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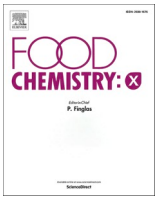

Microbial metabolism during the thermophilic phase promotes the generation of aroma substances in *nongxiangxing Daqu*

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ABSTRACT

The thermophilic phase of *Daqu* fermentation is considered the key period for aroma production in *Daqu*, but little is known about the changes in substances during this phase. In this study, we combined a metabolomics approach with high-throughput sequencing to analyze the metabolic profiles and identify metabolism-associated microbes during the thermophilic phase of *Daqu* fermentation. The results revealed that the metabolic sets after 5 and 9 days of fermentation in the thermophilic phase were similar, and several amino acid and biosynthesis-related metabolic pathways were significantly enriched. In addition, pyrazines and alkanes increased and esters decreased significantly after the thermophilic phase. The metabolism of substances during the thermophilic phase involved 38 genera, and the main metabolic pathways involved were glycolysis, TCA cycle, butyric acid metabolism, and five amino acid metabolic pathways. In summary, this study points in the direction for unravelling the mechanism of aroma production in *Daqu*.

1. Introduction

Jiuqu (a saccharification fermentation agent) is the soul of Chinese *Baijiu*, and the quality of *Jiuqu* determines the quality and yield of *Baijiu*. *Daqu* (a saccharification fermentation agent shaped like a brick) is formed by brewing wheat and other raw materials through grain wetting, crushing, press molding, and fermenting in a *Qu* room (a cultivation chamber) for approximately 30 days and then storing the product in another *Qu* room for 3–6 months. The core process of *Daqu* production is fermentation in the *Qu* room. In the *Daqu* fermentation process, workers manage the parameters such as temperature and humidity in the *Qu room* by turning the *Daqu*, opening and closing the doors and windows, etc., and then regulate the growth of *Daqu* microorganisms, which makes its community directional succession and forms a mature *Daqu* with a more stable the microbial profiles, the substance profiles, the enzyme profiles at the end of fermentation.

According to the rules of *Daqu* temperature variation, the *Qu* room fermentation period is subdivided into three stages: slowly increasing the temperature (heating phase, 30–40℃), maintaining the peak temperature (thermophilic phase, 50–60℃), and gradually decreasing the temperature (cooling phase, 40–45 °C) (Xia, Luo, Wu, & Zhang, 2023). Large quantities of yeast and mold, lactic acid bacteria, and other microbes grow during the heating phase. These microorganisms secrete hydrolytic enzymes, which degrade large molecules, such as starch and protein; this breakdown leads to the accumulation of a wealth of intermediate metabolites for the subsequent fermentation (Xiao et al., 2017; M. Zhu et al., 2022). In the thermophilic phase, high temperatures help enrich Bacillales and thermophilic fungi. The action of microorganisms during this period contributes to the continued degradation of macromolecules and, more importantly, the conversion of intermediate metabolites into flavor substances and their precursors (C. Zhu, Cheng, Zuo, Huang, & Wang, 2022). During the cooling phase, the dominant species are similar to those in the thermophilic phase, but as the temperature of the *Daqu* decreases, some *Lactobacillus* and yeast are re-enriched. The continued action of these microorganisms results in the gradual formation of the *Daqu* flavor profile (Ma et al., 2021; Tang, Rao,

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Zou, Liao, Huang, & Luo, 2023). Therefore, the thermophilic phase is the key stage in *Daqu* fermentation, and the temperature and duration of the *Daqu* fermentation affect the quality of the final product. *Daqu* can be divided into three categories depending on the maximum temperature of the *Daqu* blocks in the thermophilic phase: high-temperature *Daqu* (60–65 °C), medium-temperature *Daqu* (50–60 °C), and low-temperature *Daqu* (45–50 °C). These are the saccharification/fermentation agents for the three main aroma types of Chinese *Baijiu*. The thermophilic phase is recognized as a critical period for the conversion of raw materials into aroma-producing substances (Shen, 1998). However, our understanding of the metabolic characteristics of substances in the thermophilic phase remains limited.

The open production environment allows for diverse microbial communities in *Daqu*, each of which has unique functions. (Cai et al., 2021) used functional prediction to investigate the microbial functions of different types of low-temperature *Daqu*; they found that amino acid transport and metabolism were enriched in *Houhuoqu* (47–49 °C), whereas carbohydrate transport and metabolism were enriched in *Hongxinqu* (45–47 °C). (Ma et al., 2022) combined functional prediction with enrichment analysis and observed differences in amino acid metabolism, fatty acid metabolism, and carbon metabolism between different types of *Daqu* from different production areas. Through the PICRUSt2 technique, (Tang, Chen, Chen, Li, Huang, & Luo, 2022) found that *Daqu* bacterial communities with fermentation abilities (the fermentation functional bacterial microbiota) showed high levels of product synthesis, whereas the bacterial communities with alcohol production abilities (the alcohol functional bacterial microbiota) showed high levels of raw material utilization and self-metabolism synthesis function. These studies have strengthened our understanding of the functions of the microbial communities in *Daqu*. However, the level and type of microbial gene expression are closely related to habitat, so there is still a gap between the functions annotated at the gene level and those actually expressed by microorganisms. In addition, *Daqu* microbial ecosystems often comprise hundreds of species; these coexisting microorganisms may perform the same metabolic functions despite their different taxonomic statuses, a phenomenon known as functional redundancy. The high prevalence of functional redundancy in microorganism ecosystems presents a challenge to the identification of microorganisms that actually perform a specific function during *Daqu* fermentation (Louca et al., 2018).

