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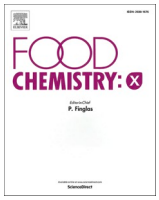

Analysis of differential metabolites in Liuyang douchi at different fermentation stages based on untargeted metabolomics approach

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ABSTRACT

The quality and flavor of Liuyang Douchi are usually closely related to the metabolites compostion. This work described the metabolic profiles of Liuyang douchi during fermentation. Obvious hydrolysis of carbohydrates, proteins and slight lipids degradation were observed. Notably, the qu-making and pile-fermentation stage of douchi could be easily distinguished according to their metabolites profile, and pile-fermentation stage showed the most abundant metabolites. Specifically, organic acid, such as succinic acid and lactic acid, accumulated during pile-fermentation, as well as amino acids and derivatives. Especially glutamate (Glu), which contributed to the umami taste, increased form 0.82 mg/g to 15.90 mg/g after fermentation. Meanwhile, metabolisms related to amino acids were also the main enrichment metabolic pathways. Among them, some flavor compunds such as phenylacetaldehyde might drived from phenylalanine metabolism. These results could provide a new understanding on the metabolic characteristics during Liuyang douchi fermentation.

1. Introduction

Soybeans are widely consumed for its nutritional and healthy ingredients, such as dietary fiber, protein, vitamins, and isoflavonoids (Liu et al., 2022). It is usually utilized to produce fermented food including soy sauce, soybean paste and douchi in east and south-east Asia. After fermentation, the nutritional value of soybean is enhanced by eliminating anti-nutritional factor (e.g. trypsin inhibitors), degrading protein and promoting the production of aglycone isoflavones (Samtiya, Aluko, & Dhewa, 2020). Additionally, various enzymes produced by microbes could catalyze the proteins, carbohydrates and lipids during fermentation (Lioe et al., 2018). It not only provides energy sources for the proliferations of microbiota, and also to offers the substrates for the formation of flavor substances such as organic acids, alcohols, ketones, esters, and amino acids, finally contributed to the characteristic tastes and odors (Park & Kim, 2020).

Usually, *Aspergillus-type* and *Mucor-type* douchi are more popular in China. Both of them involved in two-step fermentation processes, fungal solid-state fermentation (Koji-making), followed by postfermentation (maturation with/without salting) (He, Huang, Liang, Wu, & Zhou, 2016). Liuyang douchi is a kind of *Aspergillus-type* douchi originated from Hunan province of China. Compared with other fermentation processes of douchi, liuyang douchi needs less time (4–10 Days) to matured which benefited from its higher temperature (40–55 °C) in pile-fermentation and low salinity. Traditionally, manual processing relies on uncontrolled and spontaneous fermentation, which influences the stability and safety of products (Chen et al., 2022). Additionally, the quality of liuyang douchi is essentially determined by the varied metabolites which are mediated by microbial diversity during fermentation. Despite some reports pointed out the microbial diversity (Yu, Tao, Hongwei, Jiajia, & Huayi, 2020) and changes of certain components (e.g. organic acids, amino acids and volatile components) during douchi fermentation (He et al., 2019), these metabolites might be insufficient to reflect the general metabolites characteristics during fermentation of douchi, and there is limited report on the dynamic metabolite profiles of liuyang douchi during fermentation.

Untargeted metabolomics approach, which could provide an overall view of metabolites, was widely used in quality analysis, safety properties explanation and mechanism research of characteristic substance formation (Park & Kim, 2021). Usually, metabolic fingerprints can be obtained from nuclear magnetic resonance (NMR) spectroscopy, gas chromatography–mass spectrometry (GC–MS), and liquid

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chromatography-mass spectrometry (LC-MS). Among them, LC-MS was more popular due to its high sensitivity, high reproducibility, high resolution and high-throughput analysis (Yaxin et al., 2021). Thus, metabolomics was widely applied in metabolites or nutrition research of fermented foods, such as,investigations of the effects of cultivar, elevation and process variations on the metabolites of green tea (Wang, Cao, Yuan and Guo, 2021), analysis of the changes and metabolic pathways of koumiss fermented by different *Lactobacillus* (Xia, Yu, Miao, & Shuang, 2020) and study of the nutrient metabolites changes during vegetables fermentation(Kim, Choi, Ju, & Kim, 2019).

In our previous study, the changes of volatile components has been elucidated by GC–MS and GC-IMS (Chen et al., 2021). Non-volatile metabolites not only make important contributions to the taste of Liuyang douchi, but are also closely related to the formation of volatile compounds. In order to better understand the changes of non-volatile metabolites during Liuyang douchi fermentation, the further study on the degradation of protein, sugar and fatty acids, as well as metabolites formation during liuyang douchi fermentation was analyzed by the combination of targeted and untargeted metabonomics. Multivariate analysis based on result of UPLC-*Q-TOF-MS* was further applied to elucidate the differential metabolites and metabolic pathways in different fermentation stage of Liuyang douchi.

2. Materials and methods

2.1. Douchi production

Douchi samples were prepared in the workshop of Liangjia corporation (Hunan province, China). The producing and sampling methods were according to Chen et al. (2022) and showed in Fig. 1S. Firstly, black beans after soaking and steaming were fermented for 12 days in the *Qu*-making room (relative humidity 65 %, 28–32 °C). Then the mycelium covered on the surface of black beans were removed via washing (*Qu*-washing). Then, in pile fermentation stage, the black been Qu was further fermented for 2 days without salt and fermented for another 6 days with 3 % salt (*w*/w) in a big bamboo basket. Douchi samples (100 g, triplicate) at different periods of fermentation were collected everyday from *Qu*-making (D1-D12), *Qu*-washing (D12—W) and pile fermentation (D14 and D20), respectively and immediately stored at −80 °C for analysis.

