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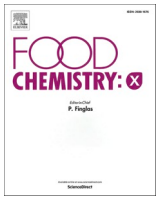

Exploring jujube wine flavor and fermentation mechanisms by HS-SPME-GC–MS and UHPLC-MS metabolomics

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ABSTRACT

The fermentation metabolites significantly influence the quality of jujube wine. However, the dynamics of these metabolites during fermentation are not well understood. In this study, a total of 107 volatile and 1758 non-volatile compounds were identified using a flavor-directed research strategy and non-targeted metabolomics. The increase in esters and alcohols during fermentation shifted the aroma from grassy, mushroomy, and earthy to a floral and fruity flavor in the jujube wine. Leucine and phenylalanine were notably enriched during fermentation, potentially benefiting human health and enriching the flavor of fruit wines. Moreover, pathway analysis identified four key metabolic pathways and two crucial metabolic substrates, pyruvate and L-aspartate. This study provides a theoretical reference for optimizing the fermentation process and enhancing the quality of jujube wine.

1. Introduction

Jujube (*Ziziphus jujube* Mill.) belonging to the Rhamnaceae family in the Ziziphus genus is a natural medicinal food, which is rich in carbohydrates, dietary fiber, vitamins, and other bioactive components such as polysaccharides, flavonoids, and polyphenols (Wang, Liu, Huang, & Luo, 2020). The special climatic and environmental conditions in the Xinjiang province provide basic quality conditions for the development of jujube wine improving total sugar content, sugar-to-acid ratio, and good fruit quality. Jujube wine, a fermented low-alcohol beverage is becoming a high-value-added product in the jujube industry. Compared with other fruit wines, the jujube wine, which has antioxidant, sugar, and lipid-lowering health effects, is produced by low-temperature light fermentation technology to maintain the original health properties (Zhao et al., 2022). To enhance the sensory quality and flavor complexity of red date fruit wine, wolfberries, and grapes are used in a certain proportion as auxiliary ingredients. This also complements the nutritional value of jujube wine and opens up paths for the industrial development of jujube wine.

Fermentation is an essential part of fruit wine production. The yeast flora converts the substrate into different flavor components and bioactive compounds during the fermentation phase. Numerous studies have explored the flavor substances of specific fruit wines. For example, Wattanakul, Morakul, Lorjaroenphon, & Jom, (2020) found that acetate and ethyl esters were the key aroma groups during the fermentation of mango wine, meanwhile, fatty acid methyl esters were positively correlated with sugars, volatile acids, higher alcohols, aldehydes, and ketones. Yang et al. (2020) reported that benzaldehyde, 3-methylbutanol, vanillin, and sotolon were the key flavor compounds, meanwhile, 1,1-diethoxyethane, 3-methylbutanol, benzaldehyde, vanillin, and sotolon significantly contributed to the overall aroma of yellow wine during the aging process. However, the dynamics of material metabolites during fermentation are not well understood. Traditional techniques struggle to comprehensively detect all fermentation metabolites due to the complexity of the process. In contrast, metabolomics enables qualitative and quantitative analysis of these metabolites, including those that fail to be detected by conventional methods. Moreover, the differences in metabolites between different samples can be examined to

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explain the related material changes in their fermentation processes. In recent years, with the advancement of metabolomics, metabolomics tools have been extensively used for the identification and analysis of key substances in foods to establish their relationship with functional activity. For example, Chen et al. used ultraperformance liquid chromatography-quadrupole/time of flight mass spectrometry (UPLC-Q/TOF-MS) to explore the fermentation process of ginkgo wine and reported that cysteine and histidine were the key metabolites responsible for flavor changes and the fermentation enhanced amino acid biosynthesis improving the quality of ginkgo wine. Moreover, the group found significant changes in the amounts of fresh taste substances (L-glutamic acid), bitter taste substances (xanthine), and acidic substances (malic acid and citric acid) during the fermentation of ginkgo wine, which enhanced protein absorption and digestion, amino acid biosynthesis, and galactose metabolism (Chen, Li, & Rong, 2022). Ai et al. examined the metabolic profiles of roselle wine at different fermentation times using ultra-high-performance liquid chromatography-mass spectrometry and found that the anthocyanin metabolic pathways were mainly related to the biosynthesis of flavonoids (Ai, Wu, Battino, Bai, & Tian, 2021). However, the changes in metabolites of jujube wine during fermentation have been rarely examined using metabonomics, especially from the perspective of transformation between the non-volatile and volatile components.

In this study, jujube wine was brewed using jujube as the main raw material. Headspace solid-phase microextraction-gas chromatography-mass spectrometry (HS-SPME-GC–MS) and ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) were employed to fully explore the metabolic profile changes during the fermentation of date wine. Combined with chemometric analysis, highly variable metabolites and significant time points were identified. This study aimed to identify the metabolite modules influencing the quality of date wine and predict related metabolic pathways. Additionally, the results help understand the metabolic characteristics of yeast, determine the fermentation stage of date wine, and provide a theoretical foundation for enhancing the quality of date wine.

2. Materials and methods

2.1. Materials and chemicals

Jujube (Zizyphus jujuba cv. Junzao, length 32–35 mm) was sourced from Aksu City, Xinjiang Province, China. Grapes and wolfberries were obtained from Shihezi City, Xinjiang, China, and Jinghe County in Bortala Mongol Autonomous Prefecture, respectively. Active dry yeast (*Saccharomyces cerevisiae* BV818) was purchased from Anqi Yeast Co., Ltd (Hubei, China). 2-Octanol and L-2-chlorophenylalanine standards (≥98 %) were purchased from Yuanye Biotechnology Co. (Shanghai, China). 2-Chloro-L-Phenylalanine (≥98 %) was purchased from Maclean Biochemical Technology Co. (Shanghai, China), and other chemicals were purchased from Macklin (Shanghai, China).

2.2. Fermentation

The samples were prepared following the previously established fermentation process by the group, with slight modifications (Zhao et al., 2022). High-quality jujubes, free from spoilage, were boiled in water (100 mg/L) for 10 min, pitted, and set aside. Wolfberries were soaked in warm water at 45–50 °C for 10 min, their stems removed, and set aside. Grapes were washed, seeded, and reserved for later use. Pretreated jujube, grapes, and wolfberries were mixed at a 5:4:1 ratio and pulped in distilled water (250 g/L) using a high-speed wall breaker (SP907R, Supor Co., Ltd., Zhejiang Province, P.R. China). The resulting fermentation broth was enzymatically digested with 0.5 g/L pectinase (Lallemand Group Co., Ltd., France, activated: 0.83 mkat/g) at 50 °C for 2 h. Sugar was added to the enzymatic digest to achieve an initial soluble solids content of 12 mg/mL, and the broth's pH was adjusted to 4.0 with food-grade citric acid. SO_2 (28 mg/L) was then added. Subsequently, the broth was inoculated with BV818 (10 g/L) and fermented at 20 ± 0.5 °C. Fermentation was stopped when the residual sugar level remained constant for 72 h in the jujube wine (approximately after 12 days). During fermentation, samples were collected at 0, 2, 4, 6, 8, 10, and 12 days, and stored at −80 °C for further analysis.