Metabolomics can provide information about genetic information flow end, and combined with multivariate statistical analyses, this technique can provide insight into the true functions of microbial communities (Kang, Xue, Chen, & Han, 2022). (He, Jin, Zhou, Zhao, Zheng, & Wu, 2022) studied *Daqu* through a combination of high-throughput sequencing and metabolomics and found a significant correlation between the synthesis of most esters and fungal communities. (Yang et al., 2021) studied *Daqu* with metagenome analysis combined with HS-SPME-GC–MS and found that three species of fungi were the major taxa expressing genes for various enzymes involved in flavor-related pathways. These studies have enhanced our understanding of the metabolic functions of the *Daqu* microbial community. However, little is known about the metabolic functions of microbial communities during the thermophilic phase specifically.

In this study, after the heating phase, *Daqu* was fermented at a high temperature (thermophilic phase) for 5 days or 9 days. We used metabolomics to analyze the metabolic profiles of *Daqu* during the thermophilic phase and assess the effects of the duration of high-temperature exposure on the metabolic profiles of *Daqu* microbial communities. In addition, we identified the key species involved in the metabolism of substances during the thermophilic phase through correlation analysis and performed metabolic mapping of the microbial communities with the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. This study points in the direction for unravelling the mechanism of aroma production in *Daqu*.

2. Materials and methods

2.1. Experimental design

After the heating phase, *Daqu* was transferred to an extendable test chamber with a controlled *Daqu* block temperature of 60°C for the thermophilic phase. Samples were taken on days 0, 5, and 9 of the thermophilic phase (days 7, 12, and 16 of fermentation, respectively). At each time point, three *Daqu* blocks were sampled and crushed, and 50 g of *Daqu* was placed into sterile bags. The samples were stored at − 80℃ for subsequent DNA extraction and compound analysis.

2.2. DNA extraction, PCR amplification, and high-throughput sequencing

The DNA in the *Daqu* samples was extracted through a modified cetyltrimethylammonium bromide extraction method (Ni, Xu, Dou, Xu, & Xu, 2010). To ensure the quality of the DNA, a fluorescence spectrophotometer (Quantifluor-ST fluorometer, Promega, E6090) was used to determine the DNA concentration and purity. The bacterial V3–V4 region was amplified by the universal primer pair 338F/806R, and the fungal ITS region was amplified by the ITS5F and ITS1R primers. Polymerase chain reaction (PCR) products were recovered on 2 % agarose gel. A NEXTFLEX Rapid DNA-Seq Kit (Bioo Scientific, USA) was used for library construction. Sequencing was performed on Illumina's Miseq PE300 platform.

2.3. Sequence data processing

Fastp software (v0.20.0), FLASH software (v1.2.7), and UPARSE software (v 7.1) were used for sequence quality control, sequence splicing, and sequence rejection of chimeras, respectively (Chen, Zhou, Chen, & Gu, 2018; Edgar, 2013; Magoc & Salzberg, 2011). According to the Sliva 138 and UNITE 8.0 databases, the classification confidence was set to 0.7, and the amplicon sequence variants (ASVs) were annotated for substances using the Naive Bayes classifier in QIIME2 (Bolyen et al., 2019).

2.4. Determination of volatile organic compounds

The volatile organic compounds (VOCs) in the *Daqu* samples were extracted by headspace solid-phase microextraction (HS-SPME) and separated and identified by gas chromatography–mass spectrometry (GC–MS) (7890B GC, 5977A MS, Agilent Technologies Inc., Santa Clara, California, USA). The following procedure was used: 3 g of the *Daqu* sample was weighed and placed in a headspace vial, and 10 μL of *n*-pentyl acetate (0.12 mL/100 mL) was added as the internal standard. The vial was immediately placed in a heater and equilibrated at 60 °C for 10 min, and extraction was performed with divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) solid phase micro-extraction (SPME) fibers (2 cm, 50/30 μm, Supelco, Pennsylvania, USA) for 50 min at 60 °C. The compounds in the SPME fibers were resolved at the GC–MS inlet for 4 min at 250 °C. The heating procedure was as follows: maintaining 40℃ for 1 min, increasing to 180℃ at 5℃/min, increasing to 230℃ at 10℃/min, and maintaining 230℃ for 10 min. The VOCs were identified by comparing the detection mass spectra plots with the standard mass spectral library of the National Institute of Standards and Technology (NIST). The relative concentration of VOCs was calculated according to the ratio of the peak area of the internal standard to the peak area of the volatile metabolite.

2.5. Determination of non-volatile organic compounds

Ultra-high performance liquid chromatography tandem Fourier transform mass spectrometry (UHPLC Q Exactive HF-X system, Thermo Fisher Scientific Inc., Massachusetts, USA) was used to separate and identify the nonvolatile organic compounds (NVOCs) in the *Daqu*