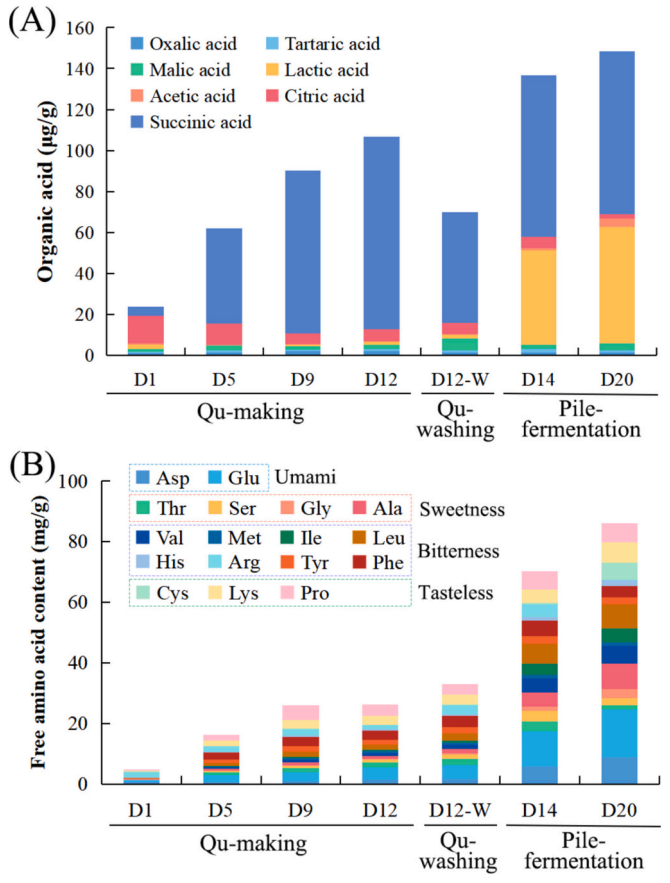

Fig. 1. Changes in composition of organic acids **(A)** and free amino acids **(B)** during Douchi fermentation.

2.2. Total protein and water-soluble protein content analyses

Douchi was lyophilized and crushed, 5 g Douchi powder were mixed with 50 mL distilled water and shaking for 1 h at 20 °C, and the extraction was repeated thrice after the precipitates were collected by centrifugation (2000 r/min, 10 min). The resultant supernatants were combined with the first supernatant to obtain water-soluble protein. Both the total protein content of Douchi powder and water-soluble protein were evaluated using the Kjeldahl nitrogen analysis.

2.3. General characteristics analysis

The total sugar was measured by using phenol-sulfuric acid method, the reducing sugar content was determined by using 3, 5-dinitrosalicylic acid (DNS) method and crude fat was analysis by using Soxhlet extraction method. For total acid and amino nitrogen content determinations, *Douchi* powder (1 g) were dispersed in 60 mL distilled water. The total acid was measured by titration with 0.01 N NaOH at pH 8.3 and the amino nitrogen content was analyzed using the formaldehyde titration method.

2.4. Organic acid composition analysis

After lyophilizing and crushing, 2.5 g of Douchi powder were dispersed in 25 mL distilled water and extracted by ultrasonics for 30 min at 20 °C, and the extraction was repeated thrice after the precipitates were collected by centrifugation (4000 r/min, 20 min). Then collected supernatants were adjusted to 100 mL and 1 mL of it was filtered using a 0.22 μm filter membrane. Organic acid composition was analyzed by HPLC (Agilent Corporation, USA) with an C18 column (4.6 mm × 250 mm, 5 μm, Agilent Corporation, USA). The mobile phases consisted of 50 % phosphate solution (0.1 % *w/v*, pH 3.0) and 50 % methanol was eluted at a flow rate of 1 mL/min. A standard solution of 7 organic acids (Shanghai Aladdin Biochemical Technology Co., Ltd., Shanghai, China) was used as external standard.

2.5. Free amino acid (FAA) analysis

The FAA content of *Douchi* with different fermented times were determined by using an A300 auto amino acid analyzer (Membra Pure, Bodenheim, Germany) according to Zhao, Zhang, Wang, et al. (2018). Free amino acid composition of the test samples was determined based on the free amino acids in the supernatant generated through centrifugation (16,770 *g* and 4 °C for 10 min) of the precipitate from the mixture (mixing time 1 h) of 15 % sulphosalicylic acid (1 mL) and test samples (4 mL). A standard solution of 17 amino acids was used as external standard (Sigma-Aldrich Corporation, America).

2.6. Metabolomic analysis based on UPLC-Q-TOF-MS

2.6.1. Metabolites extraction

Fifty mg of sample after liquid nitrogen grinding were mixed with 1000 μL extract solution (acetonitrile: methanol: water = 2: 2: 1, with isotopically-labelled internal standard mixture) after 30 s vortex in an EP tube, followed by homogenizing at 35 Hz for 4 min and sonicating for

5 min in an ice-water bath. The homogenization and sonication cycle was repeated for 3 times. Then the samples were incubated for 1 h at −40 °C and centrifuged at 12000 rpm for 15 min at 4 °C to obtain the resulting supernatant for analysis. The quality control (QC) sample was prepared by mixing an equal aliquot of the supernatants from all of the samples.