2.3. Headspace-solid phase microextraction-gas chromatography-mass spectrometry (HS-SPME-GCMS)

HS-SPME: 5 mL of wine sample was weighed in a headspace vial and added with 10 μL of 2-octanol internal standard (50,000 μg/L) and 1 g NaCl. The mixture was evenly mixed and equilibrated at 45 °C for 15 min. Extraction head 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) (Supelco., PA, USA) was aged at 250 °C for 20 min and then placed in the top headspace of the headspace vial and adsorbed at 45 °C for 20 min.

GC–MS: Volatile compounds were analyzed using an HP-INNOWAX analytical fused silica capillary column (30 m × 0.25 mm × 0.25 μm, Agilent, CA, USA). The programmed temperature ramp-up was as follows: 40 °C held for 2 min, increase to 80 °C at 3 °C/min, increase to 105 °C at 2 °C/min, increase to 180 °C at 3 °C/min, and final increase to 230 °C (held for 5 min) at 10 °C/min. The flow rate of carrier gas helium (>99.99 %) was 1.0 mL/min. The mass scan range was 35–350 *m/z* in full scan mode, and the solvent delay was 3 min.

Characterization and quantification of volatile compounds: unknown compounds were analyzed by mass spectrometry by comparing with retention indices of identified compounds in the National Institute of Standards and Technology database (NIST) 17 database, supplemented with references of qualitative analysis. Quantitative analysis was performed using internal standards and compound peak areas. The calculation formula was as follows:

Unknown compound concentration (μg/L) = peak area of the unknown compound × internal standard concentration (μg/L) / internal standard peak area × sample content (L).

2.4. Identification of aroma-active substances

The degree of contribution of an aroma component to the overall odor of the sample is expressed as the odor activity value (OAV; OAV = mass concentration/ odor threshold) (Zhang, Qin, Zhang, Jiang, & Zhu, 2023). Substances with OAV ≥ 1 are the key aroma compounds and those with 0. 1 ≤ OAV < 1 are modified aroma components.

2.5. Ultra-high performance liquid chromatography-mass spectrometry analysis

Sample pretreatment: 200 μL of the sample in an EP tube was thoroughly mixed with 800 μL of methanol–acetonitrile extract containing 0.02 mg/mL L-2-chlorophenylalanine as internal standard. The mixture was placed in an ice-water bath for 25 min and then centrifuged at 15000 × *g* and 4 °C for 20 min to obtain the supernatant, which was added with 120 μL of 50 % aqueous acetonitrile for extraction at 5 °C and 40 kHz for 5 min. The prepared extract was used for LC-MS analysis; equal volumes of experimental samples were mixed to make quality control samples.

LC-MS conditions: Chromatographic column, ACQUITY UPLC HSS T3 (100 mm × 2.1 mm i.d., 1.8 μm; Waters, Milford, USA); column temperature, 40 °C; mobile phase A, 5 % acetonitrile aqueous solution containing 0.1 % formic acid; mobile phase B, 47.5 % isopropanol + 47.5 % acetonitrile + 5 % water (containing 0.1 % formic acid), flow rate, 0.4 mL/min. The gradient was programmed as follows: 100 % A, 0–3.5 min; 24.5 %-65 % B, 3.5–5 min; 65 %-100 % B, 5–7.4 min; 100 % A, 7.4–10 min. The injection volume was 2 μL. Sample detection was performed in electrospray ionization (ESI) positive and negative ion modes with an ESI spray voltage of 3.5 kV.

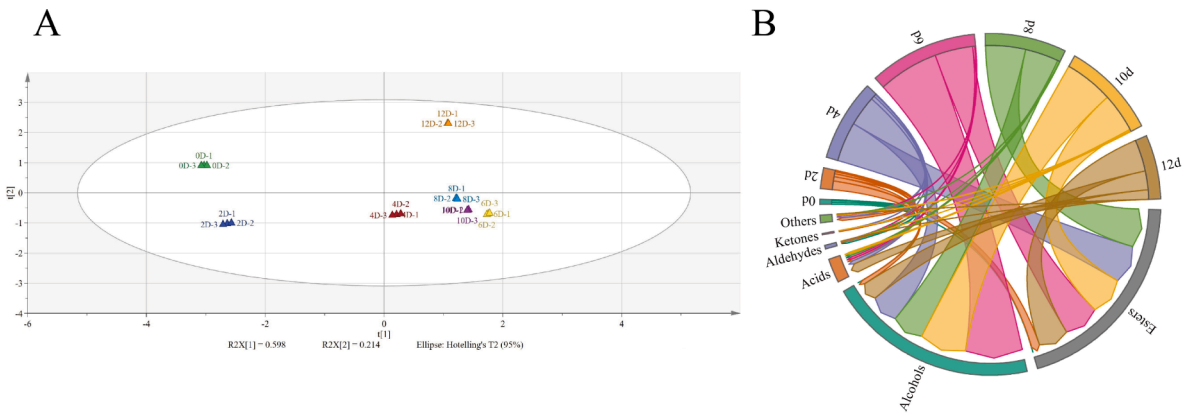

Fig. 1. Partial least squares analysis of changes in volatile metabolites during fermentation (A); trends of different types of volatile compounds (B). Concentrations are expressed as the mean ± standard deviation of three replicates.

Identification of non-volatile compounds: The Progenesis QI software (Waters Corporation, Milford, USA) was utilized for peak alignment, retention time correction, and extraction of peak areas. Metabolites obtained through Progenesis QI were identified based on matching scores. Metabolite identification involved exact mass number matching and secondary spectrum matching, as well as searching metabolomic databases and previous literature.

2.6. Data processing

Partial least squares discriminant analysis (PLS-DA) was used for the multidimensional statistical analysis of the data. Metabolites showing significant differences between groups were screened based on projection variable importance (VIP) > 1.2 and a *p*-value < 0.05 in the *t*-test. These metabolites were then subjected to the Kyoto Encyclopedia of Genes and Genomes (KEGG, https://www.genome.jp/KEGG/pathway.html) metabolic pathway analysis. Subsequently, the key metabolite-related metabolic pathways underwent enrichment and topological analyses (https://www. metaboanalyst.ca/). All analyses were performed in triplicate to ensure accuracy.

3. Results and discussion

PLS-DA is currently a widely used classification method in metabolomics data analysis. It combines regression modeling with dimensionality reduction and uses a specific discriminative threshold for analyzing regression results. In this study, the PLS-DA model characterized changes in volatile metabolites during date wine fermentation (Fig. 1A). The R^2X (0.598), R^2Y (0.214), and Q^2 (0.95) values of the model indicate that the model is stable, reliable, and has high predictive power. The volatile metabolites detected in different stages of fermentation were divided into three stages: i) 0 and 2 d for the pre-fermentation period, ii) 4, 6, 8, and 10 d for the mid-fermentation period, and iii) 12 d for the post-fermentation period. The results showed a significant effect of the fermentation process on volatile compounds ($p < 0.05$); 4 and 12 d were considered as two cut-off points for significant changes in volatile metabolites. The changes in volatile metabolites during the fermentation of jujube wine can be attributed to a series of chemical reactions, including catalytic conversion and enzymatic and oxidative reactions (Wibowo, Grauwet, Kebede, Hendrickx, & Van Loey, 2015).

