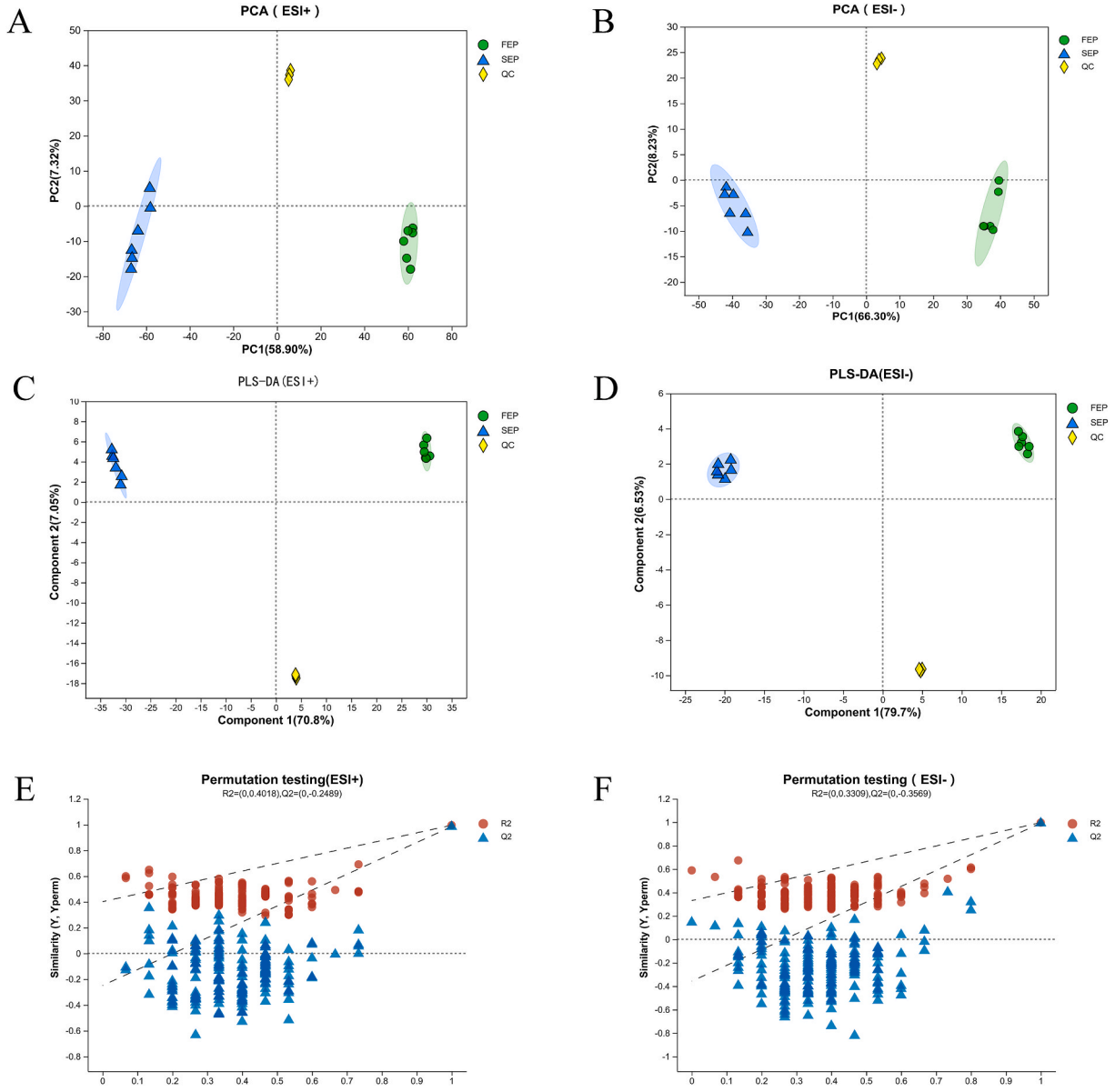

Fig. 1. PCA score plots、PLS-DA score plots and replacement test plots of all samples in the positive ionization mode (A/C/E) and negative ionization mode (B/D/F).

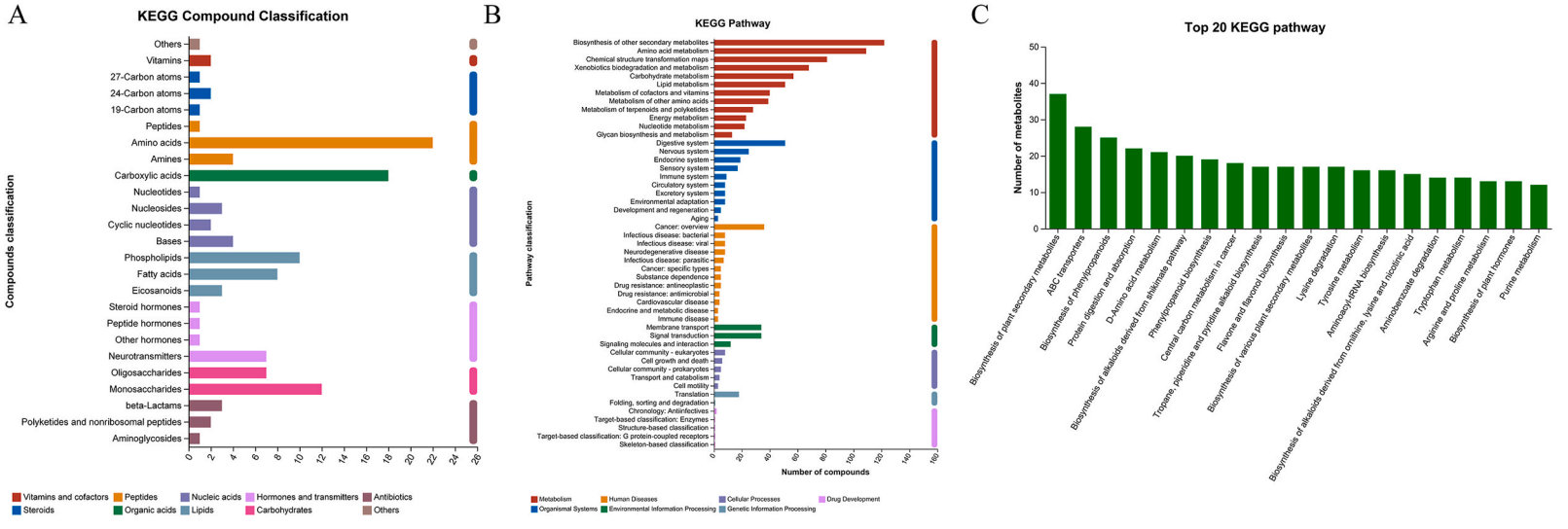

Fig. 2. Based on the identification of all metabolites by mass spectrometry, the metabolites were compared with KEGG to obtain the statistics of compound classification in the database (2A), the related pathways (2B) and the statistics of the top 20 significant pathways (2C).

the MS/MS resolution was 17,500. The Data Dependent Acquisition (DDA) mode was utilized for data collection. The detection spanned the mass range of 70–1050 *m/z*.