samples. First, 50 mg of the *Daqu* sample was placed in a 2 mL centrifuge tube, and grinding beads and 400 μL of an extraction solution (methanol and water in a 4:1 (V: V) ratio, containing L-2-chlorophenylalanine as the internal standard) were added separately. The solution was ground for 6 min, and extraction was performed by low-temperature ultrasonication (5℃, 40 kHz) for 30 min. The sample was stored at − 20℃ for 30 min and then centrifuged for 15 min, and the supernatant was pipetted into an injection vial for analysis. The chromatographic conditions were as follows: mobile phase A was 95 % water and 5 % acetonitrile (containing 0.1 % formic acid); mobile phase B was 47.5 % acetonitrile, 47.5 % isopropanol, and 5 % water (containing 0.1 % formic acid); the flow rate was 0.40 mL/min; the column temperature was 40℃; and the injection volume was 3 μL. After the samples were ionized by electrospray, the mass spectrometry signals were collected in positive and negative ion scanning modes. The raw data were subjected to baseline filtering, peak identification, integration, retention time correction, and peak alignment with Progenesis QI (Waters Corporation, Milford, USA) software, and the compounds were identified by searching the Human Metabolome Database (HMDB) database. The relative quantification of each metabolite was performed by peak area normalization, and the ratio of peak area to total peak area was calculated for each metabolite and used for the subsequent analysis of each compound. The method has been supplemented with lines in the text.

2.6. Data analysis

Orthogonal partial least squares discriminant analysis (OPLS-DA) was performed using the ropls package (v1.6.2) in R (v4.1.0) to determine the differences in metabolic profiles during the thermophilic phase, and the variable importance in prediction (VIP) coefficient and Student's *t*-test p-value ($VIP > 1$, $p < 0.05$) were used to identify differential compounds in the samples at different time points. The Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https://www.kegg.jp/kegg/pathway.html) was used for metabolic pathway annotation of differential compounds to obtain the first and second metabolic pathways in which each compound was involved. The Python (v3.6.6) scipy.stats package (v1.6.0) was used for the pathway enrichment analysis of the differential compounds. A clustering heatmap analysis was performed using the pheatmap package (v 1.0.12) in R to determine the pattern of change of the differential metabolites. The igraph package (v3.6.1) in R was used to construct co-occurrence networks (Spearman algorithm, $R > 0.7$, $p < 0.05$) to determine the correlation between differential compounds and microbes.

3. Results and discussion

3.1. Species diversity of Daqu in the thermophilic phase

This study used high-throughput sequencing to investigate and understand the diversity of microbial communities in *Daqu* during the thermophilic phase (Fig. S1ab) and identified 141 bacterial genera and 103 fungal genera. A total of 39 genera of bacteria and 38 genera of fungi were shared at the three fermentation time points (day 7, day 12, and day 16); 33 bacterial genera were unique to day 7, 27 bacterial genera were unique to day 12, and 6 bacterial genera were unique to day 16. These findings indicate that the number of species substitutions declined during fermentation, and the community structure tended to stabilize. We identified 15 genera of endemic fungi on day 7, 17 genera of endemic fungi on day 12, and 12 genera of endemic fungi on day 16, suggesting that the number of intermediate substitutions was more balanced and that the community was in a state of dynamic change. According to the Chao1 index (Fig. S1cd), the community richness of the bacterial community increased and then decreased during the thermophilic phase, whereas the richness of the fungal community gradually increased. Similarly, the Shannon index indicated that the community diversity of the bacterial community increased and then decreased during the thermophilic phase, whereas the diversity of the fungal community steadily increased (Fig. S1ef). These results suggest that the bacterial community was more sensitive to temperature, whereas the fungal community was somewhat resistant to high temperatures; this could be because fungi have evolved mechanisms to cope with stressful environmental factors (Luláková, Perez-Mon, Šantrůčková, Ruethi, & Frey, 2019). For instance, it is established that fungi can maintain cellular structure and function in response to high temperatures stress through activation of heat shock proteins (Bui et al., 2016; Verghese, Abrams, Wang, & Morano, 2012).

3.2. Metabolic profile characteristics of Daqu in the thermophilic phase

3.2.1. Non-volatile organic compounds

Changes in the NVOCs of *Daqu* during the thermophilic phase were evaluated with LC–MS, and 1013 and 754 compounds were identified in the positive and negative ion modes, respectively (Table S1-S2). OPLS-DA was used to identify significant differences in NVOCs before and after the thermophilic phase. As shown in Fig. 1ab, significant differences were observed between day 7 and day 12 of fermentation in both positive ($R^2Y = 0.994$, $Q^2 = 0.951$) and negative ($R^2Y = 0.994$, $Q^2 = 0.96$) ion modes. Similarly, as shown in Fig. 1cd, significant differences were observed between day 7 and day 16 of fermentation in both positive ($R^2Y = 0.996$, $Q^2 = 0.955$) and negative ($R^2Y = 0.991$, $Q^2 = 0.942$) ion modes, which suggests that microbial metabolism significantly altered the metabolic profiles under temperature stress. Volcano plots were used to characterize the differences in the expression of NVOCs before and after the thermophilic phase. A total of 272 compounds were upregulated and 127 compounds were downregulated on day 12 compared with day 7 (Fig. 1e). In addition, a total of 274 compounds were upregulated and 174 compounds were downregulated on day 16 compared with day 7 (Fig. 1f), suggesting that a reasonable peak temperature and duration of fermentation contributes to the transformation of NVOCs.