2.6.2. UPLC-MS/MS analysis

An UPLC system (Vanquish, Thermo Fisher Scientific) coupled to Q Exactive HFX mass spectrometer (Orbitrap MS, Thermo) was used for identifying metabolites. The extraction of samples were separated using a UPLC BEH Amide column (2.1 mm × 100 mm, 1.7 μm), with a mobile consisting of solvent A (25 mmol/L ammonium acetate and 25 ammonia hydroxide in water, pH = 9.75) and B (acetonitrile) in the following gradient: 0–0.5 min, 95 %B; 0.5–7.0 min, 95 % ~ 65 % B; 7.0–8.0 min, 65 % ~ 40 % B; 8.0–9.0 min, 40 % B; 9.0–9.1 min, 40 % ~ 95 % B; 9.1–12.0 min, 95 % B. The column temperature was 30 °C. The auto-sampler temperature was 4 °C, and the injection volume was 3 μL.

The QE HFX mass spectrometer was used for its ability to acquire MS/MS spectra on information-dependent acquisition (IDA) mode in the control of the acquisition software (Xcalibur, Thermo). In this mode, the acquisition software continuously evaluates the full scan MS spectrum. The ESI source conditions were set as following: sheath gas flow rate as 50 Arb, Aux gas flow rate as 10 Arb, capillary temperature 320 °C, full MS resolution as 60,000, MS/MS resolution as 7500, collision energy as 10/30/60 in NCE mode, spray Voltage as 3.5 kV (positive) or − 3.2 kV (negative), respectively. Peak detection, extraction, alignment, and integration were performed and MS2 database (BiotreeDB) was applied in metabolite annotation. The cutoff for annotation was set at 0.3.

2.7. Statistical analysis

Student *t*-tests were employed to identify differences in metabolite concentrations between samples. The partial least squares discriminate analysis (PLS-DA) was performed via SIMCA 13.0 to distinguish variables between groups using a calculated VIP value. A VIP cut-off of 1.0 was used to choose significant compounds. Data were presented as "mean ± standard deviation". The results obtained were subjected to one-way analysis of variance (ANOVA). Duncan's new multiple range test was performed to determine the significant difference among samples at the 95 % confidence interval using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1. The degradation of proteins, carbohydrates and lipids of douchi during fermentation

Usually, the degradation of carbohydrates, proteins and lipids by enzymatic hydrolysis could be the basis for the generation of special flavor metabolites during fermentation(Shukla et al., 2015). As shown in Table 1, the small decrease of fatty acid content (from 18.16 % to 16.84 %) during fermentation indicated an inactive degradation of lipids, which might result from low lipase produce capacity of superiority strains (such as the predominant isolates *Aspergillus flavus*) in *Liuyang douchi* (Chen et al., 2022). The total sugar exhibited notably diminution during the early stage of *qu*-making and pile-fermentation period while the reducing sugar gradually increased from 1.11 % to 3.27 % after fermentation. Carbohydrate in soybean are usually hydrolyzed to small molecule sugars by microbes, hence the decrease of total sugar would led to an enhancement of reducing sugar (Elfalleh, Sun, He, Kong, & Ma, 2017). Further, the content of reducing sugar of D5 was higher than that of D9, it might be resulted form the consumption of reducing sugar caused by proliferation of microbes, similar result has been observed in high-concentration glutinous rice fermentation.

With the processing of fermentation, some insoluble soybean protein have been hydrolyzed to peptides and amino acids, hence the soluble protein of douchi showed a significant increase, with the maximum of 13.80 % in D20 samples (Table 1). Similarity, the amino-acid nitrogen increased with the extentio of fermentation time, especially in the pile-fermentation period (from 0.57 % to 2.18 %). The high temperature (50–55 °C) during pile-fermentation was conducive to the maintenance of protease activity and further promoted protein degradation. Evidently, carbohydrate and protein metabolism usually contribute to the enhancement of total acid which including organic acids (Zha, Li, Zhang, Sun, & Chen, 2020). According to Table 1, the total acid increased 7.87 times after fermentation. As Elfalleh et al. (2017) reported that the pH of natural fermented soybean paste decreased with the increase of time. Additionally, the notable decrease in soluble protein, amino-acid nitrogen and total acid of D12—W should be caused by washing.

3.2. The changes of organic acids and free amino acids during fermentation

Sugars could be catabolized by microbes via glycolysis and the tricarboxylic acid cycle, resulting in the formation of organic acids (such as succinic acid and lactic acid) (Chen, Chen, Chen, Zhang, & Chen, 2018). The changes in the content of organic acids during the douchi fermentation were illustrated in Fig. 1a. Similar to the variation tendency of total acids, a large enhancement of organic acids (form 23.59 to 148.45 μg/g) was observed after fermented for 20 days, except organic acids of D12—W which showed a decrease. Detailedly, succinic acid gradually increased to 75.27 μg/g during whole fermentation. Since succinic acid is the intermediate of the tricarboxylic acid cycle, the sugar metabolism of microbe would lead to the large amount of succinic acid excretion during fermentation. Similar findings was reported in beer by Li and Liu (2015). Lactic acid and acetic acid were maintained at a low level in *qu-making* stage while they rapidly increased to 56.73 and 4.04 μg/g after 20 days fermentation, respectively. It indicated that lactic acid and acetic acid were mainly produced in the pile-fermentation stage. The production of lactic acid was generally accompanied by an increase of acetic acid, which resulted from the heterolactic fermentation of bacteria via hexose monophosphate pathway (Zhang et al., 2022). In addition, the accumulation of lactic acid in pile-fermentation period might be due to the anaerobic environment and the proliferation of *Pediococcus* (Chen et al., 2022) that belonged to lactic acid bacteria. On the contrary, an obviously decrease (form 13.38 to 2.30 μg/g) of

Table 1
The changes of proteins, sugar, fatty acid and their derivative of douchi during fermentation.