3.1. Overall evaluation of volatile compounds in the fermentation process

The change in volatile aroma components (VOCs) of jujube wine during fermentation was determined by HS-SPME-GC–MS. In total, 107 substances (including 32 esters, 20 alcohols, 19 acids, 21 aldehydes and ketones, and 15 other compounds) were detected (Table S1). A heat map (Fig. S1) was used to visualize the changes in the concentrations of 107 volatile compounds, and a chord diagram (Fig. 1B) was used to indicate the trends of different volatile compounds. The types and contents of volatile aroma compounds in the jujube wine varied widely during fermentation; the 0 d volatiles were low (255.80 μg/L) and mainly dominated by acids (106.02 μg/L) and aldehydes (103.40 μg/L). Due to microbial metabolism, the content of volatile compounds in jujube wine increased rapidly with the increase in fermentation time and the aroma became more complex and intense; esters (10667.29 μg/L) and alcohols (10634.68 μg/L) were the dominating volatile substances.

Higher alcohols are important secondary metabolites produced during *Saccharomyces cerevisiae* fermentation, and their right amounts can make date wine taste mellow and harmonious (Zhang, Zhang, & Xu, 2016). In this study, the highest alcohol content (3277.77 μg/L) was at 6 d of fermentation and significantly ($p < 0.05$) differed from the fermentation process of other jujube wines (Fig. 1B, S1), followed by a decrease to 1813.85 μg/L (8 d) and then to 1014.74 μg/L (12d) after another increase at 10 d (2647.90 μg/L). Notably, hash oil has higher levels of alcohols, such as phenylethanol and isoamyl alcohol, which provide a distinct dual sensory effect of elegant floral aromas such as rose at low concentrations and undesirable irritating odors at high concentrations. The results showed that the phenylethanol and isoamyl alcohol contents were significantly higher at 10 d of jujube wine fermentation than the other periods ($p < 0.05$), which imparted floral and grassy aromas to the wine samples and were possibly produced by yeast metabolism through the Ehrlich pathway (El-Dalatony, Saha, Govindwar, Abou-Shanab, & Jeon, 2019). However, their content decreased significantly ($p < 0.05$) at 12 d of fermentation, mainly due to esterification. It is suggested that higher alcohols, such as phenylethanol, can be the main bitter substance in jujube wine. High-voltage pulsed electric field pretreatment of date pulp can significantly reduce the content of higher alcohols in the finished jujube wine enhancing floral and fruity flavors (Xu, Tang, Wen, Zeng, Brennan & Niu, 2019). Although *n*-hexanol, with an herbal and raw green odor, occupies a high proportion of the varietal aroma, it contributes insignificantly to the aroma of jujube fruit wine due to its high olfactory threshold (Oliva et al., 2015).

Esters are the main aromatic substances in jujube wine, mostly produced in the fermentation stage, and have a pleasantly fruity odor. The formation of esters is influenced by the physicochemical composition of the fermentation broth and the fermentation conditions. During the ethanol fermentation stage, catalyzed by esterases acyl-Coenzyme A (CoA) and ethanol produce fatty acid ethyl esters, and acetyl CoA and higher alcohols produce acetate esters (Tufariello, Capone, & Siciliano,

Table 1
Highly variable metabolites during the fermentation of jujube wine.

No.	Compounds	VIP score	Class
Volatile compounds			
A18	Methyl salicylate	1.78	Ester
D5	Benzaldehyde	1.74	Aldehyde
B19	Phenylethyl alcohol	1.74	Alcohol
C10	Heptanoic acid	1.72	Acid
A24	Benzenepropanoic acid ethyl ester	1.72	Ester
D1	Hexanal	1.71	Aldehyde
B11	1-Octen-3-ol	1.71	Alcohol
C14	Nonanoic acid	1.71	Acid
D9	Safranal	1.71	Aldehyde
D11	2,4-Dimethylbenzaldehyde	1.60	Aldehyde
A9	ethyl (Z)-oct-4-enoate	1.52	Ester
D3	1-Nonanal	1.50	Aldehyde
A27	Tetradecanoic acid ethyl ester	1.41	Ester
F2	2,2,3,4-Tetramethylphentane	1.33	Other
A21	Dodecanoic acid, ethyl ester	1.34	Ester
D2	Trans-2-hexenal	1.28	Aldehyde
C17	U-ecanoic acid	1.27	Acid
C7	Pentanoic acid	1.26	Acid
A10	Nonanoic acid ethyl ester	1.23	Ester
F11	(2,2-diethoxyethyl)benzene	1.21	Other

No.: Volatile compounds are numbered as in Table S2.

2012). With the extension of fermentation time, the number and content of VOCs peaked at 8 d (21 species, 2167.97 μg/L) and then decreased slightly thereafter, indicating that most esters were produced in the middle stage of the fermentation. As shown in Fig. 1B, S1, the ester content in jujube juice increased significantly ($p < 0.05$) after ethanol fermentation and was dominated by ethyl esters, such as decanoic acid ethyl ester, octanoic acid ethyl ester, benzoic acid ethyl ester, dodecanoic acid ethyl ester, and hexanoic acid ethyl ester. This is consistent with Xu et al. (2022) showing that ethyl decanoate, ethyl laurate, ethyl acetate, and ethyl octanoate were present in higher amounts in the alcoholic fermentation stage of coconut water vinegar, these esters were mainly formed by the metabolic activity of the yeast.

Acids are by-products of yeast fatty acid metabolism and have a cheese or cream flavor at low concentrations. Acids add complexity to fruit wine aromas, while their high levels can produce undesirable flavors such as acetic acid, rancidity, and irritation (Tufariello, Capone, & Siciliano, 2012). Capric acid is one of the main aroma components in fresh dates (Wang, Wang, Wang, Zheng, & Chen, 2019). In this study, 12 d jujube wine samples exhibited the highest variety and content of acid compounds (9, 508.11 μg/L) and the lowest were in the 10d wine sample (8 species, 83.98 μg/L ($p < 0.05$). Oxalic and caprylic acids were the most dominant acids in jujube wine, with fruity, cheesy, and fatty flavors, and were highest at the 12th (420.41 μg/L) and 4th d (77.90 μg/L), respectively (Fig. 1B, S1).

Carbonyl compounds, mainly aldehydes, ketones, and acetals, have relatively low OAVs but are important contributors to the fruit aroma of wine (Tufariello, Capone, & Siciliano, 2012). Hexanal and benzaldehyde were identified as typical aroma compounds in jujube wine (Wang, et al., 2019). In this study, hexanal and benzaldehyde were present only in 0, 2, and 4 d wine samples (Fig. 1B, S1), indicating that the retention of these aromas was not favored during the late stages of fermentation. Aldehydes and ketones are chemically unstable and are easily oxidized to carboxylic acids by microorganisms. 3-hydroxy-2-butanone produces milk flavor and is formed by the oxidation of 2,3-butanediol and α-acetolactic acid (Al-Dalali, Zheng, Sun, Rahman, & Chen, 2022). Its content decreased from 20.88 μg/L (2 d) to 4.42 μg/L (4 d) during fermentation.

