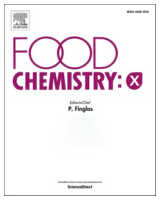
Food Chemistry: X 23 (2024) 101531

Contents lists available at ScienceDirect

Food Chemistry: X

journal homepage: www.sciencedirect.com/journal/food-chemistry-x



Phenolic metabolites changes during baijiu fermentation through non-targeted metabonomic

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ARTICLE INFO

Keywords:
Chinese baijiu
Fermented grains
Phenolic
Non-targeted metabonomic
Metabolites

ABSTRACT

To investigate the changes of phenolic metabolite during different grains fermentation stages of Chinse Baijiu, the ultra-performance liquid chromatography-quadrupole time of-flight mass spectrometry (UHPLC-QTOF-MS) was applied to identify and analyze the different phenolic metabolites, combined with principal component analysis and partial least squares discriminant analysis. Results indicated that significant differences in phenolic metabolites during different fermentation stages were found. Among the 231 phenolic metabolites detected, 36, 31, 19, 23, 14, and 50 differential phenolic metabolites were screened between different groups using partial least squares discriminant analysis. Twelve metabolic pathways with high correlation of differential phenolic metabolites and 23 main participating differential metabolites were identified through KEGG metabolic pathway enrichment analysis. The present study preliminarily revealed the differences of phenolic metabolites at different fermentation stages, and providing a theoretical basis for the further improving of the taste and quality of Chinese Baijiu.

1. Introduction

As one of the world's renowned distilled spirits, Chinese Baijiu boasts a long history and rich cultural heritage in China. Its unique quality and flavor have increasingly captivated consumer (Wang, Chen, Wu, et al., 2022). The brewing process of Maotai-flavored Baijiu is particularly intricate, involving multiple complex steps including feeding twice, cooking nine times, stacking fermentation eight times at high temperatures, and extracting Baijiu seven times. An essential substrate in this process, fermented grains, refers to grains that are well fermented after cooking but before Baijiu extraction. Throughout the brewing process, these grains undergo various changes in metabolites and microorganisms, spanning from raw material control and fermentation to entering and exiting the cellar (Wang, Lu, Xu, et al., 2022).

Phenolic substances in fermented grains are primarily derived from brewing raw materials such as wheat and sorghum (Hao, Tan, Lv, et al., 2021). During fermentation, these substances could produce a series of new active phenolic compounds through chemical structural transformations and degradation, significantly influencing the flavor and taste of the final Baijiu. For example, phenolic substances in wheat, such as ferulic acid, transform into 4-methylguaiacol, 4-ethylguaiacol, 2,3-dihydrobenzofuran, and other phenolic compounds during fermentation, imparting the distinctive flavor and taste of Maotai-flavored liquor (Wang, Wang, Liao, et al., 2022). Similarly, phenolic substances in sorghum, such as sorghum tannin, underwent enzymatic reactions, catalysis, and decarboxylation during cooking and fermentation, producing aromatic substances like syringic acid (Li, Han, Liu, et al., 2021). Identifying these phenolic substances in the brewing raw materials of Maotai-flavored liquor and analyzing their changes during fermentation could provide crucial scientific support for optimizing the liquor manufacturing process.

Metabolomics is an emerging technology that utilizes high-

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https://doi.org/10.1016/j.fochx.2024.101531
Received 23 April 2024; Received in revised form 24 May 2024; Accepted 2 June 2024
Available online 5 June 2024

throughput detection and multivariate data processing to qualitatively and quantitatively analyze small molecular metabolites in organisms, thereby exploring specific metabolic mechanisms (Jian, Qixia, Battino, et al., 2021). Untargeted metabolomics, in particular, could characterize all metabolite information in biological samples, facilitate the discovery of new metabolites and metabolic pathways (Pan, Gu, Lv, et al., 2022). In the field of liquor production, untargeted metabolomics has mainly been applied to identify the quality and authenticity of liquors. For example, Tufariello et al. (2021) compared and analyzed the chemical components of brewing liquor and blending liquor using non-targeted metabolomics combined with stoichiometric methods, identifying characteristic markers for authenticity verification. Ehlers, Uttl, RiedL, et al. (2023) developed a method to identify the quality grade of Mao-Xiangxi liquor using non-targeted metabolomics, while Luo, Zhang, Yang, et al. (2020) applied the same technique to verify the authenticity of Maotai liquor. However, there are few reports on using non-targeted metabolomics to analyze changes in phenolic metabolites in fermented grain samples at different fermentation stages.

In present study, samples of fermented grains at 15 different points during the brewing stages of Maotai-flavored liquor were selected based on their total phenolic content. Through non-targeted metabolomics, the changes in phenolic metabolites in these samples at various fermentation stages were analyzed. By combining multivariate statistical analysis, we identified differential phenolic metabolites and examined the changes in these metabolites and related metabolic pathways. Those analyses aim to provide new insights into improving the comprehensive utilization rate of fermented grains and promoting the development of liquor industry.

2. Materials and methods

2.1. Materials and reagents

Fermented grains samples (numbered A ~ O) were provided by China Kweichow Moutai Distillery (Group) Co., LTD. Where A ~ G were represented the fermented grains during *Xiasha* process (A: raw sorghum; B: fermented grains before mixed *Muqu*(Wheat fermentation retained in Maotai from the previous year's production); C: grains before mixing with koji; D: *Daqu*; E: fermented grains after mixing; F: fermented grains before entering the cellar; G: Out of the cellar fermented grains), H ~ K were the samples of fermented grains during the *Zaosha* process (H: fermented grains before mixing; I: fermented grains after mixing; J: fermented grains before entering the cellar; K: Out of the cellar fermented grains), L ~ O were the samples of fermented grains during one round (L: fermented grains before mixing; M: fermented grains after mixing; N: fermented grains before entering the cellar; O: Out of the cellar fermented grains).

Water (MS pure) was purchased from Merck (Germany); Methanol, formic acid, and ammonium acetate (mass spectrometric pure) were purchased from Thermo Fisher, USA.

2.2. Instruments and equipment

Cytocentrifuge (D3024R, Scilogex, USA), UHPLC liquid chromatograph (Vanquish, Thermo Fisher, Germany), mass spectrometer (Q Exactive™ HF-X, Thermo Fisher, Germany), vacuum freeze dryer (FD5–2.5, SIM, USA).

2.3. Methods

2.3.1. Sample source and storage conditions

The samples of fermented grains were collected from the Kweichow Moutai Production Base in 2022 in three stages of *Xiasha*, *Zaosha* and one round, three samples were taken from each sample and stored at −80 °C prior to analysis.