Classification annotations were performed for 399 compounds in the 5-day thermophilic phase group (T5) (comparing day 12 with day 7 of fermentation) and 448 compounds in the 9-day thermophilic phase group (T9) (comparing day 16 with day 7 of fermentation). The classification criteria employed were the Sub Class (Amino acids, peptides, and analogues) of the HMDB structural types. The compounds in T5 comprised 84 classifications, and the compounds in T9 comprised 88 classifications. Fig. 2ab shows the top 20 compound classifications (those with ≥ 3 compounds annotated); amino acids, peptides, and their analogs and carbohydrates and carbohydrate conjugates were the classifications with the highest numbers of compounds annotated. Amino acids, peptides, and analogs accounted for 23.72 % and 27.07 % of the compound classifications for T5 and T9, respectively, and carbohydrates and carbohydrate conjugates accounted for 14.1 % and 11.11 % of the compound classifications for T5 and T9, respectively. Thus, the sum of these two classifications reached nearly 40 % in each group, which indicates that the most active metabolism-related pathways during the thermophilic phase were those related to amino acid and carbohydrate metabolism. Similarly, in a study on microbial functions utilizing metagenome sequencing, (J. Zhang et al., 2022) found that the amino acid and carbohydrate metabolism were the most important functions of both *Daqu* and wheat-*qu*.

Next, we performed KEGG functional pathway annotations for the differential compounds in the two groups. Fig. 2cd displays the seven first metabolic pathways annotated in both groups. The Metabolism section is the most annotated section of primary metabolic pathways for different compounds. Within the Metabolism section, there are 11 secondary metabolic pathways, specifically: amino acid metabolism, chemical structure transformation maps, biosynthesis of other secondary metabolites, carbohydrate metabolism, xenobiotics biodegradation and metabolism, metabolism of other amino acids, metabolism of cofactors and vitamins, lipid metabolism, nucleotide metabolism,

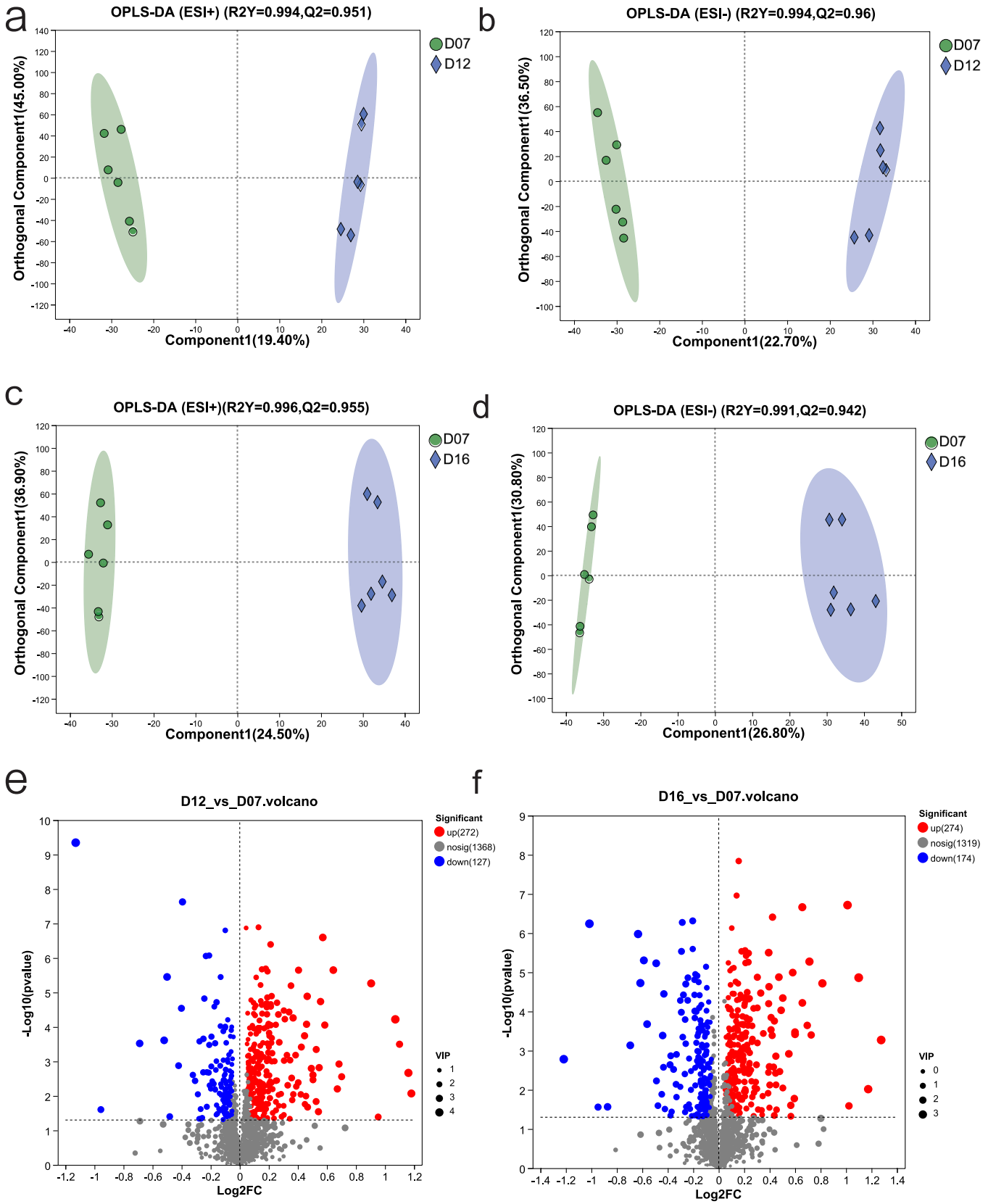

Fig. 1. Differential expression of NVOCs during the thermophilic phase. OPLS-DA on day 7 vs. day 12 of fermentation: positive ion mode (a) and negative ion mode (b); OPLS-DA analysis on day 7 vs. day 16 of fermentation: positive ion mode (c) and negative ion mode (d). Volcano plots of day 12 vs. day 7 (e) of fermentation and day 16 vs. day 7 (e) of fermentation. Red dots indicate significantly up-regulated compounds, blue dots indicate significantly down-regulated compounds, and grey dots represent compounds that were not significant between the two groups.