In general, octanoic acid ethyl ester, decanoic acid ethyl ester, isopropyl alcohol, phenylethyl alcohol, and 3-methyl-1-butanol showed a significant upward trend at the beginning of fermentation (after 2 d of fermentation) (Fig. S1). These compounds can be used as indicator compounds for aroma changes in the pre-fermentation jujube wine. In addition, the loss of volatile compounds occurred mainly after the 10th d of fermentation; more than 32 % of volatile compounds decreased significantly at the 12th d of fermentation, mainly showing the reduction in alcohols (62 %) and aldehydes (51 %).

3.2. Key aroma components and OAVs during fermentation

To identify key volatile compounds and significant aroma changes during the fermentation of jujube wine, combined significance analysis

Table 2
Odor activity values (OAV ≥ 1) of volatile compounds during the fermentation of jujube wine.

Compounds	Threshold (μg/L)[1]	0d	2d	4d	6d	8d	10d	12d	Description[2]
Acetic acid, 2-phenylethyl ester	0.25	–	–	–	59.72	59.20	–	96.48	rose, flower
1-Butanol, 3-methyl-, acetate	30	–	0.31	1.04	1.05	0.92	0.55	0.22	banana
Hexanoic acid, ethyl ester	14	–	6.13	17.03	13.81	–	7.27	–	apple, strawberry
Octanoic acid, ethyl ester	240	0.02	0.62	2.81	2.79	3.20	3.01	0.09	fruity, sweet
Isopentyl hexanoate	1	–	5.47	4.61	3.00	4.49	2.89	10.53	apple, pineapple
Nonanoic acid, ethyl ester	0.85	–	9.27	16.39	12.55	13.66	22.34	8.98	grape
Benzoic acid, methyl ester	0.016	–	95.00	–	–	–	–	–	flower, fruity
Decanoic acid, ethyl ester	200	0.04	0.00	3.83	2.86	2.36	3.65	0.01	fatty, fruity
Octanoic acid, 3-methylbutyl ester	0.125	–	–	–	–	110.80	–	43.76	fruity, brandy
Benzoic acid, ethyl ester	60	–	2.49	3.65	4.04	3.42	2.63	10.99	flower, fruity
Methyl salicylate	0.071	30.99	21.27	8.59	–	–	–	–	pine tree
Dodecanoic acid, ethyl ester	83	0.02	0.03	0.95	2.69	2.57	2.66	0.11	flower, fruity
Pentadecanoic acid, 3-methylbutyl ester	1	–	–	–	–	20.13	17.81	2.76	rose
3-Methyl-1-butanol	300	–	0.70	1.40	1.89	2.35	1.41	0.01	apple brandy, spicy
1-Octen-3-ol	0.02	304.00	265.50	–	–	–	–	–	mushroom, fresh
1-Heptanol	200	–	–	–	–	–	–	2.83	grape
Citronellol	0.1	–	–	–	5.80	–	–	–	rose
9-Decenoic acid	4	–	–	–	–	1.89	1.04	–	fatty
Heptanoic acid	0.28	38.00	21.25	10.82	4.50	3.25	–	1.93	fatty
Hexanal	0.1	315.10	233.90	–	–	–	–	–	green, apple
Trans-2-hexenal	0.284	111.90	–	–	–	–	–	–	apple
Decanal	1	5.66	4.71	8.59	–	–	3.53	–	citrus, lemon oil
6-Methyl-5-hepten-2-one	1	4.38	–	1.45	–	–	–	–	citrus, lemon oil
2-Methoxy-4-vinylphenol	0.4	–	–	–	3.80	2.88	5.28	7.68	fruity
Indole	0.33	–	1.94	–	–	–	–	–	malt

-: Cannot be calculated due to lack of concentration.
[1] Odor thresholds in alcohol as cited from the literature (van Gemert, 2011; Xu et al., 2022).
[2] Odor description taken from previous work (Sun et al., 2023; Feng et al. 2018; Cuevas et al., 2017).

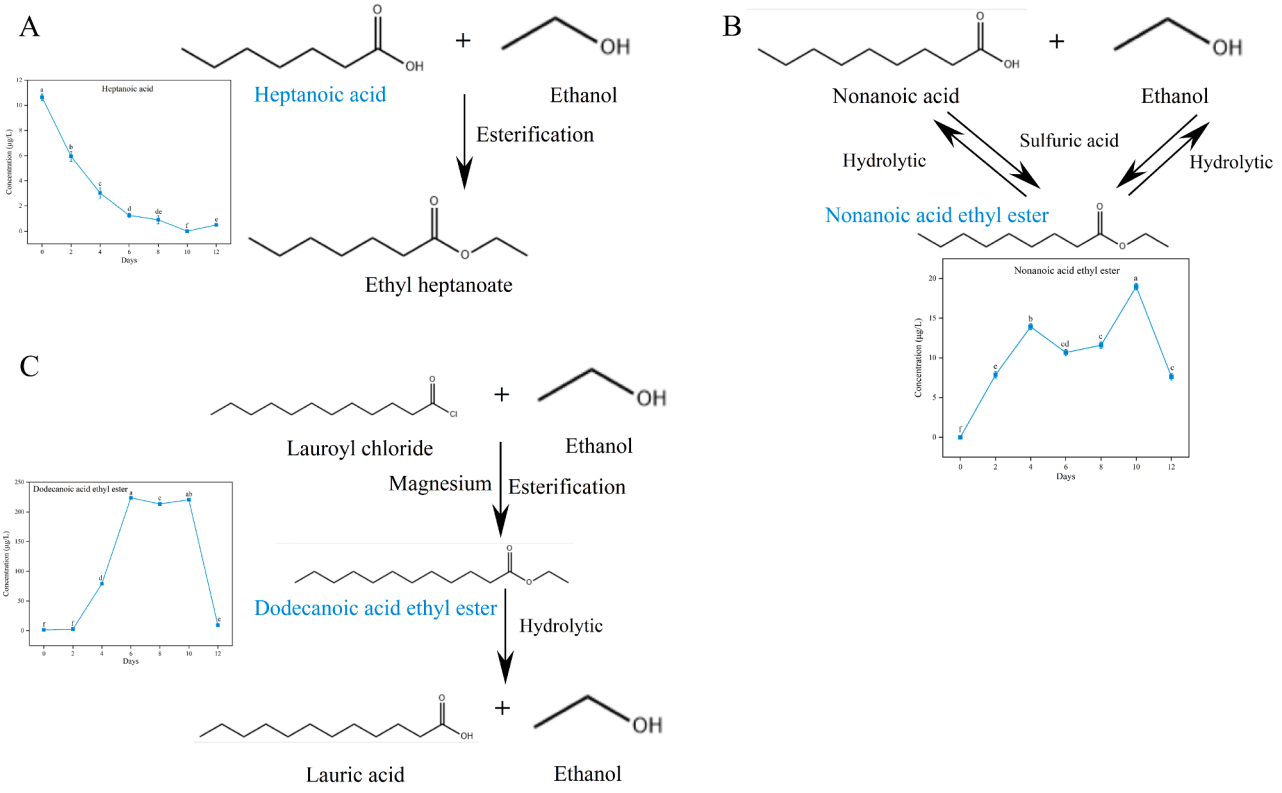

Fig. 2. Changes in the content of highly variable volatile compounds and their reaction pathways. Concentrations are the mean ± SD of three replicate trials; significant differences were found for different letter metabolisms ($p < 0.05$).