Sample		Fatty acid (%)	Total sugar (%)	Reducing sugar (%)	Soluble protein (%)	Amino-acid nitrogen (%)	Total acid (%)
Qu-making	D1	18.16 ± 0.91^{b}	18.22 ± 2.51^{d}	1.11 ± 0.11^{a}	3.74 ± 0.08^{b}	0.39 ± 0.02^{a}	0.39 ± 0.01^{a}
	D5	17.51 ± 0.88^{ab}	14.10 ± 1.40^{c}	2.13 ± 0.27^{b}	5.10 ± 0.32^{c}	0.57 ± 0.06^{b}	1.32 ± 0.02^{c}
	D9	17.37 ± 0.77^{ab}	13.18 ± 1.43^{bc}	1.25 ± 0.30^{a}	8.05 ± 0.53^{d}	0.76 ± 0.11^{c}	1.44 ± 0.02^{d}
	D12	16.23 ± 0.67^{a}	12.47 ± 1.95^{bc}	$1.22 \pm 0.0.26^{a}$	7.43 ± 0.61^{d}	0.68 ± 0.12^{bc}	1.74 ± 0.03^{e}
Qu-washing	D12-W	16.61 ± 0.71^{a}	13.38 ± 0.23^{bc}	1.96 ± 0.14^{b}	2.70 ± 0.42^{a}	0.57 ± 0.08^{b}	1.01 ± 0.02^{b}
Pile-fermentation	D14	16.34 ± 0.62^{a}	11.25 ± 0.72^{b}	2.12 ± 0.29^{b}	11.20 ± 0.38^{e}	1.60 ± 0.08^{d}	3.15 ± 0.07^{f}
	D20	16.84 ± 0.62^{ab}	9.08 ± 0.59^{a}	3.27 ± 0.17^{c}	13.80 ± 0.84^{f}	2.18 ± 0.17^{e}	3.46 ± 0.06^{g}

citric acid was found after fermenting, which agreed with the founding of fermented soy whey (Zhang et al., 2022) and fermented bog bilberry juice (Wei et al., 2018). Usually, citric acid is a key intermediate in the tricarboxylic acid cycle, and the ATP produced from its degradation might serves as an important source of energy for microbial proliferation and metabolism.

Free amino acids contribute to the formation of special aroma and taste of fermented food, as well as have an important role in microbes metabolism. In present study, amino acids increased during fermentation, especially in pile-fermentation stage (Fig. 1b), At the end of douchi fermentation (D20), the content of total free amino acids increased to 81.02 mg/g compare with that of D1. As Zhou et al. (Zhou et al., 2019) mentioned, protein hydrolysis which reflected by the increase in soluble protein and amino-acid nitrogen (Table 1) could lead to a rapid increase of free amino acids during fermentation. Umami taste amino acids (Glu and Asp), which was the most abundant amino acid in mature douchi (D20), accounted for 28.65 % of the total free amino acids, followed by Ala and Leu. Glu was also found to be the most abundant free amino acid in *doenjang* (Shukla, Bahuguna, Park, Kim, & Kim, 2020) and soybean sauce (Zhou et al., 2019), it might due to the high level of Glu in soybean proteins. Differently, Phe and Tyr displayed a decline in the late of plie-fermentation stage. Microbes such as lactic acid bacteria could catabolize aromatic amino acids (including Phe and Tyr) to generate distinct flavor compounds phenylacetaldehyde, and phenylethanol, which were considered as the important flavor volatile compounds in Liuyang douchi (Chen et al., 2021; Loh, Ng, Toh, Lu, & Liu, 2021). The accumulation of lactic acid bacteria (Chen et al., 2022) also could accelerated the metabolism of aromatic amino acids to produce energy (Wei et al., 2018). In the early stage of fermentation (D1), bitter taste amino acids (Val, Met, Iso, Leu, Phe, His, Tyr and Arg) accounted for the highest proportion (46.27 %) of free amino acids while content of sweet taste amino acids (Thr, Ser, Gly, Ala, Lys and Pro) was the lowest. Then bitter taste amino acids, sweet taste amino acids and umami taste amino acids are similar in content (28.65 % ~ 32.62 %) after fermenting for 20 days. It was partly responsible for the flavor of Liuyang douchi which exhibited a balanced taste of umami, sweet and bitter.

3.3. Differential analyses of Liuyang douchi with different fermentation time

In order to analyze changes of metabolites during fermentation of douchi, the PLS-DA was performed (Fig. 2), and the loading plots explaining 63.20 % and 59.10 % of the variance in the positive and negative ion modes, respectively. Obviously, symbols representing samples were located in different quadrants and showed distinct clustering pattern, indicating apparent differences in the metabolite profiles in douchi of different fermentation stages. In the positive ion modes, samples could be divided into three sections which were correlated to the three fermentation stages, respectively. Samples in the beginning of *qu-making* (D1) stage were situated in the fourth quadrant, samples in the late *qu-making* period (D5-D12-W) were clustered in the first quadrant, and samples in the *pile-fermentation* stage (D14-D20) were located in the third quadrant (Fig. 2a). Differently, samples in qu-making period (D1-D12-W) and pile-fermentation stage (D14-D20) were situated in different quadrants on the left and right sides of the origin in X-axis in negative ion modes. Similar trends were found in organic acids, free amino acids (Fig. 1) and volatile flavor compounds (Chen et al., 2021), indicating the evidently differences in metabolism by microorganisms between these two fermentation stages, which might be resulted form the changes in microbial community and and the activities of their enzymes during liuyang douchi fermentation (Chen et al., 2022).