2.6. Data analysis

The software MassHunter workstation Quantitative Analysis (version v10.0.707.0) preprocessed the raw data obtained from the mass spectrometer detection of the GC/MS. It exported a three-dimensional data matrix in CSV format. This three-dimensional matrix includes the sample details, metabolite names, and mass spectral response intensities. The data matrix underwent dereplication, peak pooling, and elimination of known false positive peaks such as noise, column bleed, and derivatized reagent peaks. The primary public databases used for metabolite identification were NIST (version 2017), Fiehn (version 2013), and MS-DIAL (version 2021).

Principal Component Analysis (PCA) and Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) using R package "ropls" (Version 1.6.2). The stability of the model was assessed using a 7-cycle interactive validation. Metabolites with VIP>1 and a p value < 0.05 were considered significantly distinct based on the Variable Importance in Projection (VIP) values derived from the OPLS-DA model and the p values obtained through a Student's t-test.

Metabolic enrichment and pathway analysis were conducted using the KEGG database to map different metabolites between the two groups into their respective biochemical pathways. The metabolites were categorized based on the pathways they were associated with and the tasks they performed. Enrichment analysis utilized the Python package "scipy.stats" to identify the most relevant biological pathways for the experimental treatments.

3. Results

3.1. Overview of sample grouping using unsupervised multivariate analysis

Following data processing, 5605 mass spectral peaks were identified in ESI + mode, while 1912 peaks were identified in ESI- mode. Out of these peaks, 781 can be annotated in ESI + mode and 684 in ESI- mode to public databases like HMDB and Lipidmaps. Furthermore, 427 peaks in ESI + mode and 374 peaks in ESI- mode can be identified and matched to the KEGG database.

Principal Component Analysis (PCA) is an unsupervised multivariate statistical analysis technique used to measure the overall differences between sample groups and the variability within groups. The proximity of the spots in the PCA plot indicates a higher degree of similarity in the metabolite expression patterns between the groups of Dendrobium flower EPJ (FPJ) and Dendrobium stem EPJ (SPJ).

Fig. 1A and B demonstrate satisfactory aggregation of quality control (QC) samples in both electrospray ionization positive (ESI+) and negative (ESI-) modes, indicating instrument stability, consistent signals, and experimental reproducibility. The experimental groups SPJ and FPJ demonstrated close clustering in both ESI+ and ESI- PCA plots, with no overlap, suggesting minimal variation within the groups but notable metabolic distinctions between SEP and FEP groups when subjected to the same EPJ preparation and fermentation conditions. This serves as a prerequisite for further analysis of the differential pathways. Nevertheless, the PCA plots of the experimental group revealed a distinct separation between the SPJ and FPJ groups, suggesting that Dendrobium's unique raw material components were instrumental in distinguishing between the two groups.

Partial Least Squares Discriminant Analysis (PLS-DA) is a commonly employed multivariate data analysis method for addressing classification and discriminant issues. By appropriately rotating the principal components, PLS-DA can effectively differentiate between groups of observations. The PLS-DA score plots (Fig. 1C and D) and replacement test plots (Fig. 1E and F) exhibit consistency with the PCA findings, offering clear evidence of a substantial demarcation between the SPJ and FPJ groups, devoid of any overlap. This confirms the outcomes observed in the PCA plots. A Q2 regression line with an intercept lower than 0.05 in the two replacement test plots suggests a robust and reliable model that avoids overfitting.

3.2. KEGG compound classification and pathway statistics

The KEGG database helps conduct metabolic analysis and investigate metabolic networks in living organisms. Cross-referencing it with the KEGG compound database allows us to access the KEGG compound ID numbers for metabolites. These ID numbers offer insights into the biological pathways in which a metabolite is involved and its classification based on biological function [12]. Comparing the identified metabolites with the KEGG compound database yielded metabolite classification profiles, and the associated statistics are depicted in Fig. 2A.

Fig. 2A illustrates that all samples contained a high abundance of amino acids, carboxylic acids, and monosaccharides, with 22, 18, and 12 species, respectively. This finding is consistent with the results. Feng et al.'s [13] study focused on the fermentation of elderberry juice with Lactobacillus bulgaricus and Streptococcus thermophilus. The study demonstrated an increased diversity of amino acids, sugars, flavonols, organic acids, and derivatives during the later stages of fermentation. In addition, Fig. 2A and B provide some information on the pathways linked to the metabolites and emphasize the significance of plant secondary metabolite biosynthesis, phenylpropanoid biosynthesis, D-amino acid metabolism, flavone, and flavonol biosynthesis, and related pathways in the fermentation of Dendrobium species as depicted in EPJ. Therefore, this paper will focus on these metabolites and their related pathways.

3.3. Overview of dendrobium stem EPJ and dendrobium flower EPJ differential compounds

473 differential metabolites were identified in both ESI+ and ESI- modes with statistical significance ($P < 0.05$ & VIP >2). Among these, 152 compounds exhibited up-regulation in SEP expression compared to FEP expression, while 321 compounds showed down-regulation in SEP expression compared to FEP expression. Fig. 3A depicts the volcano plot, highlighting the metabolite variations between the two sample groups and identifying the 10 compounds with the lowest *p* values labeled. Table 1 presents 93 compounds that have been up- or down-regulated and are linked to significant pathways outlined in section 3.2. Out of these, 6 compounds exhibited significant up-regulation, while 23 compounds showed significant down-regulation. The 93 compounds were grouped using heat maps (Fig. 3B), classifying them into 10 groups. This approach provided a more comprehensive insight into each compound's expression in the Dendrobium flower EPJ and Dendrobium stem EPJ.

3.4. Differential compound changes

Fig. 3C shows the VIP bar graph for the metabolite. The length of each bar represents the contribution of the metabolite to the difference between the two groups, with larger values indicating more significant differences. The color of the bars indicates the significance of the metabolite difference in the two sample groups, specifically the *p* value. A smaller *p* value corresponds to a higher -log10 (*p* value) and a darker color.

3.4.1. Change of phenylpropanoids and polyketides

Most of the phenyl compounds and polyketides that exhibited notable expression changes in this investigation were flavonoids. Furthermore, several compounds were classified under cinnamic acid, cinnamyl alcohol, coumarin and its derivatives, and tannins. Phenylpropanoids and polyketides exhibiting notable changes in expression were consistently found to be down-regulated in Dendrobium stem EPJs in comparison to Dendrobium flower EPJs.