2.3.2. Sample processing

According to the method of Xu et al. (2022), an appropriate number of samples were ground with liquid nitrogen, 100 mg of the ground sample powder was accurately weighed, placed in a centrifuge tube, and 500 μL of 80% methanol aqueous solution was added and mixed with a vortex oscillator. The metabolite extracts of fermented grains samples were placed in an ice bath for 5 min, centrifuged for 20 min at 15,000 ×*g* and 4 °C, then appropriate amount of supernatant was taken and placed in a centrifuge tube, and diluted with a certain volume of mass spectrometry water to the methanol content of 53%. The supernatant was collected by centrifugation for 20 min (15,000 ×g, 4 °C) and filtered with 0.22 μm organic phase filter membrane before liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis. An equal volume of samples was sucked from each sample and mixed evenly as a quality control (QC) sample to test the stability of the instrument during the whole experiment.

2.3.3. Chromatographic conditions

The chromatographic conditions were set as fellows, column temperature: 40 °C, Flow rate: 0.2 mL/min. The mobile phase of positive ion mode: A was 0.1% formic acid, B was methanol; Mobile phase in negative ion mode: A was 5 mmol/L ammonium acetate (pH 9.0), B was methanol; The chromatographic gradient elution program was 0–1.5 min, 2%–2%B; 1.5–10 min, 2%–100% B; 10 to 12 min, 100% to 2% B.

2.3.4. Mass spectrum conditions

Mass scanning range of positive and negative ion mode and detail conditions as fellows: *m/z* 100–1500; Spray voltage: 3.5 kV, Sheath gas flow rate: 35 psi, Auxiliary gas flow rate: 10 L/min; Ion transfer tube temperature: 320 °C, Iontophoresis RF level: 60, Auxiliary gas heater temperature: 350 °C, the MS/MS secondary scan is a data-dependent scan.

2.3.5. Qualitative and quantitative metabolites

The raw data of samples were processed using the CD 3.1 library search software. Firstly, the retention time and mass-charge ratio of each metabolite were simply selected, and then the peak alignment was carried out among different fermented grains samples according to the mass deviation (5 ppm) and retention time deviation (0.2 min). The metabolites in the samples of fermented grains were compared with the databases of Beijing NOWO Zhisource Technology Co., LTD (mzCloud (https://www.mzcloud.org/), mzVault and Masslist) to identify the metabolites in the samples, and each chromatologic peak was corresponding to one metabolite. Finally, the chromatographic peaks in the samples of fermented grains were standardized and integrated to obtain the relative peak area.

2.3.6. Data analysis

KEGG database (https://www.genome.jp/kegg/pathway.html) is used for marking on fermented grain samples. Principal component analysis (PCA) and partial least square discriminant analysis (PLS-DA) were performed to analyze phenolic metabolites in fermented grains samples at different stages. VIP value, Fold change (FC) and P value were used to analyze the differential metabolites of fermented grains at different stages. The differential metabolites should meet the conditions of VIP value >1 and FC > 0.05 or FC < 0.833 and *P*-value<0.05. The significant differences of metabolites in the samples of fermented grains at different fermentation stages were screened using R language, and volcanic maps and clustering heat maps were drawn. KEGG database was used to perform metabolic pathway enrichment analysis of significantly different metabolites.

3. Results and analysis

3.1. Analysis of QC samples

QC samples were used to balance the chromatography-mass spectrometry system and instrument status to obtain reliable and high-quality metabolomics data. As shown in Fig. 1, the ion peak baseline of QC samples was stable, indicating that the stability of the data was in high quality. In addition, the peak number, peak area and response intensity of ion peaks in positive and negative ion modes were different, indicating that the modes and contents of different metabolites were different.

The coefficient of variation (CV) of QC samples was screened, and the chromatographic peaks with CV > 30% in QC samples were deleted. The differences between groups and within groups were analyzed by PCA, and the stability of the system was evaluated. As shown in Fig. 2, the within-group samples of 15 groups of samples were close to each other, indicating that the within-group differences of the samples were not significant. The QC samples were located in the middle of the 15 groups and clustered closely, indicating that the experiment showed good stability and reproducibility for subsequent experiments.

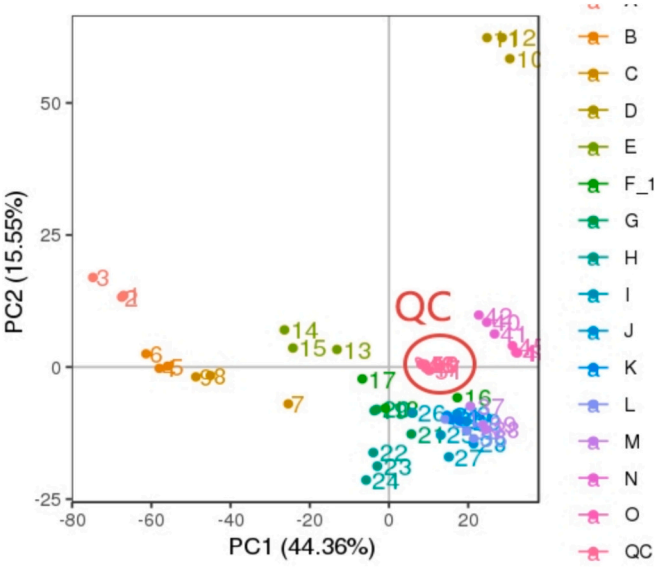

Fig. 2. PCA score chart for QC and experimental groups.

3.2. Multivariate statistical analysis of metabolites at different fermentation stages

The ultra-performance liquid chromatography-quadrupole time of-flight mass spectrometry (UHPLC-TOF-MS) assay was applied to detect the metabolites in fermented grains samples at different fermentation stages. Fig. 3a was the first-order classification pie chart of all metabolites detected in 15 sets of samples, with a total of 2009 metabolites were detected. Those metabolites mainly included 667 lipids and lipid-like molecules, 291 organic acids and their derivatives, 282 phenylpropanes and polyketones, 250 organic heterocyclic compounds, 170 benzene ring compounds, 161 organic oxygen compounds, 66 nucleotides and their analogues, 54 alkaloids and their derivatives, 32 organic nitrogen compounds, 19 lignins and 17 other compounds other metabolites. In order to reduce the data dimension and improve the interpretability and validity of the data, PCA multivariate statistical analysis technique in unsupervised mode was applied to the processed metabolites for further analysis. PCA analysis could reflect the variability of inter-group and intra-group samples, reveal the distribution trend between different samples, and determine the possible discrete points (Greno, Plaza, Luisa, et al., 2023). The PCA results of 15 groups (Fig. 3b) showed that the contribution rates of PC1 and PC2 were 45.03% and 15.96%, respectively. Among them, the samples of group A were clustered into one class, the samples of group B and C were clustered into one class, the samples of group D were clustered into one class, the samples of group E were clustered into one class, the samples of group F, G, H, I, J, K, L, M were clustered into one class, and the samples of group N and O were clustered into one class. Those results indicated that the three key steps leading to the changes of metabolites in the whole fermentation process were grain moistening, koji mixing and stacking fermentation (Wang, Qin, Wang, et al., 2024).