and VIP values to determine the magnitude of changes in each volatile component along with OAV tests. Based on the criteria VIP > 1.2 and $p < 0.05$, a total of 20 compounds with significant changes were identified, including 6 esters, 6 aldehydes, 4 acids, 2 alcohols, and 2 other substances (Table 1). Although the reported odor thresholds may vary between different studies, the horizontal comparison of OAVs in different fermentation stages of jujube wine had a positive impact on the characteristic flavor of the final product. According to past literature and GC–MS results, floral and fruity aromas are typical of jujube wine. There are around 25 key aroma compounds (OAV > 1), mainly including fruity esters (Table 2).

Additionally, 6 volatile compounds were identified: hexanal (grassy, apple flavor), 1-octen-3-ol (mushroom, earthy flavor), safranal, *trans*-2-hexenal (apple flavor), undecanoic acid and pentanoic acid (cream, cheese). These contributed to the aroma of pre-fermentation jujube wine with OAV > 1 and VIP > 1.2 (Table 2). Among them, hexanal is the key compound that provides the green and apple flavors to the pre-fermentation jujube wine.

The remaining 14 volatile compounds (Table 1), namely methyl salicylate (OAV > 1), benzaldehyde, phenylethyl alcohol, heptanoic acid (OAV > 1), benzenepropanoic acid ethyl ester, nonanoic acid, 2,4-dimethylbenzaldehyde, ethyl (Z)-oct-4-enoate, 1-nonanal, tetradecanoic acid ethyl ester, 2,2,3,4-tetramethylpentane, dodecanoic acid ethyl ester (OAV > 1), nonanoic acid ethyl ester (OAV > 1) and (2,2-diethoxyethyl) benzene had an OAV value of > 0.1 and some even > 1 for at least one fermentation stage. These contributed to the pleasant and complex aromatic flavor of jujube wine (Table 2). The increase in phenethyl alcohol during fermentation can be attributed to amino acid catabolism and sugar metabolism (Styger, Jacobson, Prior, & Bauer, 2013). Heptanoic acid, which provides a rancid fatty taste, showed a decreasing trend from 10.64 to 0.91 μg/L after the 2nd d of fermentation, and then a slight increase (0.50 μg/L) at 12th d of fermentation (Fig. 2A). This may be due to the esterification of heptanoic acid forming ethyl heptanoate in lower amounts, reducing the undesirable odors in jujube wine. In contrast, ethyl nonanoate (grape flavor) and ethyl laurate (floral and fruity aroma) showed the opposite trend. They first increased from 0 to 10 d and then decreased by 96 % after the 10th d of fermentation (Fig. 2B, C). This decrease can be attributed to the hydrolysis of esters, producing corresponding acids and alcohols (Zhu et al., 2020). Combined with the changes in the 20 highly variable volatile compounds, the significant decreases in hexanal, 1-octen-3-ol, and nonanoic acid in the pre-fermentation stage decreased the fruity flavor of jujube wine (Table S1). It has been found that the floral and fruity flavors in rose wine accompany the increase in the contents of esters and volatile substances, mainly isoamyl caprylate and diethyl succinate (Li, Wang, Xu, Li, & Tao, 2020). In this study, floral and fruity aromas (such as rose, green apple, strawberry, and grape) in the jujube wine increased with an increase in esters and alcohols. This rise of esters and alcohols during fermentation facilitated the aroma shift from grassy, mushroom, and earthy to floral and fruity aromas in the jujube wine. After the 10th d of fermentation, the content of most substances such as phenylethyl alcohol, heptanoic acid, dodecanoic acid, benzenepropanoic acid ethyl ester, ethyl ester, and nonanoic acid ethyl ester decreased, while the content of ethyl (Z)-oct-4-enoate and tetradecanoic acid ethyl ester increased. Such changes may have reduced the fatty aromas and increased the apple and coconut aromas in the jujube wine.

3.3. Changes in non-volatile compounds during fermentation

The nutritional value of food depends on its trace substances with physiological activity. Therefore, to understand the effect of fermentation on the nutritional value of jujube wine determined key metabolites by UPLC-MS non-target metabonomics and comprehensively investigated the dynamic changes in nonvolatile metabolites. In total, 1758 non-volatile metabolites were detected and classified into 11 Superclasses, 92 Classes, and 151 subclasses (Fig. 3A). At the Superclass level,