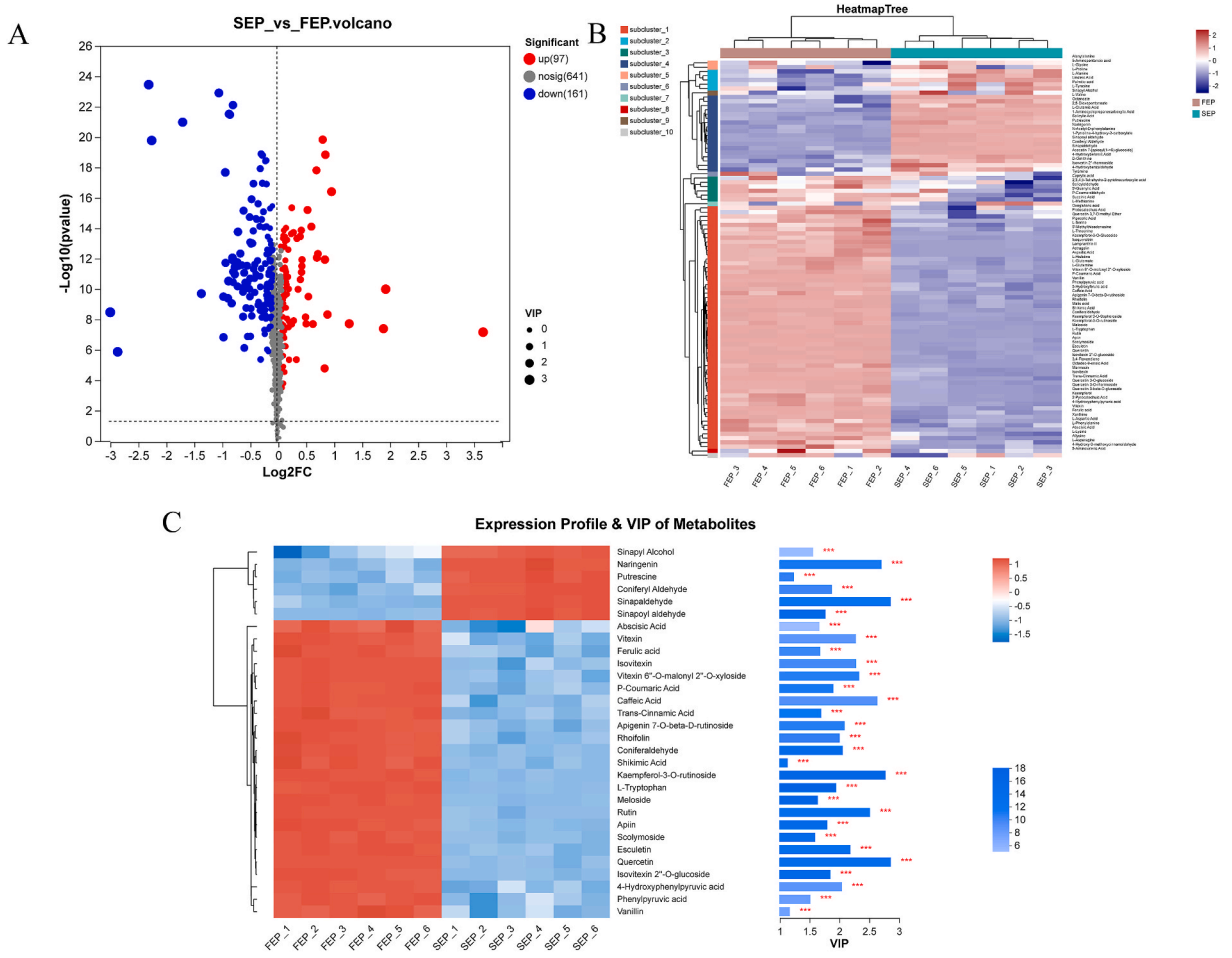

Fig. 3. Differential volcano plots (3A), clustered heat maps (3B) and VIP bar graphs (3C) for SEP vs. FEP.

Table 1
Differential metabolites in the fermented samples of FEP and SEP.

Metabolite	Significant	Regulate	level	KEGG Compound ID	HMDB Superclass	HMDB Class	HMDB Subclass
Quercetin	yes	down	B(i)	C00389	Phenylpropanoids and polyketides	Flavonoids	Flavones
Sinapaldehyde	yes	up	B(ii)	C05610	Benzenoids	Phenols	Methoxyphenols
Kaempferol-3-O-rutinoside	yes	down	B(i)	C21833	Phenylpropanoids and polyketides	Flavonoids	Flavonoid glycosides
Naringenin	yes	up	B(i)	C00509	Phenylpropanoids and polyketides	Flavonoids	Flavans
Caffeic Acid	yes	down	B(i)	C01197	Phenylpropanoids and polyketides	Cinnamic acids and derivatives	Hydroxycinnamic acids and derivatives
Rutin	yes	down	B(ii)	C05625	Phenylpropanoids and polyketides	Flavonoids	O-methylated flavonoids
Vitexin 6″-O-malonyl 2″-O-xyloside	yes	down	B(ii)	C01460	Phenylpropanoids and polyketides	Flavonoids	Flavonoid glycosides
Isovitexin	yes	down	B(i)	C01714	Lipids and lipid-like molecules	Fatty Acyls	Fatty amides
Vitexin	yes	down	B(i)	C01460	Phenylpropanoids and polyketides	Flavonoids	Flavonoid glycosides
Esculetin	yes	down	B(i)	C09263	Phenylpropanoids and polyketides	Coumarins and derivatives	Hydroxycoumarins
Apigenin 7-O-beta-D-rutinoside	yes	down	B(ii)	C12627	Phenylpropanoids and polyketides	Flavonoids	Flavonoid glycosides
Coniferaldehyde	yes	down	B(ii)	C02666	Benzenoids	Phenols	Methoxyphenols
4-Hydroxyphenylpyruvic acid	yes	down	B(ii)	C01179	Benzenoids	Benzene and substituted derivatives	Phenylpyruvic acid derivatives
Rhoifolin	yes	down	B(ii)	C12627	Phenylpropanoids and polyketides	Flavonoids	Flavonoid glycosides
L-Tryptophan	yes	down	B(ii)	C00078	Organoheterocyclic compounds	Indoles and derivatives	Indolyl carboxylic acids and derivatives
P-Coumaric Acid	yes	down	B(i)	C02646	Phenylpropanoids and polyketides	Cinnamyl alcohols	Not Available
Coniferyl Aldehyde	yes	up	B(i)	C02666	Benzenoids	Phenols	Methoxyphenols
Isovitexin 2″-O-glucoside	yes	down	B(ii)	C04199	Phenylpropanoids and polyketides	Flavonoids	Flavonoid glycosides
Apiin	yes	down	B(ii)	C04858	Phenylpropanoids and polyketides	Flavonoids	Flavonoid glycosides
Sinapoyl aldehyde	yes	up	B(i)	C05610	–	–	–
Trans-Cinnamic Acid	yes	down	B(i)	C00423	Phenylpropanoids and polyketides	Cinnamic acids and derivatives	Cinnamic acids
Ferulic acid	yes	down	B(ii)	C01494	Phenylpropanoids and polyketides	Cinnamic acids and derivatives	Hydroxycinnamic acids and derivatives
Abscisic Acid	yes	down	B(i)	C06082	Lipids and lipid-like molecules	Prenol lipids	Sesquiterpenoids
Meloside	yes	down	B(ii)	C04199	Phenylpropanoids and polyketides	Flavonoids	Flavonoid glycosides
Scolymoside	yes	down	B(ii)	C12630	Phenylpropanoids and polyketides	Tannins	Hydrolyzable tannins
Sinapyl Alcohol	yes	up	B(i)	C02325	Benzenoids	Phenols	Methoxyphenols
Phenylpyruvic acid	yes	down	B(ii)	C00166	Benzenoids	Benzene and substituted derivatives	Phenylpyruvic acid derivatives
Putrescine	yes	up	B(i)	C00134	Organic nitrogen compounds	Organonitrogen compounds	Amines
Vanillin	yes	down	B(ii)	C00755	Benzenoids	Phenols	Methoxyphenols
Shikimic Acid	yes	down	B(i)	C00493	Organic oxygen compounds	Organooxygen compounds	Alcohols and polyols
L-Glutamic Acid	no	up	B(i)	C00025	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides, and analogues
N-Acetyl-D-phenylalanine	no	up	B(i)	C05620	–	–	–
Marmesin	no	down	B(ii)	C09276	Phenylpropanoids and polyketides	Coumarins and derivatives	Furanocoumarins
Kaempferol 3-O-Sophoroside	no	down	B(i)	C12634	Phenylpropanoids and polyketides	Flavonoids	Flavonoid glycosides
Lampranthin II	no	down	B(ii)	C12634	Phenylpropanoids and polyketides	Flavonoids	Flavonoid glycosides
3,4-Flavandione	no	down	B(ii)	C01495	Phenylpropanoids and polyketides	Flavonoids	Flavones
Malic acid	no	down	B(i)	C00149	Organic acids and derivatives	Hydroxy acids and derivatives	Beta hydroxy acids and derivatives
Kaempferol-3-O-Glucoside	no	down	B(i)	C12249	Phenylpropanoids and polyketides	Flavonoids	Flavonoid glycosides
Quercetin 3-beta-D-glucoside	no	down	B(i)	C05623	Phenylpropanoids and polyketides	Flavonoids	Flavonoid glycosides
L-Glutamine	no	down	B(ii)	C00064	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides, and analogues
Astragalin	no	down	B(i)	C12249	Phenylpropanoids and polyketides	Flavonoids	Flavonoid glycosides