3.3. PLS-DA analysis of phenolic metabolites of fermented grains at different stages

Phenolic compounds (such as flavonoids, phenolic acids and tannins) were the most abundant secondary metabolites in the samples of fermented grains Chetrariu and Dabija (2021). It has been reported that phenolic compounds have a considerable impact on the overall sensory quality of finished baijiu, such as the quality and taste (Hou, Chen, Pu, et al., 2023). In order to reveal the changes of phenolic metabolites of fermented grains at different fermentation stages, it was necessary to

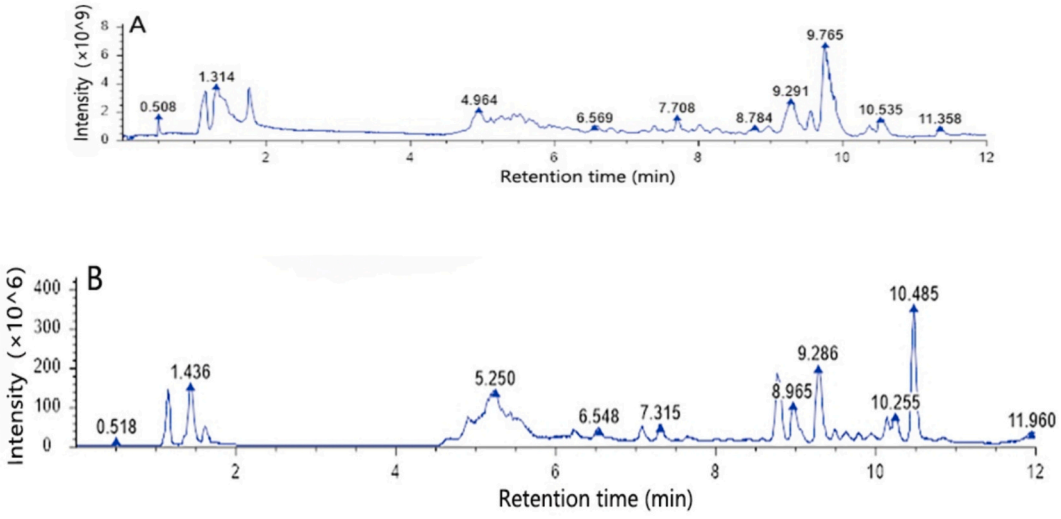

Fig. 1. Base peak plots of positive ions (A) and negative ions (B) in QC samples.

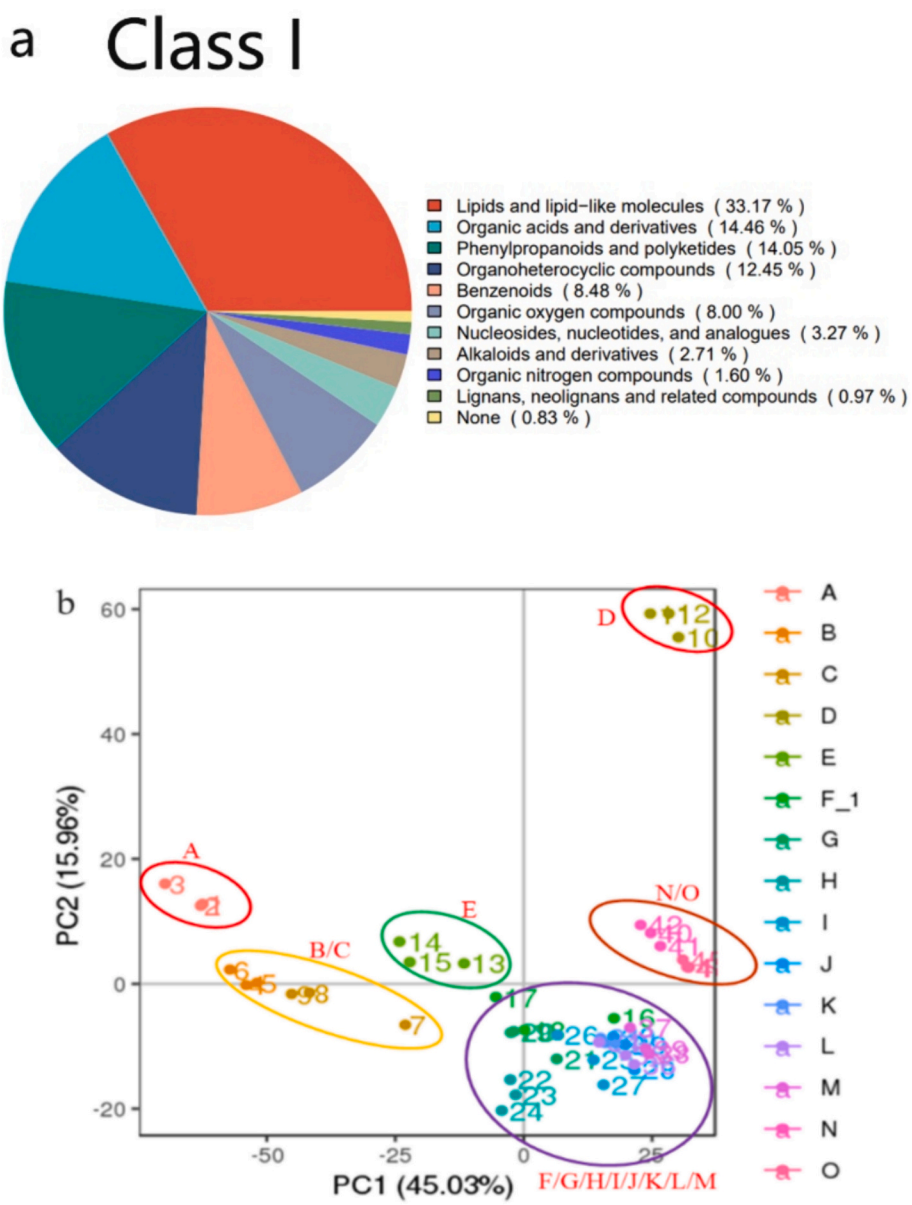

Fig. 3. a. A primary classification diagram of all metabolites; b. PCA score chart for 15 sample groups.

screen out phenolic metabolites and analyze them emphatically. A total of 228 phenolic metabolites were screened from 15 groups of samples (Supplementary Table 1), and all the phenolic metabolites detected in the 15 groups of samples were classified according to ClassyFire's secondary classification criteria. The results were shown in Fig. 4a, which mainly include 90 kinds of flavonoids, 32 kinds of cinnamic acid and its derivatives, 30 kinds of phenols, 23 benzenes and their substituted derivatives, 17 heteroflavones, 14 stilbenes, 8 coumarin and their derivatives, 5 tannins, 4 high isoflavones and 3 organic oxygen compounds. A more detailed classifications of these phenolic metabolites were also carried out, as shown in Fig. 4b, which was the third-level classification pie chart of all phenolic metabolites. These mainly include 12 flavonoids, 12 *O*-methyl flavonoids, 5 hydrolyzed tannins, 5 *O*-methyl isoflavones, 4 phenyldiols, 4 hydroxycoumarin, 4 isoflavone-2-enes, 4 biflavonoids and cluster flavonoids, 4 cinnamate esters, 4 isoflavone O-glycosides, 4 stilbene glycosides and other compounds.