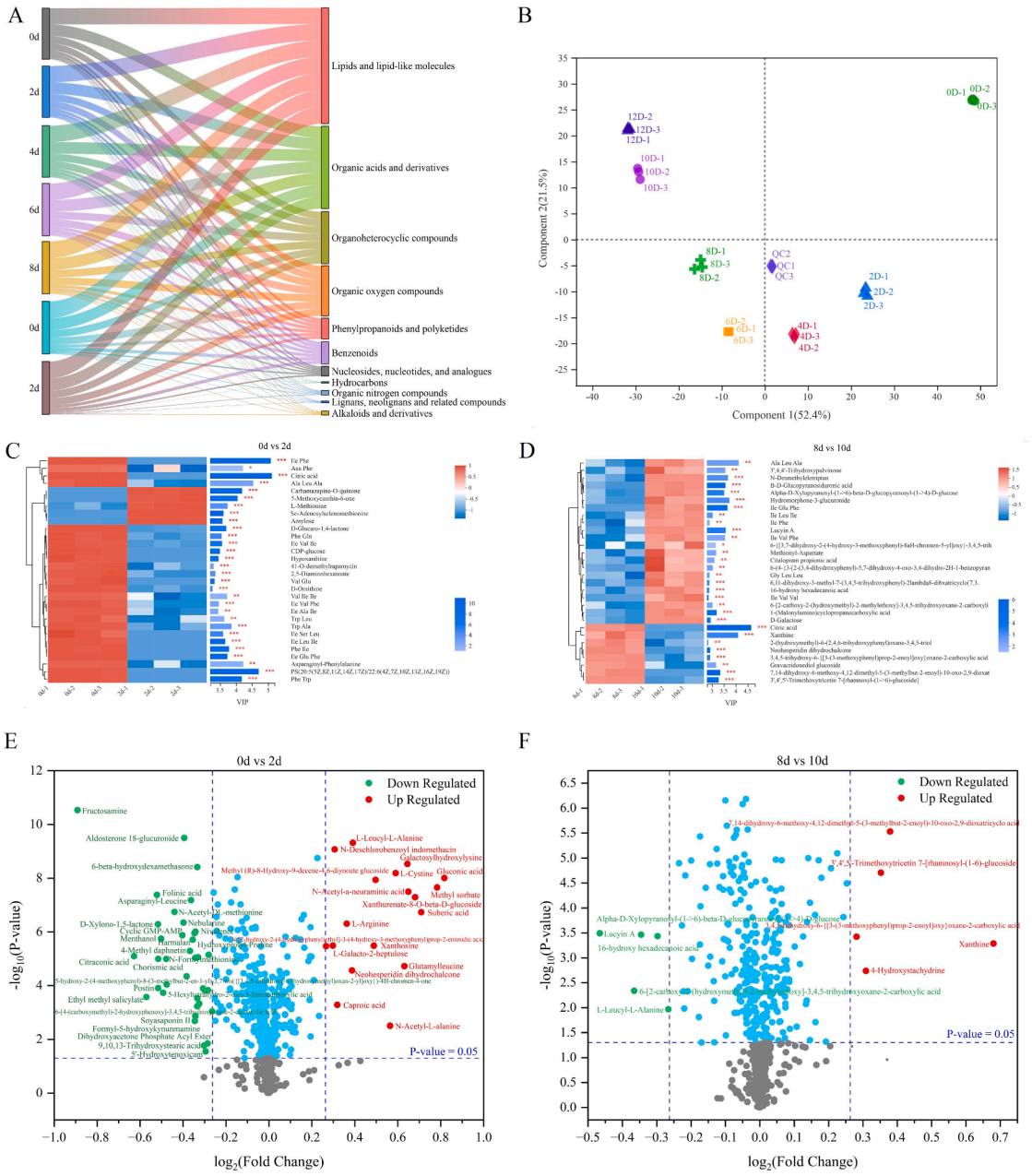

Fig. 3. A sankey diagram of the dynamics of non-volatile metabolites in jujube wine based on HMDB super class (A); partial least squares analysis of changes in non-volatile metabolites during fermentation (B); 0d vs 2d expression profiles of important non-volatile metabolites (VIP > 2) with VIP (C); 8d vs 10d expression profiles of important non-volatile metabolites (VIP > 2) with VIP (D); volcanogram of non-volatile metabolites in the fermentation of steamed jujube wine (E) and (F).

the non-volatile metabolite classes with an average relative content > 10 % were lipids, while the others were lipid-like molecules (31.78 %), organic acids and derivatives (22.61 %), organoheterocyclic compounds (14.28 %), and organic oxygen compounds (13.79 %). A supervised PLS-DA was used to model the relationship between the content of non-volatile metabolite and fermentation stages to identify the variables causing such changes. "R^2X" refers to the model's explanatory rate for the X variable; "R^2Y" for the Y variable; and "Q^{2}" for the model's predictive power (Chen et al., 2022). In this study, the values of R^2X (0.524), R^2Y (0.215), and Q^2 (0.89 > 0.5) indicate that the model is robust and reliable without overfitting. As shown in Fig. 3B, based on fermentation stages, the non-volatile metabolites were divided into three stages: i) 0 d for the pre-fermentation period, ii) 2, 4, 6, and 8 d for the mid-fermentation period, and iii) 10 and 12d for the post-fermentation period. The results show that the fermentation process significantly affected the amount of non-volatile compounds ($p < 0.05$) and differed from the changes in volatile metabolites (Fig. 1A). 2nd and 10th d were considered as the two cut-off points for significant changes in non-volatile metabolites.

The flavor contribution of the content of a non-volatile metabolite to the corresponding fermentation stage was evaluated using the OPLS-DA model based on VIP values. In total, 52 significant non-volatile metabolites were screened based on the threshold VIP > 2. Their contents significantly differed ($p < 0.05$) between the pre-, mid-, and post-fermentation stages (Fig. 3C, D). Among the 52 compounds, 11 non-volatile metabolites had VIP > 4. Citric acid, carbamazepine-o-quinone, 5-methoxycanthin-6-one, and asparaginyl-phenylalanine were the high VIP representative metabolites in the 0 d vs 2 d and

citric acid and xanthine in 8 d vs 10 d of fermentation.

Differential metabolites in different fermentation stages of jujube wine were screened using volcano plots with the criteria of $p < 0.05$ and fold change (FC) ≥ 1.2 (Fig. 3E, F). The number of differential metabolites between two adjacent cut-off points was 18 (37) and 5 (5), respectively; the number of compounds outside parenthesis (18 and 5) were upregulated and those in parenthesis (37 and 5) were downregulated. The differential metabolites were mainly amino acids and peptides, sugars and glycosides, and organic acids and lipids. The upregulated metabolites in 0 d vs 2 d were l-leucyl-l-alanine, *n*-deschlorobenzoyl indomethacin, and gluconic acid and the downregulated metabolites were fructosamine, d-xylono-1,5-lactone, and ethyl methyl salicylate, etc. (Fig. 3E); the upregulated metabolites in 8 d vs 10 d were 3,4,5-trihydroxy-6-{[3-(3-methoxyphenyl) prop-2-enoyl] oxy} oxane-2-carboxylic acid, xanthine and 4- hydroxystachydrine, etc., and the downregulated metabolites were lucyin A, alpha-d-xylopyranosyl-(1->6)-beta-d-glucopyranosyl-(1->4)-d-glucose and l-leucyl-l-alanine, etc. (Fig. 3F). Changes in these metabolites were mainly due to amino acid metabolism, carbohydrate metabolism, citric acid cycle (TCA cycle), and lipid metabolism. Notably, most organic compounds increased significantly during 0-2d and were at significantly higher levels than at 8–10 d ($p < 0.05$). This suggested that the most significant changes occurred in the pre-fermentation stage rather than in the post-fermentation stage.

3.4. Key dynamic differences in non-volatile metabolites at different fermentation stages of jujube wine

3.4.1. Amino acids, peptides, and analogs

Amino acids are not only important in the regulation of human health but also contribute to the flavor and taste of fruit wine; they are important precursors of flavor substances such as esters, higher alcohols, and pyrazines (Liu, et al., 2018). Amino acids in jujube are decomposed into free amino acids after microbial metabolism and then deaminated to form keto acids, further producing carbonyl compounds, higher alcohols, and other flavor substances (Yin, et al., 2017). Table S2 shows that jujube wine is rich in amino acids and has a high content of peptides. The contents of eight amino acids, peptides, and analogs differed significantly ($p < 0.05$) between the pre and post-fermentation stages of jujube wine: L-leucyl-l-alanine, L-cystine, L-arginine, leucine, and *N*-acetyl-l-alanine were significantly enriched in the pre-fermentation stage, while xanthine, L-phenylalanine, and 4-Hydroxystachydrine were significantly enriched in the post-fermentation stage. Amino acid degradation is one of the important metabolic pathways for the synthesis of higher alcohols during fruit wine brewing. Leucine and phenylalanine are the precursors for the formation of isoamyl alcohol and 2-phenyl ethanol, respectively. Phenylalanine is oxidized to L-tyrosine by hydroxylase in the human body and the generated L-tyrosine, phenylalanine synthesized hormones and neurotransmitters participate in fat and glucose metabolism. L-arginine through the arginine deiminase pathway produces ethyl carbamate, which is a common by-product in alcoholic beverages and a potentially hazardous ingredient in terms of food quality and safety. Ethyl carbamate is removed either by adding acid urease to the fermentation broth or using resin adsorbent materials (Bu et al., 2020). In addition, L-valine, L-lysine, and phenylalanine have been associated with bitterness. Their levels decreased significantly during fermentation ($p < 0.05$). Overall, leucine and phenylalanine produced by yeast metabolism may have potential health benefits for humans while enriching the flavor and reducing the bitterness of fruit wines.