3.4. Differential abundant metabolites identified in Liuyang douchi with different fermentation time

According to Fig. 3a, a total of 654 differential metabolites which having a VIP value greater than 1 from the PLS-DA model were identified by MS2. The differential metabolites could be classified into 15 categories such as organic acids and derivatives (147), lipids and lipid-like molecules (112), organic oxygen compounds (71). Further, there are over 1000 differential metabolites in the early stages of qu-making (D1 vs D5), the washing of qu (D12 vs D12—W), and the early stages of pile-fermentation (D12 vs D14), while the number of differential metabolites in the other groups ranges from 668 to 860, indicating more notable difference in microbial metabolism during these periods. There were 99 common differential metabolites observed in all six groups, including organic acids and derivatives (31), lipids and lipid-like molecules (20), organic oxygen compounds (18).

Among acids and derivatives, the major metabolic differences were reflected in 120 kinds of amino acids and related derivatives. Most of them reached the peak in D14 and/or D20. For the common differential metabolites, 21 kinds of them such as N-Acetyl-L-alanine and Prolyl-Arginine accumulated in pile-fermentation stage, while just 7 kinds of them (e.g. and Prolyl-Threonine) reached a higher level during qu-maiking period, indicating that amino acid metabolism is more vigorous during pile-fermentation stage.

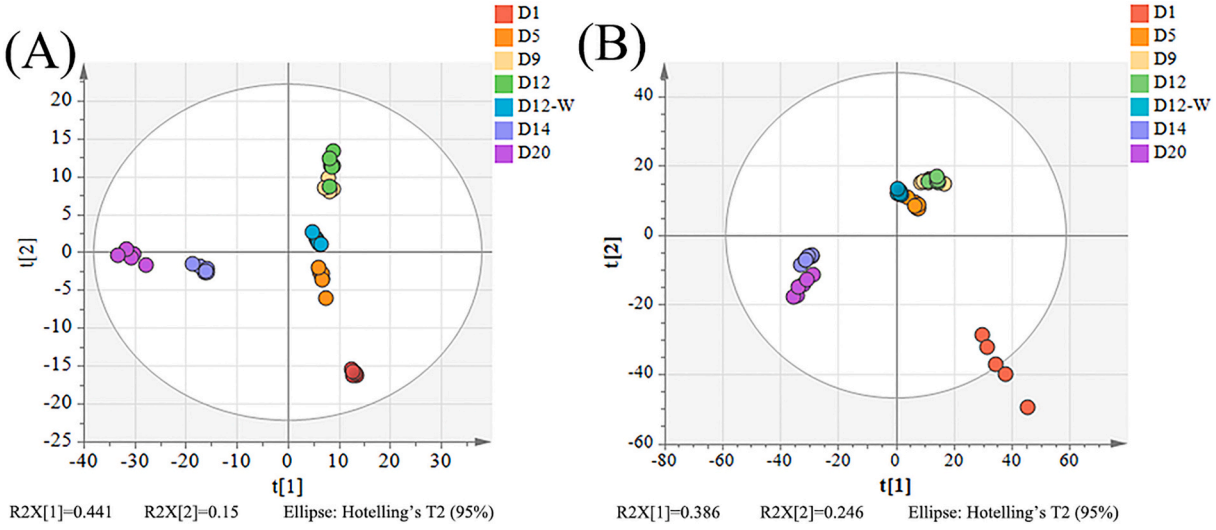

Fig. 2. PLS-DA of identified metabolites from Liuyang douchi during fermentation. Metabolomes were obtained by positive ion mode **(A)** and negative ion mode **(B)** of UPLC-Q-TOF-MS.