Unsupervised PCA could intuitively represent the grouping, change rule and similarity between samples, but the difference variables were distributed on PC, and the influence of unrelated variables cannot be excluded. PLS-DA is a supervised statistical method that could use multivariate data to distinguish two or more groups (classes) and exclude the influence of variables unrelated to experimental conditions, to achieve effective separation of samples between groups (Cao, Shu, Wen, et al., 2023; Wang, Wang, Zhang, et al., 2022. The results of PLS-DA multivariate statistical analysis of phenolic metabolites were shown in Fig. 5, a clear separation between group B samples and group A samples were observed, indicating that *Runliang* had a significant effect on the phenolic metabolites of the samples (Song, Das, Zhu, et al., 2021). There was also a significant separation between the samples of group C and the samples of group B, as well as the samples of group E and the samples of group C, indicating that the mixing of *Muzao* (Sorghum fermentation left after Maotai production last year), cooking and the addition of *Daqu* had significant effects on the phenolic metabolites (Ofosu, Elahi, Daliri, et al., 2022; Ren, Sun, Yang, et al., 2024). The separation of the samples from groups F and group E, and the separation of the samples from groups N and O and the samples from groups L and M showed that the stacking fermentation had a significant effect on the phenolic metabolites (Jiang, Mu, Wei, et al., 2020). The separation of samples from group G, H and group F indicated that the fermentation in the cellar had a significant effect on the phenolic metabolites of samples.

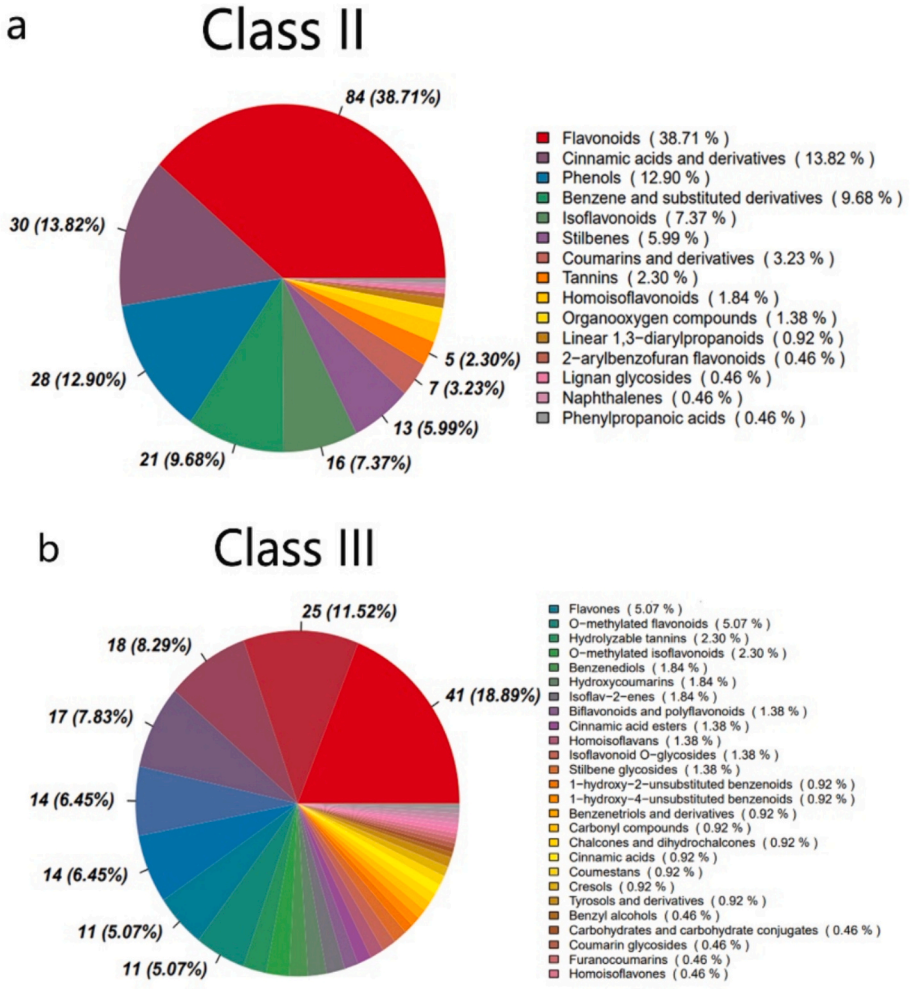

Fig. 4. Secondary classification diagram (A) and tertiary classification diagram (B) of phenolic metabolites.

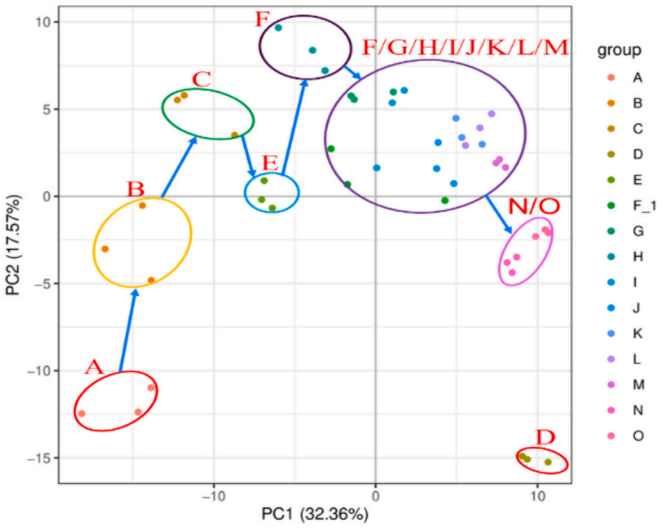

Fig. 5. PLS-DA scores of phenolic metabolites in different fermented grains samples.

Similar results were also observed by Seo, Park, Kim, et al. (2020).

3.4. Screening and analysis of different phenolic metabolites of fermented grains at different stages

The VIP value from PLS-DA analysis was considered to be an effective parameter to evaluate the strength and interpretability of the influence of expression patterns between groups. A higher VIP value indicates a greater contribution of the variable to grouping. Usually, metabolites with VIP values >1 were considered differential metabolites. In addition, *t*-tests were used to further verify the significance of metabolites between groups, and the *P* values derived from t-tests were usually used to assess the likelihood of differences between groups. The screening criteria of PC1 were VIP >1 and FC > 0.05 or FC < 0.833 and *P-value* < 0.05 in PLS-DA model. The differential phenolic metabolites of the B vs A, C vs B, E vs C, F vs E, G vs F, and N vs M comparison groups were screened, and results were shown in Supplementary Table 2. Volcano plot analysis was performed on the samples of B vs A, C vs B, E vs C, F vs E, G vs F, N vs M comparison groups respectively. As showed in Fig. 6, red, green, and gray indicated that the relative content of metabolites was significantly up-regulated, significantly down-regulated, and no significant changes, respectively.