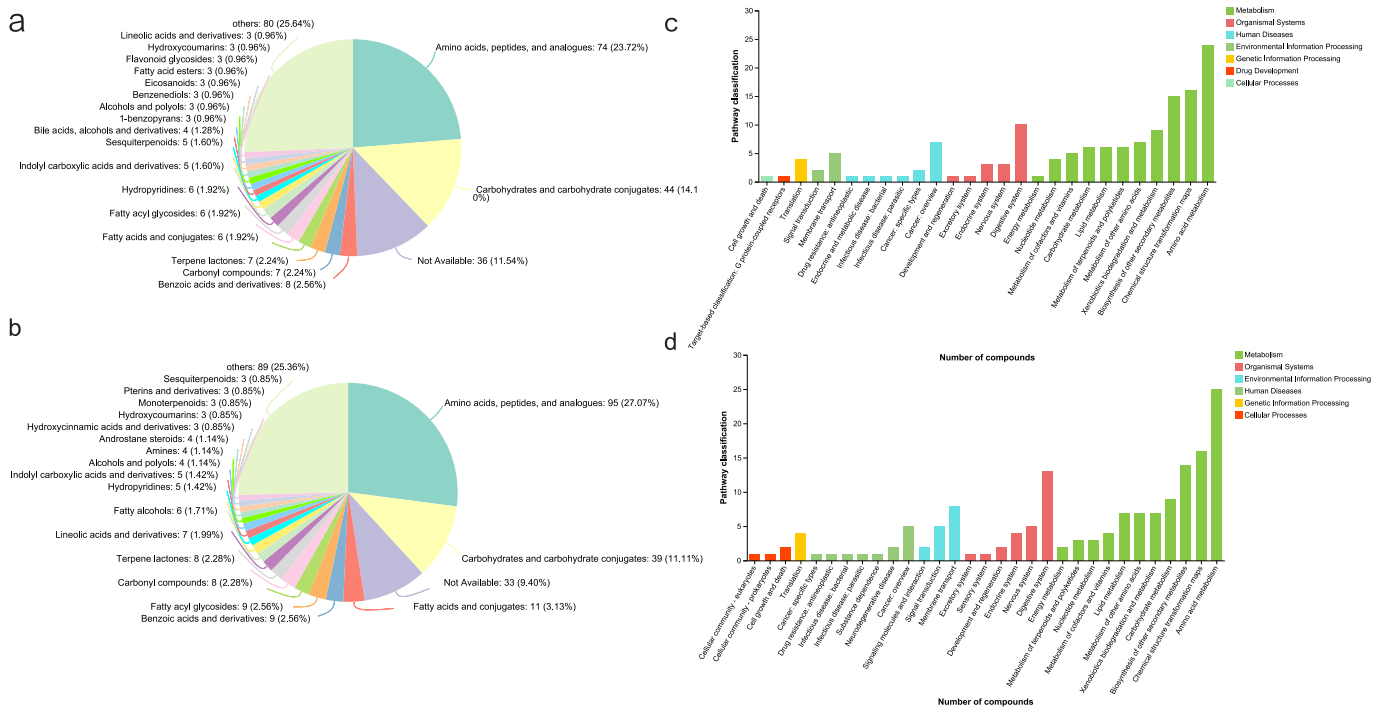

Fig. 2. Differential compound annotations during the thermophilic phase. Compound classification: (a) thermophilic phase 5-day group; (b) thermophilic phase 9-day group. Functional pathway: (c) thermophilic phase 5-day group and (d) thermophilic phase 9-day group; the upper right pathway is for first-level classification and the horizontal coordinates are for second-level classification.

metabolism of terpenoids and polyketides, and energy metabolism. Amino acid metabolism was the pathway with the highest number of annotated compounds, with 25 and 24 compounds annotated to amino acid metabolism in T5 and T9, respectively. This finding confirms that the amino acid metabolism-related pathways were very active during the thermophilic phase. It has been shown that differences in the amino acid metabolism of the microbial community in *Daqu* directly lead to the differentiation of the ecological niche, which in turn affects the sensory aspects and quality of *Daqu* (L. Yang, Fan, & Xu, 2023; Y. Zhang et al., 2022).