(*continued on next page*)

Table 1 (*continued*)

Metabolite	Significant	Regulate	level	KEGG Compound ID	HMDB Superclass	HMDB Class	HMDB Subclass
5-Hydroxyferulic acid	no	down	B(ii)	C05619	Phenylpropanoids and polyketides	Cinnamic acids and derivatives	Hydroxycinnamic acids and derivatives
Kaempferol	no	down	B(i)	C05903	Phenylpropanoids and polyketides	Flavonoids	Flavones
2-Pyrocatechuic Acid	no	down	B(i)	C00196	Benzenoids	Benzene and substituted derivatives	Benzoic acids and derivatives
5′-Methylthioadenosine	no	down	B(ii)	C00170	Nucleosides, nucleotides, and analogues	5″-deoxyribonucleosides	5″-deoxy-5″-thionucleosides
Quercetin 3-O-rhamnoside	no	down	B(ii)	C01750	Phenylpropanoids and polyketides	Flavonoids	Flavonoid glycosides
Aspartic Acid	no	down	B(i)	C00049	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides, and analogues
1-Pyrroline-4-hydroxy-2-carboxylate	no	up	B(ii)	C04282	Organoheterocyclic compounds	Pyrrolines	Not Available
1-Aminocyclopropanecarboxylic Acid	no	up	B(i)	C01234	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides, and analogues
Quercetin 3-O-glucoside	no	down	B(i)	C05623	Phenylpropanoids and polyketides	Flavonoids	Flavonoid glycosides
Octadec-9-enoic Acid	no	down	B(ii)	C00712	Lipids and lipid-like molecules	Fatty Acyls	Fatty acids and conjugates
Isoquercitrin	no	down	B(i)	C05623	Phenylpropanoids and polyketides	Flavonoids	Flavonoid glycosides
Protocatechuic Acid	no	down	B(i)	C00230	Benzenoids	Benzene and substituted derivatives	Benzoic acids and derivatives
L-Aspartic Acid	no	down	B(i)	C00049	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides, and analogues
Quercetin 3,7-Dimethyl Ether	no	down	B(i)	C01265	Phenylpropanoids and polyketides	Flavonoids	O-methylated flavonoids
L-Histidine	no	down	B(i)	C00135	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides, and analogues
L-Glutamate	no	down	B(i)	C00025	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides, and analogues
Salicylic Acid	no	up	B(i)	C00805	Benzenoids	Benzene and substituted derivatives	Benzoic acids and derivatives
Acacetin 7-[apiosyl (1->6)-glucoside]	no	up	B(ii)	C01470	Phenylpropanoids and polyketides	Flavonoids	Flavonoid glycosides
4-Hydroxybenzoic Acid	no	up	B(i)	C00156	Benzenoids	Benzene and substituted derivatives	Benzoic acids and derivatives
2,5-Dioxopentanoate	no	up	B(ii)	C00433	Organic acids and derivatives	Keto acids and derivatives	Short-chain keto acids and derivatives
Oxoglutaric acid	no	down	B(i)	C00026	Organic acids and derivatives	Keto acids and derivatives	Gamma-keto acids and derivatives
L-Alanine	no	up	B(i)	C00041	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides, and analogues
Palmitic acid	no	up	B(ii)	C00249	Lipids and lipid-like molecules	Fatty Acyls	Fatty acids and conjugates
Isowertin 2″-rhamnoside	no	up	B(ii)	C12629	Phenylpropanoids and polyketides	Flavonoids	Flavonoid glycosides
Octanoate	no	up	B(ii)	C06423	Lipids and lipid-like molecules	Fatty Acyls	Fatty acids and conjugates
Caprylic acid	no	down	B(ii)	C06423	Lipids and lipid-like molecules	Fatty Acyls	Fatty acids and conjugates
Xanthine	no	down	B(i)	C00385	Organoheterocyclic compounds	Imidazopyrimidines	Purines and purine derivatives
L-Lysine	no	down	B(ii)	C00047	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides, and analogues
Allysine	no	down	B(ii)	C04076	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides, and analogues
5′-Guanylic Acid	no	down	B(i)	C00144	Nucleosides, nucleotides, and analogues	Purine nucleotides	Purine ribonucleotides
L-Asparagine	no	down	B(i)	C00152	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides, and analogues

(*continued on next page*)

Table 1 (*continued*)