As shown in Fig. 6a (B vs A), a total of 36 differential phenolic metabolites were identified in raw sorghum after *Runliang*, of which 27 were up-regulated and 9 were down-regulated. *Runliang* was a performance that soaking the raw material (temperature ≥ 95 °C), allowing the starch in the raw material to absorb water, resulting in the rupture of

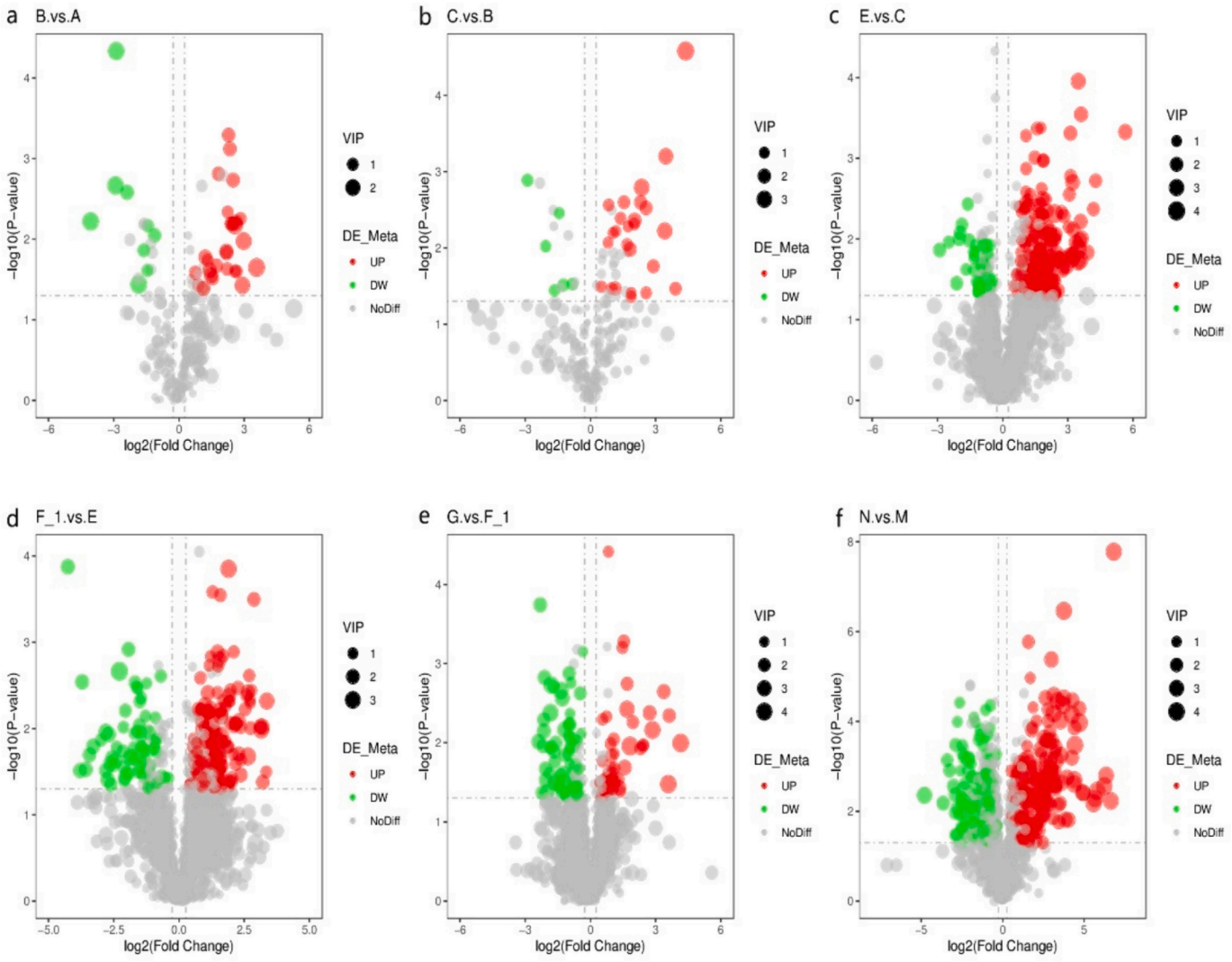

Fig. 6. Volcano diagrams of different phenolic metabolites in fermented grains samples at different fermentation stages ((a) B vs. A; (b) C vs. B; (c) E vs. C; (d) F vs. E; (e) G vs. F; (f) N vs. M).

the cell wall of the grains Dos Santos et al. (2023). After high temperature water infiltration, raw sorghum would loss some water-soluble phenolic compounds. Wu, Huang, Qin, et al. (2013) reported that heating leads to the degradation of some phenolic compounds in sorghum, such as vanillic acid and *p*-coumaric acid. Xiong, Zhang, Luo, et al. (2019) also found that soaking significantly reduced the total phenolic content in black and red sorghum ($P < 0.5$). However, high temperature also increases the extractability of some phenolic acids by destroying the cellular structure of grains and releasing non-extractable polymeric phenolic substances (Cheng, Su, Moore, et al., 2006). In addition, because flavonoid glycosides were usually related to the astringency of Maotai liquor (Scharbert, Holzmann, & Hofmann, 2004), >50% of the down-regulated metabolites belonged to flavonoid glycosides, which might be related to the hydrolysis of glycosidic bonds (You, Ahn, & Ji, 2010), indicating that *Runliang* contribute to the degradation of flavonoid glycosides, thereby reducing the astringency and bitterness of Maotai liquor. In addition, the changes of flavonoid glycosides with different bond types showed opposite trends (e.g., the relative content of flavonoid 7-O-glycosides such as galangin and acacetin decreased, and the relative content of flavonoid 3-O-glycosides such as narcimicin and quercetin 3β-D-glucoside increased), indicating that the type of glycoside linkage might affect the degradation of flavonoid glycosides (Long, Wen, et al., 2020).