To further elucidate the metabolic pathways of the substances in *Daqu* in the thermophilic phase, we enriched the differential metabolic sets of T5 (65 species) and T9 (66 species). As shown in Fig. 3, 26 metabolic pathways were enriched for T5, and 23 metabolic pathways were enriched for T9; most of the metabolic pathways were clustered into subset 3, which suggests that these two metabolic sets were similar. Arginine biosynthesis, aminoacyl-tRNA biosynthesis, mineral absorption, phenylalanine metabolism, cysteine and methionine metabolism, phenylalanine, tyrosine and tryptophan biosynthesis, D-glutamine and D-glutamate metabolism, tryptophan metabolism, aminobenzoate degradation, ABC transporters, biosynthesis of phenylpropanoids, and betalain biosynthesis were common to both groups, and notably, six amino acid metabolism-related pathways were significantly enriched. The metabolic pathways of benzoxazinoid biosynthesis, starch and sucrose metabolism, galactose metabolism, and cell cycle—yeast were enriched only in T5; three of these are carbohydrate metabolism-related pathways. Biosynthesis of alkaloids derived from ornithine, lysine, and nicotinic acid, steroid degradation, tropane, piperidine, and pyridine alkaloid biosynthesis, and arginine and proline metabolism were enriched only in T9; two of these are amino acid metabolic pathways. Differences were observed in the metabolites and pathways that were enriched after different durations of high-temperature fermentation, suggesting that the metabolic patterns of T5 and T9 differed; additionally, these differences suggest that a reasonable extension of high-temperature fermentation time would favor amino acid-related gene expression in the microbial community. Similarly, the metabolic profiles of *Daqu* at different locations within the same space showed differences due to the spatial heterogeneity in the fermentation process of high temperature *Daqu*, which resulted in different peak temperatures of *Daqu* fermentation reached in different locations of the *Daqu*, which shaped the *Daqu* in three different colors: white, yellow, and black (Shi et al., 2022; Zhao et al., 2023).

3.2.2. Volatile organic compounds

We evaluated changes in VOCs in *Daqu* during the thermophilic phase by HS-SPME-GC–MS and identified a total of 105 VOCs (Table S3). OPLS-DA was used to check for significant differences in VOCs before and after the thermophilic phase. As shown in Fig. 4ab, significant differences in VOCs were observed between day 7 and day 12 of fermentation ($R^2Y = 1$, $Q^2 = 0.998$) and between day 7 and day 16 of fermentation ($R^2Y = 1$, $Q^2 = 0.998$), which suggests that microbial metabolism altered the flavor profiles under temperature stress. The VIPs of each VOC were calculated according to the OPLS-DA model, and compounds with VIP > 1 were considered differential VOCs. A total of 24 differential VOCs were identified in T5, and 23 differential VOCs were identified in T9. A total of 13 differential VOCs were shared in both groups, whereas 11 were specific to T5 and 10 were specific to T9 (Fig. S2). These results indicated that the types of VOCs varied depending on the duration of microbial fermentation in a high-temperature environment.

We used a clustered heatmap to examine the variation patterns of the 34 VOCs. As shown in Fig. 4c, most of the VOCs, such as tetramethylpyrazine, 2-ethyl-3,5,6-trimethylpyrazine, 2,3-tetramethylpyrazine, 2-ethyl-3,5,6-trimethylpyrazine, 2,3-dimethyl-5-ethylpyrazine, ethyl nicotinoate, 3-phenylfuran, undecane, hexamethylcyclotrisiloxane, tridecane, dodecane, pyrrole-2-carboxaldehyde, cyclohexasiloxane, dodecamethyl, 2-acetyl pyrrole, and decamethylcyclopentasiloxane, showed an increasing trend, suggesting that high-temperature environments effectively stimulate the microbial community to convert substrates into flavor substances. Ethyl myristate, ethyl oleate, ethyl caproate, ethyl laurate, 6-di-*tert*-butyl-4-methylphenol, coca, and other compounds decreased during fermentation. Interestingly, all four esters