Metabolite	Significant	Regulate	level	KEGG Compound ID	HMDB Superclass	HMDB Class	HMDB Subclass
D-Ornithine	no	up	B(i)	C00515	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides, and analogues
L-Threonine	no	down	B(i)	C00188	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides, and analogues
L-Serine	no	down	B(i)	C00065	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides, and analogues
L-Phenylalanine	no	down	B(i)	C00079	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides, and analogues
L-Glycine	no	up	B(i)	C00037	–	–	–
L-Methionine	no	down	B(ii)	C00073	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides, and analogues
L-Valine	no	up	B(i)	C00183	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides, and analogues
L-Proline	no	up	B(i)	C00148	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides, and analogues
2,3,4,5-Tetrahydro-2-pyridinecarboxylic acid	no	down	B(ii)	C00450	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides, and analogues
Succinic Acid	no	up	B(i)	C00042	Organic acids and derivatives	Carboxylic acids and derivatives	Dicarboxylic acids and derivatives
P-Coumaraldehyde	no	down	B(ii)	C05608	Phenylpropanoids and polyketides	Cinnamaldehydes	Not Available
Tyramine	no	down	B(i)	C00483	Benzenoids	Benzene and substituted derivatives	Phenethylamines
Pipecolic Acid	no	down	B(i)	C00408	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides, and analogues
4-Hydroxy-3-methoxycinnamaldehyde	no	down	B(i)	C02666	Benzenoids	Phenols	Methoxyphenols
Linoleic Acid	no	up	B(i)	C01595	Lipids and lipid-like molecules	Fatty Acyls	Lineolic acids and derivatives
Salicylaldehyde	no	down	B(i)	C06202	Organic oxygen compounds	Organooxygen compounds	Carbonyl compounds
L-Tyrosine	no	up	B(i)	C00082	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides, and analogues
Alanylalanine	no	up	B(i)	C00993	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides, and analogues
5-Aminopentanoic acid	no	up	B(ii)	C00431	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides, and analogues
5-Aminovaleric Acid	no	down	B(i)	C00431	Organic acids and derivatives	Carboxylic acids and derivatives	Amino acids, peptides, and analogues
4-Hydroxybenzaldehyde	no	no change	B(i)	C00633	Organic oxygen compounds	Organooxygen compounds	Carbonyl compounds

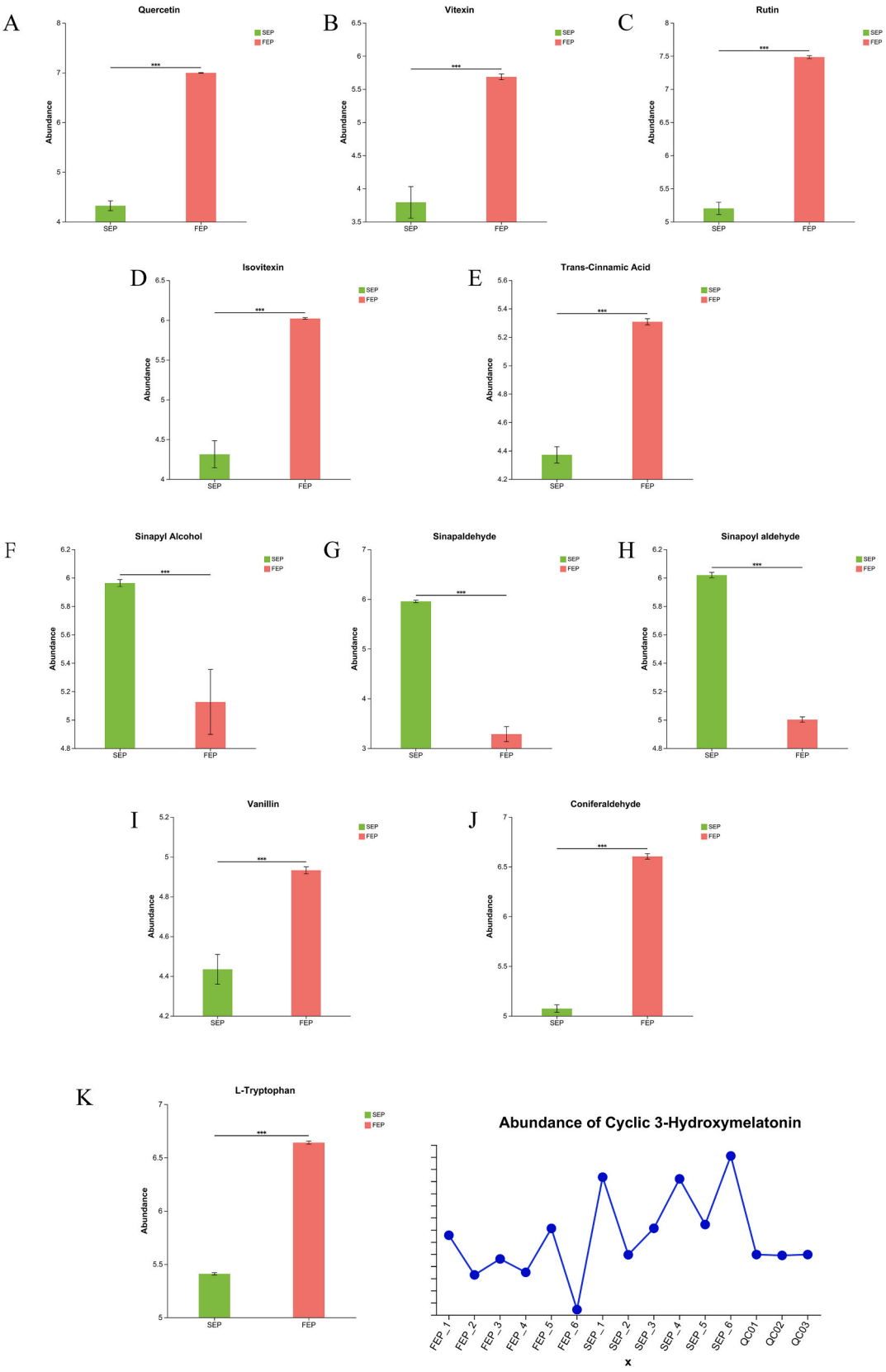

Fig. 4. Differential compound changes of Phenylpropanoids and polyketides (4A~4E)、Phenolic compounds (4F~4J) and amino acids (4K).