3.4.2. Organic acids and derivatives

Organic acids are an important part of fruit wines and the right number of organic acids impart a fresh and refreshing flavor. Also, they accelerate the conversion of polysaccharides and the decomposition of pectin substances, promoting the aging and clarification of fruit wines (Yabaya, Bobai, & Adebayo, 2016). Yeast fatty acid metabolism and the TCA cycle change the organic acid content during fermentation. The lactic acid in jujube wine is mainly derived from alcoholic and malic-lactic fermentation, which produce soft acidity increasing wine stability against deterioration (Williamson, 2016). Succinic acid, a common metabolite formed during yeast fermentation (Table S2), is one of the most important by-products of sugar-to-alcohol conversion. Succinic acid is formed from malic acid under anaerobic conditions or from the breakdown of some amino acids. The acidity of the fermentation broth affects the content of succinic acid, which not only improves wine acidity but also contributes to wine flavor by forming esters (Chidi, Bauer, & Rossouw, 2018). Moreover, succinic, citric, and fumaric acids are also important substrates in the TCA cycle, promoting lactic acid decomposition and relieving fatigue along with anti-aging, appetite improvement, and disease-prevention properties (Chidi et al., 2018).

3.4.3. Carbohydrates and carbohydrate conjugates

Sugar contents fluctuated during the fermentation of jujube wine due to the hydrolysis of fructose and sucrose into monosaccharides, which then entered the TCA cycle through the glycolytic pathway to produce organic acids and other compounds (Klosowski, Mikulski, Macko, Miklaszewska, Kotarska, & Czupryński, 2015). Also, sugars are important precursors for the formation of aromatic amino acids and phenolics (Wattanakul et al., 2020) and provide energy for microbial growth. In this study, sucrose, D-mannose, cottonseed, and glycerol were identified as the main carbohydrates during the fermentation of jujube wine. Among them, the sucrose content first increased and then decreased (Fig. S2), which is consistent with Jiang, Mu, Wei, Mu, & Zhao (2020). The decrease in sucrose content was due to its breakdown into glucose and fructose by yeast extracellular converting enzymes. During the fermentation of jujube wine, the contents of stachyose and D-mannose first increased and then decreased slightly. D- mannose can be used as an index of wine quality (Wattanakul et al., 2020). In late-stage fermentation, the sugar content decreased due to the conversion of hexokinase and adenosine triphosphate into D-mannose-6-phosphate by phosphorylation.

Cottonseed sugar is produced via the galactose metabolic pathway, which is then converted to glucose and fructose in the presence of convertase. Interestingly, sugar alcohols such as glycerol were also detected in this work. Glycerol is an important by-product of alcoholic fermentation by brewer's yeast and is thought to neutralize ethanol-induced spiciness, balancing acidity and improving the fullness of wine taste (Liu, Laaksonen, Kortesniemi, Kalpio, & Yang, 2018). The glycerol content increased in the later stages of fermentation due to the reduction of dihydroxyacetone phosphate to glycerol 3-phosphate by 3-phosphoglycerol dehydrogenase, which is converted to glycerol in the presence of 3-phosphoglycerol esterase. Notably, isomaltose content showed obvious differences in the different fermentation stages of jujube wine. Isomaltose is converted to glucose by maltase, which allows glycogen to enter the glycolytic metabolic pathway.

The sweetness of jujube wine is mainly due to its sugar content, and the wine retains most of the original nutrients of the jujube fruit. Though the type and content of carbohydrates may vary, they in total make up the sweetness of jujube wine. During the brewing process, some new ingredients different from red dates were produced in jujube wine. Also, the content of most nutritional components increased. The sugar in jujube fruit is not only a source of alcohol but also a prerequisite for the production of some other nutrients. The yeast consumes sugar to produce ethanol, CO_2, and various by-products. During the sugar fermentation process, glycerol, butylene glycol, inositol, sorbitol, and other alcohols are produced, increasing the sweetness of jujube wine. Meanwhile, the resulting alcohols and acids produce important esters contributing to the aroma of jujube wine. Therefore, appropriate carbohydrates as starting substrates or those produced during fermentation are essential components that can be tailored to improve the quality and nutritional value of jujube wine.