Fig. 6b (C vs B) showed that 31 differential phenolic metabolites were identified after mixing and cooking of fermented grains before mixing *Muzao*, among which 25 were up-regulated and 6 were down-regulated. This might attribute to the fact that the mixing of *Muzao* in fermented grains was associated with a large proportion of up-regulated phenolic metabolites, as *Muzao* were rich in microbial metabolites, such as phenolic compounds (Mao, Huang, Zhou, et al., 2023). Fig. 6c (E vs C) showed that a total of 19 different phenolic metabolites were identified in fermented grains before koji mixing and after the mixing of *Daqu*, of which 6 were up-regulated and 13 were down-regulated. It was possible that the mixing of *Daqu* down-regulated a large parts of phenolic metabolites, because *Daqu* was mainly a collection of beneficial microbial communities and contained a very small number of phenolic substances (Mao, Huang, Zhou, et al., 2022). Fig. 6d (F vs E) showed that 23 different phenolic metabolites were identified in fermented grains after stacking fermentation, of which 13 were up-regulated and 10 were down-regulated, 60% of the down-regulated metabolites belonged to flavonoid glycosides, indicating that stacking fermentation was conducive to the degradation of flavonoid glycosides. Fig. 6e (G vs F) showed that a total of 14 different phenolic metabolites were identified in fermented grains before entering the cellar, of which 9 were up-regulated and 5 were down-regulated, and 6 metabolites belonged to phenolic acids, indicating that re-oxidation and hydrolysis reactions would occur

in the cellar fermentation to promote the conversion of phenolic acids (Qian, Lu, Chai, et al., 2021).

In addition, the up-regulation of most differential phenolic metabolites after accumulation and fermentation in the cellar might relate to the activities of microorganisms. As reported by Li, Jia, and Wang (2024), microorganisms would continue to grow and multiply in the process of accumulation and fermentation in the pool (at the temperature of 23–55 °C), and produced active substances such as phenols through various enzymatic and biochemical reactions. Fig. 6f (N vs M) showed that a total of 50 different phenolic metabolites were identified after stacking fermentation after a round of mixing, of which 10 were up-regulated and 40 were down-regulated, indicating that further stacking fermentation would cause a large part of phenols to be metabolized by microorganisms.Dordevic et al. (2010) confirmed that microbial fermentation leaded to the degradation of bioactive substances such as phenols. The above results showed that the phenolic metabolites in fermented grains at different fermentation stages were significantly different, and this might attribute to the interaction among microorganisms, enzymes and sample components during fermentation (Sun, Wang, Zhang, et al., 2020). It was speculated that *Bacillus subtilis*, *Bacillus amyloolytica*, *Saccharomyces cerevisiae*, *Aspergillus Niger* and other microorganisms related to phenolic metabolism and their enzymes were different in different fermented stages, and this would lead to the structural transformation or mutual transformation of phenolic substances, and had a great impact on the relative content of phenolic metabolites at different stages (Huynh, Van Camp, Smagghe, et al., 2014).

3.5. *Variation pattern of differential phenolic metabolites*

To observe the variation trend of the relative contents of different phenolic metabolites in samples of fermented grains at different fermentation stages visually, a trend chart was drawn based on the common differences in the relative contents of phenolic metabolites in groups A, B, C, E, F, G, M and N. As shown in Fig. 7a, the relative contents of most flavonoid glycosides in the fermented grains, such as rutin, centaurin, hesperidin, isovitexin, and astilbin, did not change significantly, while the relative contents of quercetin-3β-D-glucoside showed a sharp decline in general. Fig. 7b showed that the relative contents of most flavonoids in fermented grains, such as luteolin, myricetin, apigenin, sericol, naringin, etc., did not change significantly, while the relative contents of taxifolin showed a sharp decline in general, which

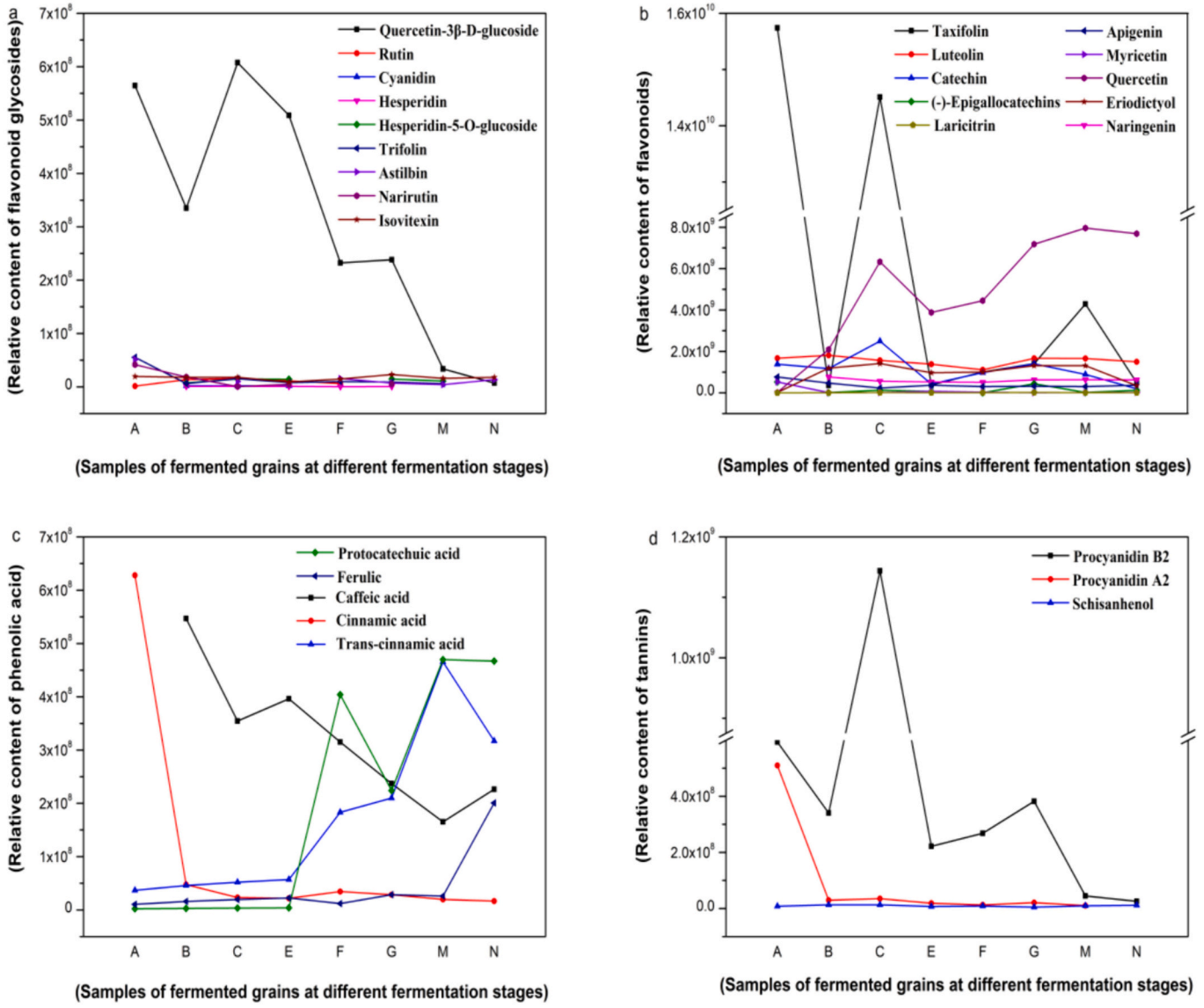

Fig. 7. Trend chart of differential phenolic metabolites in fermented grains samples at different fermentation stages. ((a) flavonoid glycosides; (b) flavonoids; (c) phenolic acid; (d) tannic acid).

might attribute to the degradation and utilization by microorganisms during the fermentation process Li, Su, et al. (2023). However, the relative content of quercetin showed a fluctuating upward trend, which might attribute to the degradation of quercetin-3β-D-glucoside under the action of glucosidase secreted by microorganisms (Fan, Terrier, Hay, et al., 2010; Xie, Chen, Huang, et al., 2021), and this was consistent with the decrease of the relative content of quercetin-3β-D-glucoside.