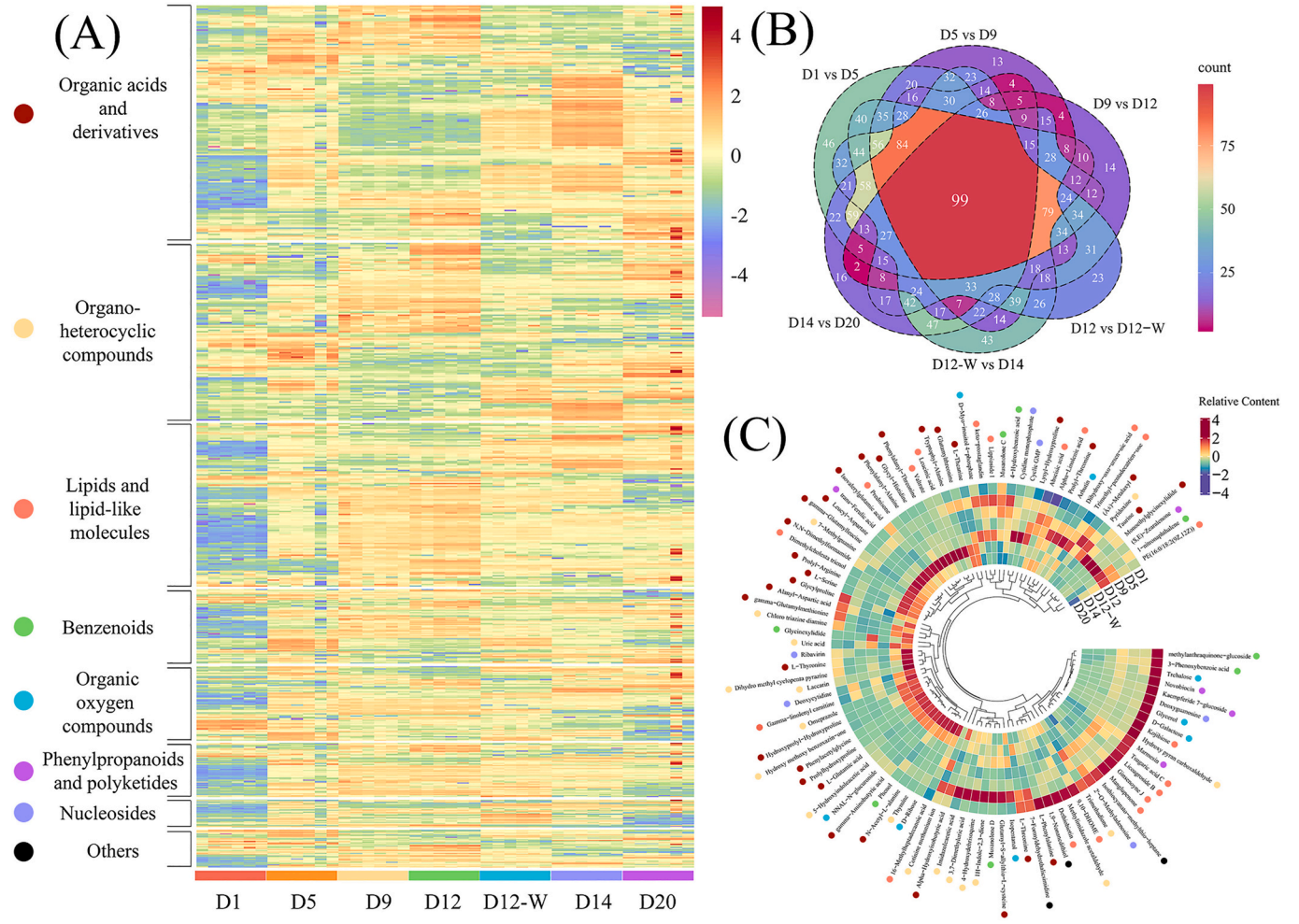

Fig. 3. Heatmap of hierarchical clustering analysis **(A)** and venn diagram **(B)** of differential abundant metabolites during fermentation, and heatmap of hierarchical clustering analysis **(C)** of common differential metabolites during fermentation. Metabolite classification are represented by the colored dots. Color coding is graded from blue to red, with relative strength increasing from low to high. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Organoheterocyclic compounds include purines, pyrimidines, indoles, amines, pyrazines and so on, most of which have high levels at the end of pile-fermentation (D20). Both indoles and amines were usually considered as the derivative of amino acid while production of purines and pyrimidines might be associated with the degradation of nucleic acid. The decrease of deoxyguanosine might led to the enhancement of 7-methylguanine and D-ribose as illustrated in Fig. 3c.

Although the degradation of lipids was inactive, there were 112 lipids and lipid-like molecules were regarded as the differential metabolites, and vast majority of them reached the maximum content after qu-making (D12). Similarity, most of Benzenoids, showed higher levels in douchi during qu-making period. Organooxygen compounds are also the important metabolites in douchi, comprising 53 carbohydrates and carbohydrate conjugates, 7 carbonyl compounds and 6 alcohols and polyols. Saccharides such as sucrose, D-galactose and trehalose were degraded with the increase of fermented time which could provide carbon source for microbial proliferation and metabolism. Similar results were found by Xiaoxing, Jiachun, Liqiang, Zhen, and Qiming (2018) and Joanna et al. (2021) in vegetable-fruit beverage fermented and brewing novel beer, respectively.

Additionally, Phenylpropanoids and polyketides mainly contain some active ingredients, such as phenolic and flavonoid compounds. Microbe such as lactic acid bacteria could contribute to the increase of them (Wang et al., 2022). However, only 46.30 % of phenolic and flavonoid compounds such as trans-ferulic acid showed a significant increase, Fang (Fang & Yi, 2019) also reported a slight decrease in flavone compounds during Yongchuang douchi fermentation.

3.5. *Differential metabolic pathway analysis of Liuyang douchi during fermentation*

Based on the aforementioned discussions (Table 1 and Fig. 1), the notable decrease of total sugar and the rapid accumulation of reducing sugar, soluble protein, amino-acid nitrogen, total acid, especially the free amino acids and organic acids indicated the deep degradation of carbohydrate and proteins. Their degradation products (including sugars and amino acids) would further generate a series of metabolites via a series of complex metabolic pathways. According to differential analyses(Fig. 2), the composition of metabolites of liuyang douchi in different fermentation stages (*qu-making* and pile-fermentation) were dramatically different. The metabolic profiles of qu-making and pile-fermentation stage were illustrated in Fig. 4, and a total of 58 and 55 metabolic pathways (including 53 common pathways) were observed in qu-making and pile-fermentation stage, respectively.

Overall, metabolisms related to amino acids were the main enrichment metabolic pathways of differential metabolites during whole fermentation, especially the Histidine metabolism in qu-making stage and Arginine biosynthesis in pile-fermentation. Amino acids could