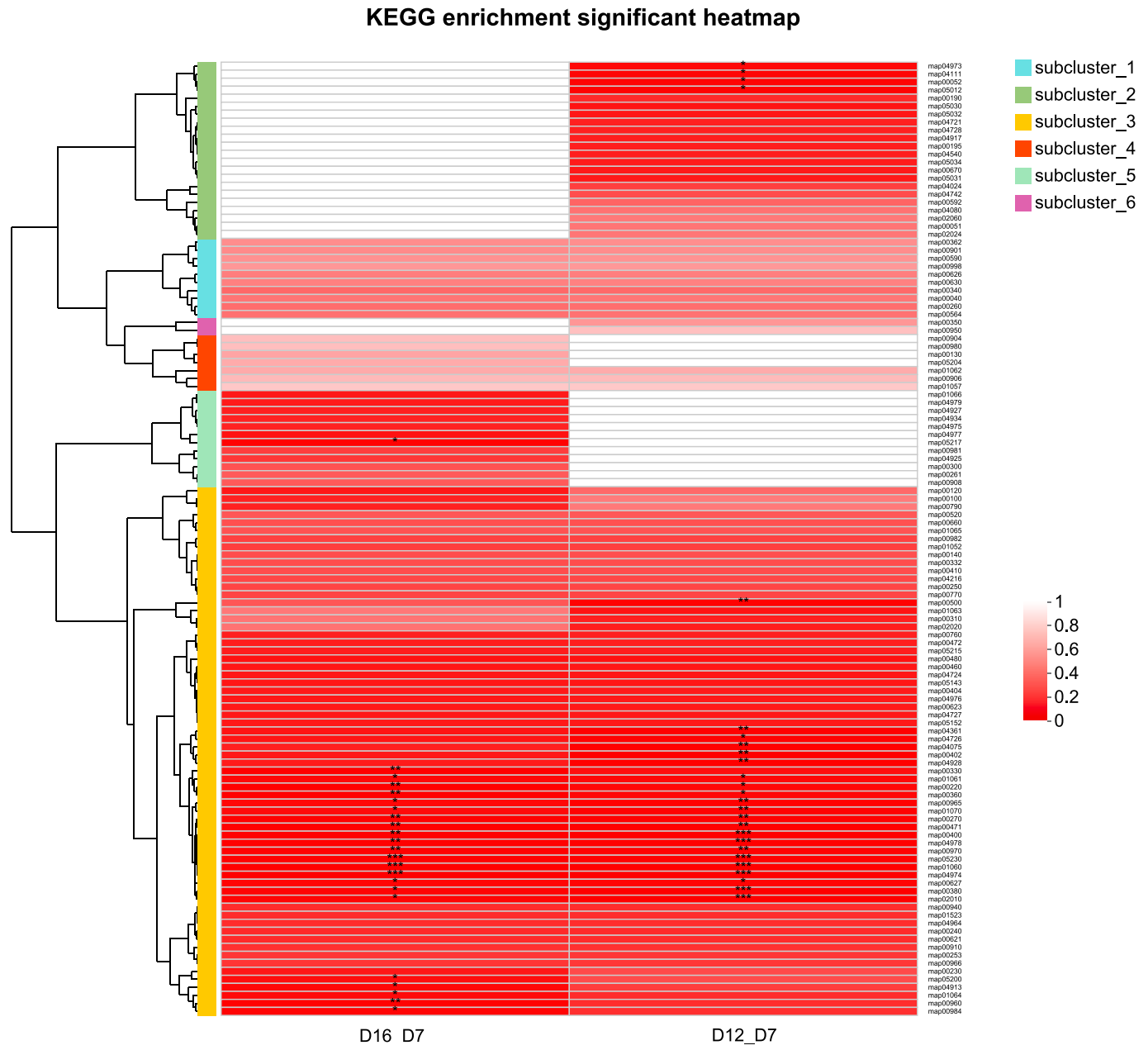

Fig. 3. Differential metabolic set pathway enrichment in the thermophilic phase 5-day group (day 12 vs. day 7) versus the thermophilic phase 9-day group (day 16 vs. day 7). The vertical left coordinate displays a dendrogram of metabolic pathway clusters, categorised into six subclusters with different colours. The vertical right coordinate features the metabolic pathway ID. The closer the two pathway branches are, the more similar the metabolites involved in these two pathways are in the metabolic set. The darker the color, the higher the enrichment rate. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

were composed of higher acids and ethanol; these esters may have been formed by the interactions between the lactic acid bacteria, yeasts, and molds that were dominant in the heating phase (Deng et al., 2021; Jin et al., 2019). However, when *Daqu* fermentation enters the thermophilic phase, the sustained high temperatures cause those microorganisms ability to synthesize higher esters to be weakened or lost, even those microorganisms are killed.

Furthermore, we examined the differential VOCs at different time points during the thermophilic phase. As shown in Fig. 4d, tetramethylpyrazine, 2,3,5-trimethylpyrazine, 2-ethyl-3,5,6-trimethylpyrazine, and 2,3-dimethyl-5-ethylpyrazine reached significantly higher on fermentation day 12 compared with fermentation day 7 ($p < 0.05$), indicating that the thermophilic phase favored pyrazine generation. Pyrazines are typically generated by a Maillard reaction or synthesized by microorganisms (Y. Xu, Wu, Fan, & Zhu, 2011). The high temperatures sustained during the thermophilic phase not only facilitate the Maillard reaction but also lead to active amino acid metabolism, which releases $NH_3{\cdot}NH_3$ is a key precursor for the microbial synthesis of pyrazines; therefore, the conditions during this stage contribute to the accumulation of pyrazines. Ethanol, 2,3-butanediol, and ethyl oleate reached a significantly lower on day 16 of fermentation compared with day 7 of fermentation (Fig. 4e), which could be because substances were assimilated by microorganisms during the fermentation process. As shown in Fig. 4f, tetramethylpyrazine, ethanol, and ethyl oleate were significantly lower in T9 than in T5 ($p < 0.05$), suggesting that prolonging the thermophilic phase would decrease the accumulation of these substances. Tetramethylpyrazine is one of the main flavor components of *Baijiu*, and it endows *Baijiu* with health benefits (Shi et al., 2022). *Daqu* is one of the main sources of tetramethylpyrazine in *Baijiu*, so it is necessary to reasonably controlled the thermophilic phase