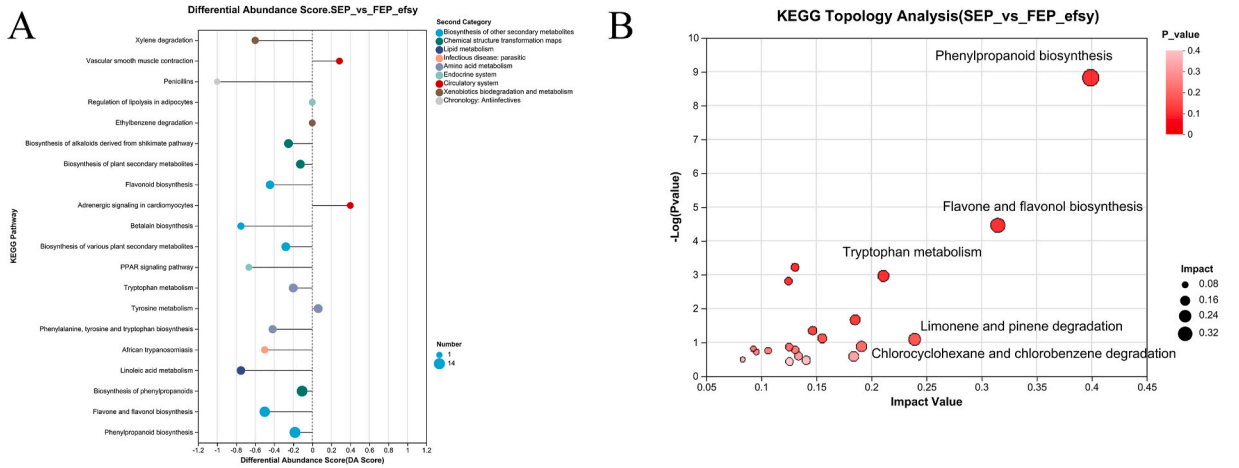

Fig. 5. KEGG pathway differential abundance score plot (5A) versus KEGG topology analysis bubble plot (5B).

The metabolism of flavonoids constitutes a significant aspect of phenylpropanoid metabolism. While not categorized as a flavonoid, mangiferic acid is a precursor for producing phenylalanine, an indispensable raw material in phenylpropanoid metabolism. Phenylalanine is a final product of the mangiferic acid pathway [14]. *Trans*-cinnamic acid, generated through the catalysis of phenylalanine, functions as the initial crucial compound, guiding the metabolic pathway from the mangiferous acid pathway toward different branches of phenylalanine metabolism [15]. Flavonoids, including naringenin, quercetin, vitexin, rutin, and their derivatives, are considered intermediate or end products within the flavonoid pathways [15]. The decreased expression of these compounds in Dendrobium stem EPJs, compared to Dendrobium flower EPJs, indicates that phenylpropanoid metabolism in Dendrobium flower EPJs is more vigorous and mainly focused on flavonoid synthesis. This observation could be attributed to the heightened sensitivity of Dendrobium flowers to relatively unfavorable fermentation conditions. In reaction, Dendrobium flowers up-regulate the expression of flavonoid structural genes to synthesize flavonoids, thereby enhancing their resistance to external oxidative damage and biological infection [16,17]. The results align with the findings presented in the VIP bar graph.

Flavonoids, functioning as antioxidants, alleviate oxidative harm resulting from the buildup of reactive oxygen species (ROS) triggered by abiotic stresses such as soil salinity, drought, and temperature fluctuations. The transamination of phenylalanine by transaminases resulted in a notable up-regulation of phenyl pyruvic acid in the EPJ of Dendrobium flowers. Moreover, phenyl pyruvic acid undergoes additional reduction to form 3-phenylacetic acid (PLA) through dehydrogenase. Polylactic acid (PLA) has been identified as an antimicrobial compound with wide-ranging activity [18], indicating a potential antimicrobial role for Dendrobium flower EPJ that merits additional investigation.

The bar graphs depicting the abundance of significant phenylpropanoids and polyketides in Dendrobium flower EPJ and Dendrobium stem EPJ are presented in Fig. 4A.~4E.

3.4.2. Change of phenolic compounds

The presence of phenolic compounds in EPJ products may be attributed to the release of polyphenolic compounds that result from the degradation of Dendrobium plant components during LAB fermentation [19]. These compounds may also be synthesized as a defense mechanism by Dendrobium plant cells in reaction to the adverse conditions resulting from bacterial fermentation [20].

Table 1 illustrates the presence of five phenolic compounds that exhibited notable regulation, with three demonstrating significant up-regulation and two showing significant down-regulation. It is important to note that pinus aldehyde, sinapaldehyde, sinapoyl aldehyde, sinapyl alcohol, and vanillin are all products of lignin hydrolysis [21]. Nevertheless, no measurable lignin was detected in either set of samples. This indicates that microorganisms completely hydrolyzed the lignin in Dendrobium flowers and stems into various lignin monomers during the later stages of fermentation. In Dendrobium stems, the degradation of lignin primarily resulted in the formation of cinnamaldehyde and its derivatives.

In contrast, in Dendrobium flowers, lignin degradation led to the production of two different lignin monomers, pinus aldehyde and vanillin. These low molecular weight phenolic compounds exhibit antioxidant, anti-free radical, anti-inflammatory, and anti-cancer properties. The bar graphs depicting the abundance of major phenolic compounds in Dendrobium flower EPJ and Dendrobium stem EPJ are presented in Fig. 4F~4G.

3.4.3. Change of amino acids

The metabolism of various amino acids exhibited less variation between SEP and FEP despite the significance of this pathway. Significant down-regulation was observed only in L-tryptophan. The observed phenomenon may be attributed to the high metabolic activity of phenylpropanoids and polyketides in the exudates of the epidermal papillae of Dendrobium flowers. In addition to its role in amino acid metabolism, L-tryptophan is also implicated in several secondary metabolic pathways, such as isoflavonoid biosynthesis,