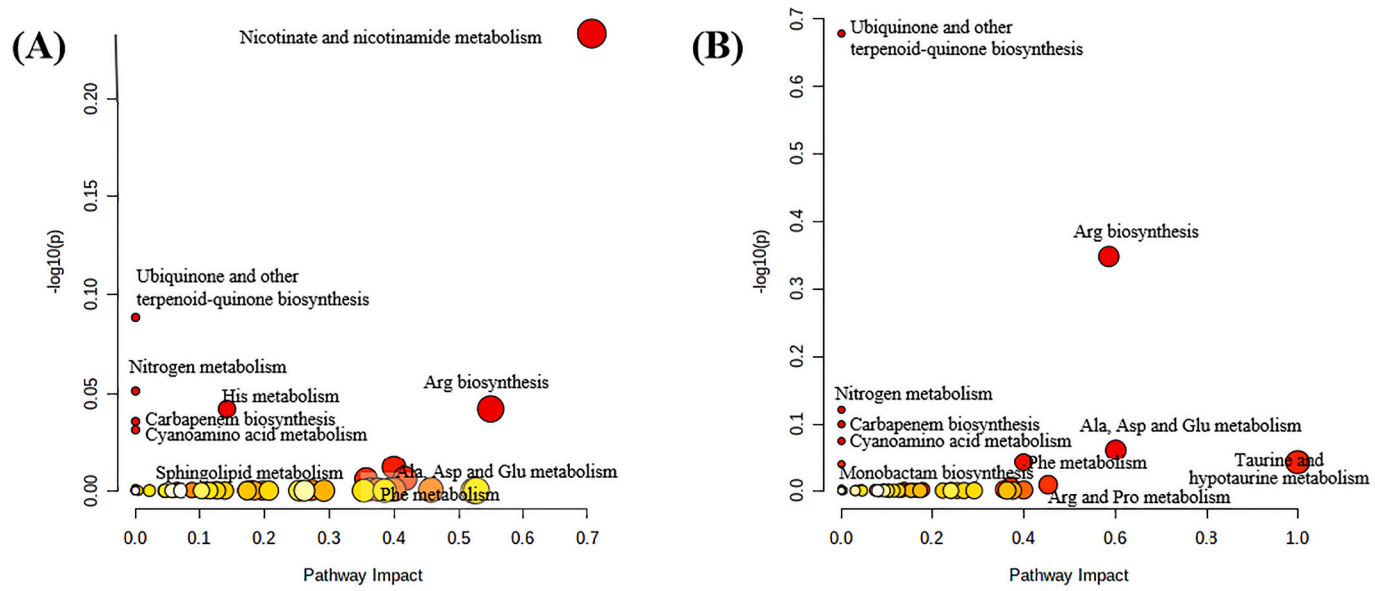

Fig. 4. Path view of Liuyang douchi during qu-making **(A)** and pile-fermentation stage **(B)** through pathway analysis (KEGG pathway). Sizes of each node represented the intensity of pathway.

participate in the formation of various secondary metabolites including aldehydes, alcohols, acids, and esters via transamination, decarboxylation, and oxidation/reduction in Ehrlich pathway(Park & Kim, 2021). Meanwhile, glycometabolism (e.g. starch and sucrose metabolism, citrate cycle and pentose phosphate pathway) that involved in carbohydrate metabolism were also the important metabolic pathways. It could provide a carbon source for microorganism growth as well as contribute for the accumulation of succinic acid and lactic acid (Fig. 1A). Ribose 5-phosphate and NADPH generated in pentose phosphate pathway could be important precursors in purines and pyrimidines metabolism which were also the vital pathways during douchi fermentation (Fig. 4 and Table 1S).

Although degradation of lipids was inactive during fermentation (Table 1), the Glycerolipid metabolism as well as glyoxylate and dicarboxylate metabolism that related to fat metabolism were also important kinds of the metabolic pathways in qu-making stage, and lipase catabolite Acetyl-CoA could be transformed to succinic acid and malic acid via glyoxylate cycle, further participated in citrate cycle (Lee, Jang, Kim, & Maeng, 2011). Similarly, the nicotinate and nicotinamide metabolism was more superior in *qu-making* period compare to pile-fermentation stage (Fig. 4). Evidently, nicotinamide originated from nicotinate could be transformed to NAD + i and affected cells proliferating (Orlandi, Alberghina, & Vai, 2020). The low level of nicotinate and nicotinamide metabolism during pile-fermentation might be due to the