Lee, Cho, Kim, et al. (2016) reported that the content of quercetin 3-O-β-D-glucopyranoside gradually decreased under the action of β-glucosidase produced by *Lactobacillus mesenterium*, while the content of quercetin gradually increased. Fig. 7c showed that the relative contents of trans-cinnamic acid, ferulic acid and protocatechuic acid in fermented grains showed a trend of fluctuation and increase, while the relative contents of cinnamic acid and caffeic acid showed a trend of sharp decline. Among them, the decrease in the relative content of cinnamic acid might due to its structural transformation to produce trans-cinnamic acid (Hanai, Kuwae, Takai, et al., 2001). Salim et al. (2013) reported that cinnamic acid was unstable and easily converted to trans cinnamic acid with stable structure at slightly higher temperatures. The results of Fig. 7d show that the relative content of all tannins showed a decreasing trend. Tannins were known to enhance the astringency of beverages by binding to salivary proteins to form insoluble complexes (Gonzalez, Hernando, & Moraga, 2021; Hamada, Hasegawa, & Ogata, 2009), and it was speculated that the reduction of tannins after fermentation might contribute to the taste of Maotai liquor positively.

3.6. Metabolic pathway analysis of differential phenolic metabolites

The fermentation processes of fermented grains were affected by many factors, and it was not possible to judge the whole process based on the change of metabolite content. Thus, further enrichment analysis of metabolic pathways was needed to discover potential metabolic pathways in the fermentation process. The enrichment of metabolic pathways in the B vs A, C vs B, E vs C, F vs E, G vs F, and N vs M comparison groups were analyzed based on KEGG database, and the results were shown in Table 1. During different fermentation stages of fermented grains, different phenolic metabolites were identified to participate in 12 metabolic pathways. The KEGG secondary classification names were tyrosine metabolism, folate biosynthesis, ubiquinone and other terpenoid quinones biosynthesis, phenylalanine and flavonol biosynthesis, flavonoid biosynthesis, phenylalanine, tyrosine and tryptophan biosynthesis, respectively. Metabolic pathways, anthocyanin biosynthesis, stilbene, dialylheptane and gingerol biosynthesis, secondary metabolite biosynthesis, phenylpropanoid biosynthesis. In addition, 23 differential metabolites were mainly involved in 12 key metabolic pathways, including 4-hydroxybenzoic acid, taxifolin,

Table 1
Metabolic pathway analysis results of differential phenolic metabolites during the fermentation process of fermented grains.

Comparison group	Metabolic pathway pathway No	Metabolic pathway	P-value	Enriched differential phenolic metabolites
B vs A	map00350	Tyrosine metabolism	0.124	3, 4-dihydroxyphenylpropionic acid; 2, 5-dihydroxybenzaldehyde
	map00790	Folate Biosynthesis	0.132	4-hydroxybenzoic acid
	map00130	Biosynthesis of ubiquinone and other terpenoid quinones	0.352	4-hydroxybenzoic acid
	map00360	Phenylalanine metabolism	0.443	4-hydroxybenzoic acid
	map00944	Biosynthesis of flavonoids and flavonols	0.588	Siberian larch flavones; Clove pavilion
C vs B	map00790	Folate Biosynthesis	0.189	4-hydroxybenzoic acid
	map00350	Tyrosine metabolism	0.235	2, 5-dihydroxybenzaldehyde; Homovanillic acid
		Flavonoid biosynthesis	0.260	Galangin; Taxifolin; 3′,5, 7-trihydroxy-3-4 ‘-methoxyflavanone; Hesperetin; Myricetin
		Phenylalanine, tyrosine, and Tryptophan biosynthesis	0.345	Protocatechuic acid
	map00130	Biosynthesis of ubiquinone and other terpenoid quinones	0.473	4-hydroxybenzoic acid
		Metabolic pathways	0.730	Taxifolin; Protocatechuic acid; 2, 5-dihydroxybenzaldehyde; 4-hydroxybenzoic acid; Homovanillic acid
	map00360	Phenylalanine metabolism	1	4-hydroxybenzoic acid
E vs C	map00942	Anthocyanin biosynthesis	0.111	Delphinidin chloride
		Biosynthesis of stilbene, diarylheptane, and gingerol	0.163	White pictaxol
	map00941	Flavonoid biosynthesis	0.238	Delphinidin chloride; galangin
	map01110	Biosynthesis of secondary metabolites	1	Delphinidin chloride; picrotaxol
F vs E	map00940	Biosynthesis of phenylpropanoid	0.009	Trans cinnamic acid; Coniferyl alcohol; Ferulic aldehyde
	map01110	Biosynthesis of secondary metabolites	0.143	Anthocyanins; Trans-cinnamic acid; Coniferyl alcohol; Ferulic aldehyde
	map00942	Biosynthesis of anthocyanins	0.147	Anthocyanins
	map00130	Biosynthesis of ubiquinone and other terpenoid quinones	0.214	Trans cinnamic acid
	map00360	Phenylalanine metabolism	0.276	Trans cinnamic acid
	map01100	Metabolic pathways	0.305	Trans cinnamic acid; Coniferyl alcohol; Ferulic aldehyde
G vs F	map00350	Tyrosine metabolism	0.403	3, 4-dihydroxyphenylpropionic acid
	map00940	Biosynthesis of phenylpropanoid	0.574	Ferulic acid
	map01100	Metabolic pathways	0.642	Ferulic acid; 4-hydroxybenzaldehyde; Catechol;
N vs M	map00945	Biosynthesis of stilbene, diarylheptane, and gingerol	0.071	5-O-caffeyl shikimic acid; resveratrol
	map00940	Biosynthesis of phenylpropanoid	0.124	Ferulic acid; 5-O-caffeyl shikimic acid; Scopolamine
		Phenylalanine, tyrosine, and Tryptophan biosynthesis	0.313	Protocatechuic acid
	map00942	Biosynthesis of anthocyanins	0.313	Delphinidin chloride
	map01100	Metabolic pathways	0.478	Ferulic acid; 5-O-caffeyl shikimic acid; 4-methylcatechol; Protocatechuic acid; Homovanillic acid
	map00350	Tyrosine metabolism	1	Homovanillic acid
	map00941	Flavonoid biosynthesis	1	Delphinidin chloride; 5-O-caffeoyl shikimic acid; 3′, 5, 7-trihydroxy-4 ‘-methoxyflavanone
	map01110	Biosynthesis of secondary metabolites	1	Delphinidin chloride; Ferulic acid; 5-O-caffeyl shikimic acid; Scopolamine; Protocatechuic acid; resveratrol

hesperetin, myricetin, protocatechuic acid, delphinidin chloride and other compounds. Among them, 5-O-caffeyl shikimic acid (4), trans-cinnamic acid (4), 4-hydroxybenzoic acid (3), protocatechuic acid (3), delphinidin chloride (3), coniferyl alcohol (3), ferulaldehyde (3), ferulic acid (3) were all involved in >3 metabolic pathways. If the same metabolite was involved in multiple metabolic pathways at the same time, those results indicated that the differential metabolites had a great impact on the metabolic pathways and could be regarded as the key metabolites.