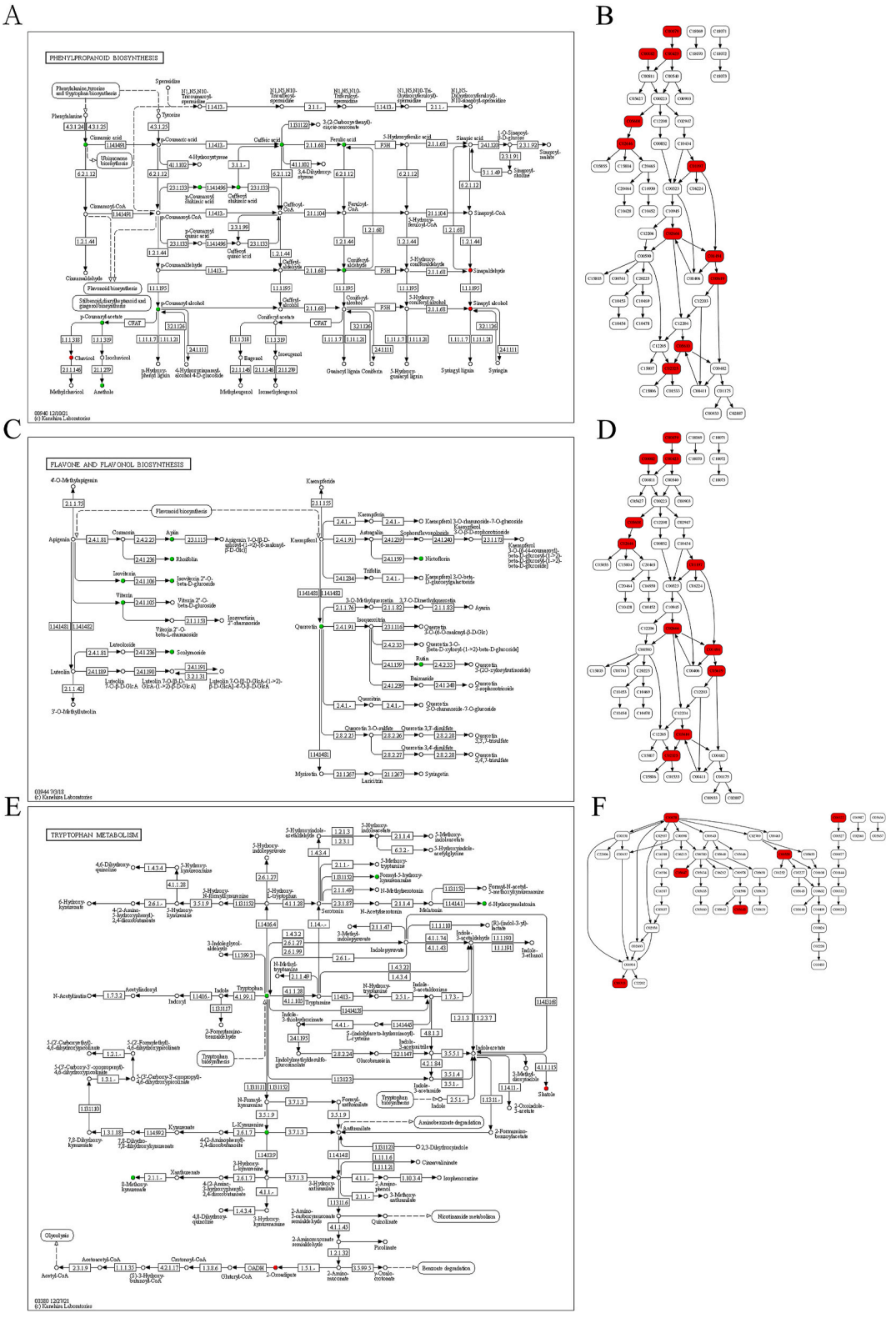

Fig. 6. Relative positions and adjustment trends of important compounds in the pathway maps of Phenylpropanoids biosynthesis (6A/6B), Flavone and flavonol biosynthesis (6C/6D) and Tryptophan metabolism (6E/6F).

flavonoid biosynthesis, and phenylpropanoid biosynthesis. L-tryptophan acts as a precursor for bioactive compounds such as melatonin (MEL), serotonin (5-HT), and 3-indoleacetic acid (3-IAA) [22]. This suggests that the up-regulated level of L-tryptophan in the Dendrobium flower FEP has the potential to promote sleep by facilitating the synthesis of melatonin in the fermentation or human environment.

Nevertheless, upon analyzing L-tryptophan metabolism, it was observed that the melatonin byproduct, cyclic 3-hydroxy melatonin, exhibited an up-regulation in Dendrobium stem EPJ compared to Dendrobium flower EPJ. The phytomelatonin pathway is crucial in removing excessive reactive oxygen species (ROS) and collaborates with other mechanisms to mitigate diverse abiotic stresses. Cyclic 3-hydroxymelatonin, a metabolite derived from phytomelatonin, is produced due to reactive oxygen species (ROS) scavenging [23]. Cyclic 3-hydroxy melatonin has demonstrated superior antioxidant efficacy to melatonin or vitamin C, as it can scavenge hydroxyl radicals (HO). Moreover, to restore oxidized horseradish peroxidase to its initial state [24]. Therefore, this indicates a potential antioxidant capacity for Dendrobium stem EPJ.

The abundance plots of L-tryptophan and Cyclic 3-hydroxy melatonin in the EPJ of Dendrobium flowers and stems are depicted in Fig. 4K.

3.5. KEGG pathway enrichment analysis

The KEGG pathway enrichment analysis was utilized to ascertain the biological pathways and roles linked to the identified differential compounds. Fig. 5B displays a bubble plot depicting the outcomes of the KEGG enrichment analysis. The x-axis denotes the significance of the enrichment *p* value, where lower values correspond to higher statistical significance. A *p* value below 0.05 is commonly regarded as indicative of a significantly enriched term. The y-axis is indicative of the KEGG pathway. The size of the bubbles in the graph reflects the degree of enrichment of the pathway with the differential compounds. The three most prominent pathways identified were phenylpropanoid biosynthesis, flavone and flavonol biosynthesis, and tryptophan metabolism.

In Fig. 5A, the x-axis denotes the Differential Abundance Score (DA Score), whereas the y-axis represents the names of the KEGG pathways. The Differential Abundance (DA) score represents the cumulative alterations in all metabolites along the pathway, while the length of the line segments signifies the magnitude of the DA score. The size of the dots reflects the quantity of annotated differential metabolites within the pathway, where larger dots correspond to a higher number of differential metabolites. The dots are positioned to the right of the central axis. As the length of the line segment increases, the overall expression of the pathway is up-regulated in the Dendrobium stem EPJ in comparison to the Dendrobium flower EPJ.

Conversely, the dots are distributed to the left of the central axis, and the longer the line segment, the more downregulated the overall expression of the pathway in Dendrobium Stem EPJ compared to Dendrobium Flower EPJ. Fig. 5A depicts the 20 pathways with the lowest *p* values, indicating that several crucial pathways are downregulated in Dendrobium stem EPJ. These pathways include phenylpropanoid biosynthesis, flavone and flavonol biosynthesis, and tryptophan metabolism.

3.5.1. The KEGG topology analyzed pathway maps of the phenylpropanoids biosynthesis

Both Fig. 6A and B depict the KEGG pathway of phenylpropanoid biosynthesis. In Fig. 6B, metabolites depicted on a white background denote metabolites within this pathway, whereas metabolites shown on a red background indicate differentially regulated compounds. In Fig. 6A, the compounds highlighted in green represent down-regulated compounds in the Dendrobium stem EPJ in comparison to the Dendrobium flower EPJ. Conversely, the compounds highlighted in red indicate up-regulated compounds in the Dendrobium stem EPJ compared to the Dendrobium flower EPJ. Significantly, the compounds that exhibited up-regulation were primarily concentrated in the middle and upper segments of the phenylpropanoid biosynthetic pathway. In contrast, those showing down-regulation were predominantly concentrated in the lower segments. This observation provides additional evidence for the presence of a heightened phenylpropanoid metabolism in the epidermal papillae (EPJ) of Dendrobium flowers, along with a more prominent breakdown of lignin, specifically sinapyl alcohol, in the EPJ of Dendrobium stems.