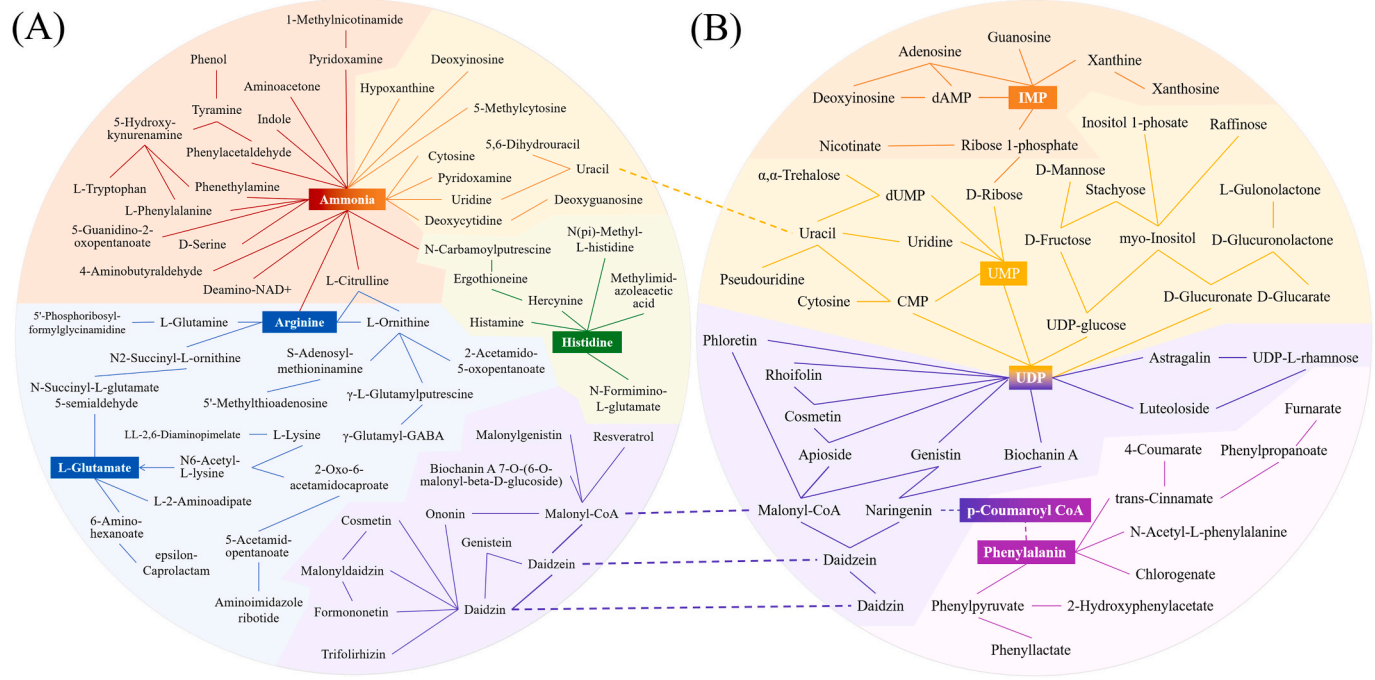

Fig. 5. Correlation network analysis based on primary metabolites identified by positive ion mode **(A)** and negative ion mode **(B)** of UPLC-Q-TOF-MS. Metabolic pathways are represented by different colors.

fact that the high temperature (50–60 °C) and salt addition inhibited microbial proliferation. Moreover, both flavone and flavonol biosynthesis, ubiquinone and other terpenoid-quinone biosynthesis, nitrogen metabolism and cyanoamino acid metabolism were also found during whole douchi fermentation.

3.6. Reconstruction of metabolic network of douchi during fermentation

According to KEGG database, the changes in metabolites, including amino acids, nucleotides and flavones, for possible metabolic pathways during douchi fermentation were reconstructed (Fig. 5). Two hundreds and ten compounds, 94 enzymes and 6 pathway are involved in the metabolism.

Specifically, differential metabolites identified in the positive ion modes were mainly related to arginine and histidine metabolism. The accumulation of intermediates such as N2-Succinyl-L-ornithine and N-Succinyl-L-glutamate 5-semialdehyde originated form arginine, as well as N-Formimino-L-glutamatecould originated form histidine resulted in the biosynthesis of glutamate which was beneficial to the taste of douchi umami. Arginine could also be changed into L-Ornithine via arginase (EC 3.5.3.1). Additionally, the active phenylalanine metabolism was also found, Phe could successively be transformed to phenylethylamine and phenylpyruvate by aromatic-L-amino-acid decarboxylase (EC 4.1.1.28) and primary amine oxidase (EC 1.4.3.21) during koji-making period. Then phenylethylamine and phenylpyruvate decreased in pile-fermentation to form phenylacetaldehyde and styrene which detected in mature douchi (Chen et al., 2021). Besides the influence of Phe metabolism, phenylacetaldehyde also could be generated due to the Strecker degradation from phenylalanine in a high temperature environment during pile-fermentation (Nakada et al., 2018). Differently, histidine metabolism showed influences on the function rather than flavor by generating ergothioneine with antioxidant activity and histamine. Other biogenic amine, tyramine and phenylethylamine were also detected.

Nucleotide metabolism is also crucial for proliferation and metabolism of microbes. A total of 36 kinds of purine, pyrimidine and their derivatives including AMP, AICAR and xanthosine were detected. Among them, AMP and AICAR were related to arginine biosynthesis and histidine metabolism, respectively. Xanthine as a kind of necessary additive for some microorganisms' proliferation and metabolism (Kim et al., 2012), showed a dramatically increase (more than 400-fold) after fermentation (Fig. 3). UDP-glucose was also the important metabolite of pyrimidine metabolism, which was generated form the reaction between UDP and sugar (such as sucrose and fructose), and then participated in flavone and flavonol biosynthesis. The glucose group of genistin and daidzin was removed and transformed to genistein and daidzein by the catalysis of isoflavone 7-O-glucosyltransferase (EC 2.4.1.170), respectively. The increase of luteolin and naringenin might be converted from tricetin and eriodictyol by flavanoid 3′,5′-hydroxylase (EC 1.14.14.81). It might be the reason for the the increase of glycoside isoflavones after *Liuyang Douchi* fermentation that found in our previous study (Chen, Liu, Jiang, Li, & Liao, 2020). Duan et al. (2023) also found an enhancement of luteolin and Naringenin levels in Goji juice after fermenting by *Lacticaseibacillus rhamnosus*.

4. Conclusion

In summary, the degradation of protein, sugar and fatty acids, as well as their metabolites formation during liuyang douchi fermentation were analyzed by the integrated targeted and untargeted metabonomics. Then the metabolic pathways-based analysis, and a correlation network analysis were also approached to illustrate the difference of metabolic profile of douchi in different fermentation stages. The results showed that protein degradation and amino acid metabolites were enriched in douchi fermentation, especially metabolism of arginine, proline and phenylalanine. It might contribute to the formation of flavor compounds such as umami amino acids (Glu and Asp) and aroma components (butyric acid and phenylacetaldehyde). Additionally, flavone and flavonol biosynthesis, such as the transformation from genistin to genistein, could help to increased bioactivities of douchi. Based on non-targeted metabolomics, present study could provide the useful information for the metabolic differences during douchi fermentation. However, the relationship between these metabolites and the flavor compounds in Liuyang douchi, as well as the key microorganisms and influencing factors that cause these metabolic changes, have not been explored yet. Next, the relationship between the formation of flavor compounds and functional microbial metabolism in Liuyang douchi would be investigate.

CRediT authorship contribution statement

Liwen Jiang: Writing – original draft, Project administration. **Yi Chen:** Data curation. **Tiantian Zhao:** Writing – review & editing. **Pao Li:** Validation, Software. **Luyan Liao:** Methodology, Formal analysis. **Yang Liu:** Writing – original draft, Visualization, Validation, Project administration.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.102097.

Data availability

Data will be made available on request.

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Further reading

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