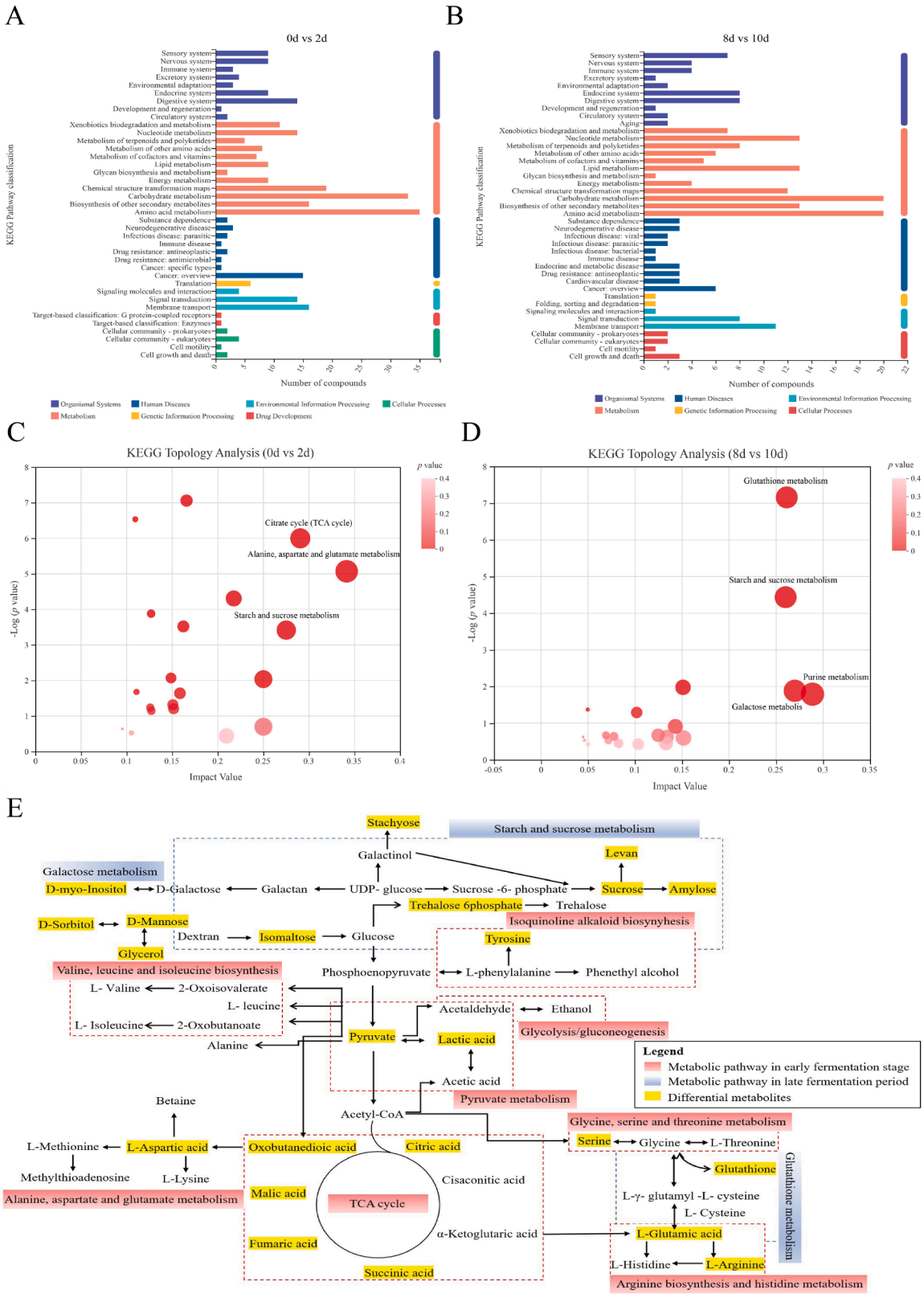

Fig. 4. KEGG metabolite statistics (A) and (B); KEGG topology analysis (C) and (D); KEGG-based pathway-module relationships for key differential metabolites of steamed jujube wine quality. Framed substances are differential metabolites in the metabolic pathway and unframed substances are intermediate metabolites in the metabolic pathway (E).

3.4.4. Polyphenols

Polyphenols, which are important secondary metabolites, mostly originate from jujube raw materials and have various physiological functions such as anti-cancer and antioxidant. In this study, 19 phenols, 12 flavonoids, 3 stilbenes, and 1 tannin were detected (Fig. S3). However, flavonoids, astragals, and tannins showed no significant changes in their content during fermentation ($p > 0.05$), while phenolic acid content increased from 97.02 (0 d) to 117.70 (12th d) μg/L. The increase mainly occurred after the 2nd d of fermentation, which can be related to the increase in phenylalanine aminolytic enzyme activity in the middle and late stages of fermentation (Yan et al., 2019). Overall, the fermentation of jujube wine improved its bioavailability and functional properties.

3.5. Key metabolic pathways analysis

Fermentation is a very complex metabolic process involving the formation of bioactive components and flavor substances, which cannot be analyzed only in terms of the types and contents of such substances. Pathway analysis can identify key metabolites and reveal substance regulation mechanisms at the metabolic level. Here, this research explored key metabolic pathways related to differential metabolites in different fermentation stages of jujube wine. In total, 39 key metabolic pathways were identified at 0 d vs 2 d and 41 pathways at 8 d vs 10 d by comparing results in the KEGG database (Fig. 4A, B). The metabolic pathways with impact values > 0.25 and |log10(*p*-value) scores| >4 at 0 d vs 2 d included alanine, aspartate, and glutamate metabolism and citrate cycle (TCA cycle) (Fig. 4C); for 8 d vs10 d, glutathione, starch, and sucrose metabolism were the key metabolic pathways (Fig. 4D). These results indicated that yeast played an important role in the formation of flavor and active substances mainly through alanine, aspartate, glutamate metabolism, and TCA cycle at the early stage of fermentation, while at the later stage of fermentation, glutathione starch, and sucrose metabolism played an important role.

The key metabolic pathways affecting the contents of different metabolites and transformation relationships during fermentation are shown in Fig. 4E. In the saccharification stage, jujube polysaccharide was first decomposed into glucose by glycolysis, and then pyruvic acid and L-phenylalanine were synthesized enzymatically. The TCA cycle is the connecting hub for sugar, amino acid, and lipid metabolism. Organic acids like oxaloacetate enter the TCA cycle via acetyl CoA to form citric acid in the pre-fermentation stage. In addition, succinic and malic acids are produced through the TCA cycle and then converted to arginine and aspartic acids. In contrast, under anaerobic conditions, lactic acid is catalyzed by lactate dehydrogenase to produce pyruvic acid, which is then converted into isobutanol, 2,3 butanediol, and other flavor substances. Aspartic acid, an important metabolite produced by the TCA cycle, forms oxaloacetate through the metabolic pathways of alanine, aspartic acid, and glutamic acids, and participates in the synthesis of various other metabolites, such as 2- methyl −1- butanol, glycine, valine, and leucine. In addition, pyruvic and L-aspartic acids also participate in multiple metabolic pathways. Moreover, the change in types and contents of key metabolites was consistent with the changes in their relative abundances.

4. Conclusion

In this study, flavor and metabolomics analyses were used to comprehensively investigate the quality of jujube wine during fermentation. The study revealed that ethyl caprylate, ethyl decanoate, and isopropanol were the indicator compounds for the aroma changes in jujube wine during the pre-fermentation period. More than 32 % of volatile compounds were significantly reduced by the 12th day of fermentation. This reduction was mainly in alcohols (62 %) and aldehydes (51 %). OAV analysis showed that the grassy, mushroomy, and earthy aroma of the initial broth shifted to a floral and fruity aroma as fermentation proceeded. Amino acids and organic acids were the main non-targeted differential metabolites at different stages of jujube wine fermentation. Especially noteworthy are leucine and phenylalanine, which enhance the nutritional value of the wine. They also enrich the flavor and reduce the bitterness of the fruit wine. The KEGG pathway enrichment indicated that the metabolic pathways primarily involved alanine and aspartate in the early stages of fermentation. In contrast, the metabolism of glutathione, starch, and sucrose played a significant role in the later stages. Pyruvate and L-aspartate were identified as important metabolites. Additionally, the phenolic content increased significantly as fermentation time extended, indicating improved antioxidant properties in jujube wine. This research provides insight into the key compounds of jujube wine at different fermentation stages, which can aid in enhancing the flavor and nutritional value of jujube wine.

CRediT authorship contribution statement

Xinxin Zhao: Data curation, Formal analysis, Software, Visualization, Writing – original draft, Writing – review & editing. **Zhouping Wang:** Investigation, Methodology. **Fengxian Tang:** Methodology, Project administration. **Wenchao Cai:** Data curation. **Bo Peng:** Methodology, Validation. **Chunhui Shan:** Conceptualization, Funding acquisition, Supervision.

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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.

Data availability

No data was used for the research described in the article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101115.

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