3.5.2. The KEGG topology analyzed pathway maps of the flavone and flavonol biosynthesis

Both Fig. 6C and D depict the KEGG pathway of flavone and flavonol biosynthesis. Metabolites with a white background indicate their presence in this pathway, while metabolites labeled in red signify significant up- or down-regulation.

The down-regulation of all significantly adjusted flavones and flavonols was observed, impacting multiple synthesis pathways of these compounds. This finding supports previous analyses indicating the abundance of flavonoid metabolism in Dendrobium flower EPJs. This metabolic activity serves to protect against external oxidative damage and biological infections.

3.5.3. The KEGG topology analyzed pathway maps of the tryptophan metabolism

Both Fig. 6E and F depict the KEGG pathway of Tryptophan metabolism. In Fig. 6F, metabolites in the white background denote metabolites within this pathway; pathways in the red background indicate differentially regulated compounds. Compounds labeled in green in Fig. 6E signify down-regulated compounds at Dendrobium stem EPJ compared to Dendrobium flower EPJ. In contrast, compounds labeled in red represent up-regulated compounds at Dendrobium stem EPJ relative to Dendrobium flower EPJ. The results obtained align with Section 3.4.3.

4. discussion

Untargeted metabolomics analyses using LC-MS were conducted in this study to assess the metabolites present in Dendrobium stem

EPJ and Dendrobium flower EPJ fermented with *Saccharomyces cerevisiae*, Lactobacillus bulgaricus, and Streptococcus thermophilus after the fermentation process. The study analyzed the differential pathways and compounds associated with metabolism in Dendrobium stem EPJ and Dendrobium flower EPJ. A total of 476 distinct compounds were identified.

The study further confirmed that the metabolic pathways involved in phenylpropanoid biosynthesis, flavonoid, and flavonol biosynthesis, and tryptophan metabolism play a crucial role in distinguishing the metabolic profiles of Dendrobium stems EPJs from those of Dendrobium flowers EPJs, as indicated by KEGG pathway enrichment analyses. Additionally, the study examined the positions and effects of the differential compounds within these three metabolic pathways using pathway maps. 93 differential compounds were identified in the three selected pathways, which exhibited the most significant differences between Dendrobium flower EPJ and Dendrobium stem EPJ. The pathways and compounds related to phenylpropanoid biosynthesis, particularly the lignin and flavonoid, were identified as the most significant differential pathways and compounds in distinguishing between Dendrobium stem EPJ and Dendrobium flower EPJ [25].

In the stem of Dendrobium EPJ, the major up-regulated metabolites associated with phenylpropanoid biosynthesis were lignin and its derivatives, including cinnamaldehyde, sinapoyl aldehyde, and sinapyl alcohol. Nevertheless, the precursor compounds in the lignin pathway exhibited down-regulation, indicating potential lignin degradation by lactic acid bacteria (LAB) during the fermentation process. The low molecular weight phenolic compounds exhibit antioxidant, anti-free radical, anti-inflammatory, and anticancer properties [26].

Conversely, there was a significant up-regulation of mangiferic acid in the Dendrobium flower EPJ compared to the Dendrobium stem EPJ. The metabolism of phenylalanine and related metabolites in the flavonoid pathway exhibited significant up-regulation in Dendrobium flower EPJ, thereby making the most substantial contribution to the observed differences between the two products. The observed up-regulation could be attributed to the over-expression of flavonoid structural genes in Dendrobium flowers, leading to heightened resistance against external oxidative damage and biological infections. Recent research indicates that flavonoids exhibit anti-inflammatory, vasodilatory, anticoagulant, cardioprotective, antidiabetic, chemoprotective, neuroprotective, anti-obesity, and anti-aging properties, and appropriate anti-aging active activities [27,28]. This statement offers a theoretical foundation and potential for advancing Dendrobium EPJ nutraceuticals.

The tryptophan pathway also plays a crucial role, as L-tryptophan is involved in amino acid metabolism and secondary metabolism, such as isoflavone biosynthesis and flavonoid biosynthesis. It is a precursor to bioactive compounds like melatonin, 5-hydroxytryptophan, and 3-indoleacetic acid [29], suggesting the potential of L-tryptophan in promoting sleep and synthesizing melatonin. In contrast, the cyclic 3-hydroxy melatonin, a metabolite generated through the metabolism of L-tryptophan, exhibited up-regulation in the Dendrobium stem EPJ compared to the Dendrobium flower EPJ. This metabolite exhibits potent antioxidant properties, surpassing melatonin and vitamin C in its ability to scavenge hydroxyl radicals and restore oxidative enzymes [24]. Therefore, the EPJs extracted from the stems and leaves of Dendrobium plants have the potential to exhibit antioxidant capacity by up-regulating cyclic 3-hydroxy melatonin.

This untargeted metabolomics study offers new insights into the distinctions between Dendrobium flowers and stems for EPJ production, which is induced by variations in the plant's raw material source. The substantial modifications in metabolic pathways and compounds contribute to the advancement of scientific knowledge and indicate potential mechanisms for the distinct bioactivities of the two EPJ products. The abundant flavonoid metabolism in flower EPJ implies potential antioxidant, anti-inflammatory, and antimicrobial properties that warrant further investigation. The elevated levels of lignin metabolites in stem EPJ, including cinnamaldehyde and its derivatives, warrant further exploration of their potential antioxidant properties. The Flower EPJ may possess supplementary antimicrobial properties due to containing phenylpyruvic acid and flavonoids. These unique metabolite compositions allow the customizing of functional food products to address particular health advantages. Incorporating Flower EPJ into nutraceuticals or functional foods may enhance antioxidant defense, immune function, or antimicrobial effects. The potential applications of Stem EPJ may be found in products aimed at reducing oxidative stress or promoting cardiovascular health. Future research endeavors may involve the isolation, identification, and quantification of essential bioactive compounds present in the two EPJ products.

5. Conclusion

This untargeted metabolomics study revealed significant differences in the metabolic profiles of edible plant Jiaosu (EPJ) products derived from Dendrobium flowers versus stems during fermentation. The flower EPJ exhibited upregulated flavonoid biosynthesis conferring potential antioxidant and antimicrobial properties, while the stem EPJ contained increased levels of lignin metabolites and cyclic 3-hydroxymelatonin with antioxidant capacities. These distinct metabolite compositions indicate opportunities for developing tailored functional EPJ products targeting specific health benefits from the different Dendrobium sources. Further research isolating and quantifying the key bioactive compounds is warranted.

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Ethical guidelines

Ethics approval was not required for this research.

Data availability statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Lihong Jiang: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Xingjiang Li:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition. **Shuo Wang:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition. **Du Pan:** Writing – review & editing, Methodology, Data curation. **Xuefeng Wu:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition. **Fengxu Guo:** Writing – review & editing, Methodology, Data curation. **Dongdong Mu:** Writing – review & editing, Resources, Funding acquisition. **Fuhuai Jia:** Writing – review & editing, Resources. **Min Zhang:** Writing – original draft, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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