During different stages of fermented grains, two metabolic pathways related to anthocyanins (markers delphinidin chloride and myricetin chloride) were identified, including flavonoid biosynthesis and anthocyanin biosynthesis (Sunil and Chetty, 2022; Liu, Liu, Wu, et al., 2021). It was well known that anthocyanin biosynthesis was derived from phenylpropanoid biosynthesis. In brief, phenylalanine produced cinnamoyl-CoA through the phenylpropane biosynthetic pathway; Cinnamyl-CoA was then metabolized through the flavonoid biosynthetic pathway to produce anthocyanins such as delphinidin chloride. Finally, secondary anthocyanin metabolites were produced through anthocyanin biosynthesis pathway (Ma, Ma, Gao, et al., 2021). In present study, during anthocyanin biosynthesis, cyanidin and delphinidin were catalyzed by anthocyanin 3-O-glucosyltransferase (3GT), and the glucosyl moiety was transferred to the 3-hydroxyl group of anthocyanin by glucuronyl transferase (UDP) to generate their respective glycosides, respectively (Fig. 8). However, the enzymes and substrates involved in the metabolism of other anthocyanins are not well understood, and the specific metabolic mechanisms are not fully understood. Therefore, future studies should aim at establishing standardized metabolic fingerprints of anthocyanins in different stages of fermented grains.

4. Conclusion

Phenolic metabolites in fermented grains samples at different fermentation stages were studied using UHPLC-TOF-MS non-targeted metabolomics technology in present study. PLS-DA analysis showed that significant differences in phenolic metabolites in different fermented grains samples were observed, including groups B and A, C and B, E and C, F and E, G and H and F, N and O and L and M. Among the 231 phenolic metabolites detected, VIP value >1 and FC > 0.05 or FC < 0.833 and P-value<0.05 were used as screening criteria. 36, 31, 19, 23, 14 and 50 differential phenolic metabolites were screened between B vs A, C vs B, E vs C, F vs E, G vs F and N vs M, respectively. Analysis of the change trend of the relative content of common differential phenolic metabolites among each group showed that phenolic metabolites would undergo structural transformation and degradation during the fermentation process. Through the cluster analysis of differential phenolic metabolites, each comparison group was obviously clustered into two categories, indicating that the change trend of the relative content of phenolic metabolites was significantly different among the comparison groups. KEGG pathway enrichment analysis and Pearson correlation analysis showed that the differential phenolic metabolites were mainly involved in 12 metabolic pathways, including phenylalanine metabolism, flavonoid and flavonol biosynthesis, flavonoid biosynthesis, anthocyanin biosynthesis, and secondary metabolite biosynthesis. Among them, 5-O-caffeyl shikimic acid, trans-cinnamic acid, 4-hydroxybenzoic acid, protocatechuic acid, delphinidin chloride, coniferyl alcohol, ferulaldehyde, ferulic acid were all involved in more than three metabolic pathways and were regarded as the key metabolites. In the future, it is necessary to further conduct absolute quantitative analysis of the differential phenolic metabolites identified based on targeted metabolomics, and to reveal the correlation between the differential phenolic metabolites and the quality and taste of Maotai-flavored liquor. This will provide a theoretical basis for deeply elucidating the formation mechanism of flavor and taste of Maotai-flavored liquor.

CRediT authorship contribution statement

Derang Ni: Writing – original draft, Formal analysis, Data curation. **Shuifang Mao:** Writing – review & editing, Validation. **Yubo Yang:** Writing – review & editing, Conceptualization. **Jinhu Tian:** Writing – review & editing. **Chao Chen:** Writing – review & editing. **Huabin Tu:** Writing – review & editing. **Xingqian Ye:** Writing – review & editing,

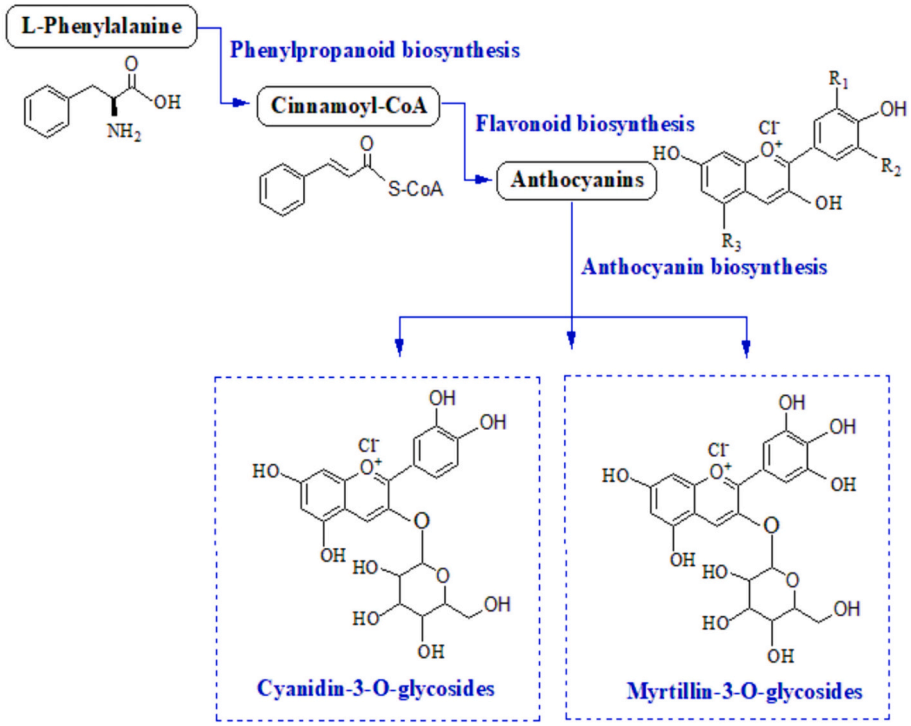

Fig. 8. The hypothetical metabolic pathways related to anthocyanins in fermenting grains.

Supervision. **Fan Yang:** Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare no competing personal interest and financial interest.

Data availability

The data that has been used is confidential.

Acknowledgement

This research was funded by the Institute of Science and Technology, Moutai Group (No. MTGF2021020), China.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101531.

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