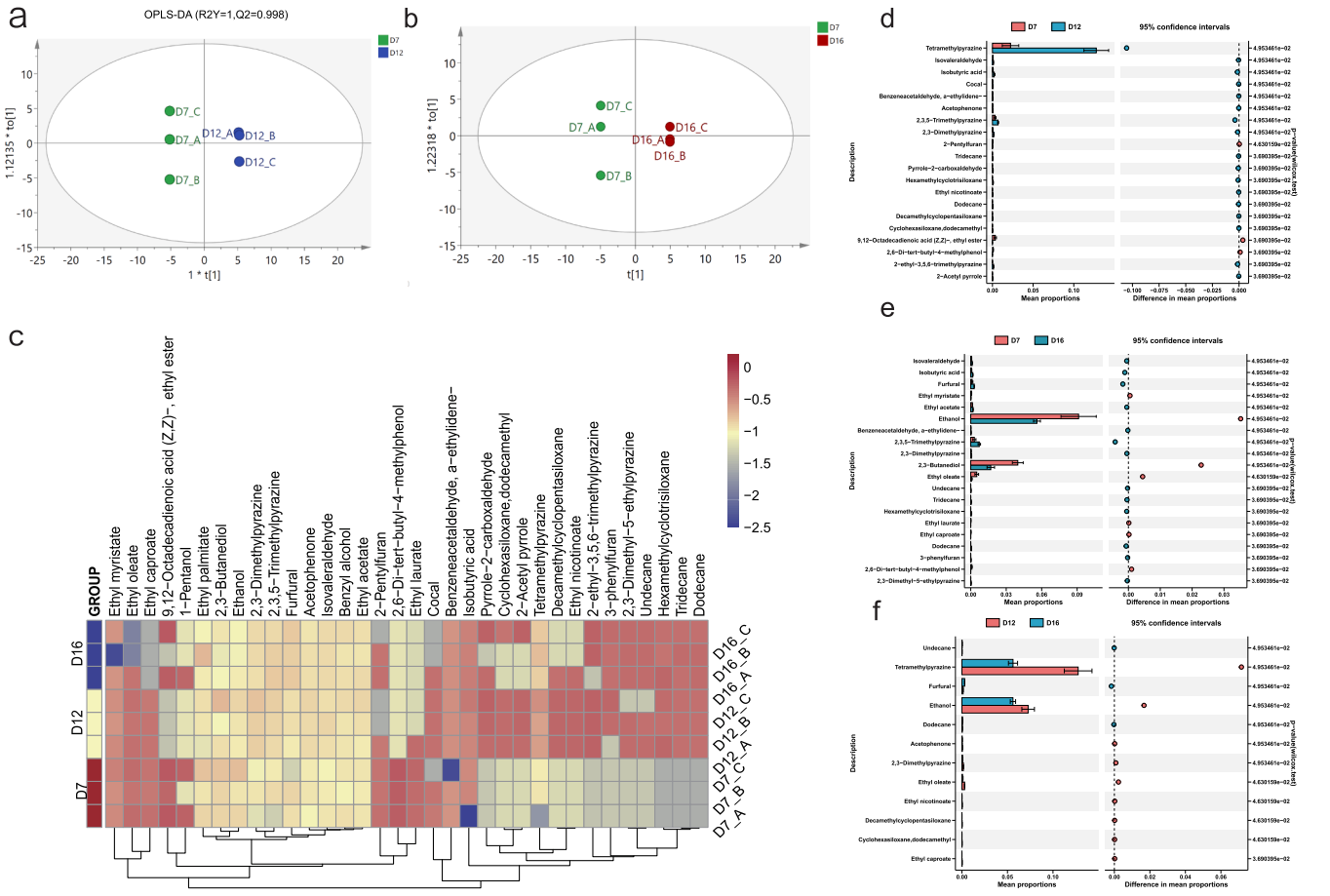

Fig. 4. Differences in volatile organic compounds during the thermophilic phase. OPLS-DA on day 7 vs. day 12 of fermentation (a); OPLS-DA on day 7 vs. day 16 of fermentation (b). Clustering heatmap of differential volatile organic compounds (c). STAMP on day 7 vs. day 12 of fermentation (d), day 7 vs. day 16 of fermentation (e), and day 12 vs. day 16 of fermentation (f).

fermentation time. The above results demonstrate that the thermophilic phase contributes to aroma generation, especially in terms of the generation of pyrazines, but is not conducive to the accumulation of esters. The results also indicate that different durations of the thermophilic phase result in different flavor profiles.

3.3. Correlation between differential metabolites and microbial communities

To identify the main microorganisms involved in the metabolism of substances in the thermophilic phase, we performed correlation analyses between differential compounds and microbial communities ($R > 0.7$, $p < 0.01$). In the bacteria–NVOCs network, 12 bacterial species were significantly correlated with 35 differential compounds, 21 of which were downregulated and 14 of which were upregulated after the thermophilic phase (Fig. 5a), suggesting that the functions of these microbial communities are conducive to assimilate these substances. *Methylobacterium/Methylorubrum* was significantly correlated with 23 NVOCs (19 correlations were positive and 4 were negative), suggesting that this genus may have a broad metabolic capacity. *Cupriavidus* and *Bosea* were significantly positively correlated with (S)-(-)-2-pyrrolidone-5-carboxylic acid, putrescine, L-glutamine, and L-cystine. Similarly, *Undibacterium* was significantly positively correlated with (S)-(-)-2-pyrrolidone-5-carboxylic acid, L-glutamine, and L-cystine. *Prevotella* was significantly positively correlated with dopamine, hydrogen phosphate, and deoxyguanosine. Seven species of fungi were significantly correlated with 40 differential compounds in the fungus–NVOCs network, 23 of which were upregulated and 17 of which were downregulated after the thermophilic phase (Fig. 5b), suggesting that the actions of these microorganisms are conducive to the accumulation of metabolites in the ecosystem. *Thermoascus* was significantly correlated with the synthesis of 18 NVOCs; 12 of these correlations were positive and 6 were negative. *Thermoascus* is a biomarker in the middle and late stages of *Daqu* fermentation (S. Ma, et al., 2021); it can express a variety of thermophilic glycoside hydrolases (McClendon, Batth, Petzold, Adams, Simmons, & Singer, 2012), and it participates in the metabolism of substances to form many intermediate metabolites, which is conducive to the formation of flavor substances. By contrast, *Neocosmospora* was significantly correlated with the synthesis of 17 NVOCs, with 6 positive and 11 negative correlations. This suggests that these two strains have a wide range of metabolic capabilities, which of course cannot be separated from their division of labor with other microorganisms. *Simplicillium* was significantly positively correlated with (S)-(-)-2-pyrrolidone-5-carboxylic acid and L-cystine, and *Thermomyces* was significantly positively correlated with L-cystine.

As shown in Fig. 5c, 17 species of bacteria in the bacteria–VOCs network were significantly correlated with 25 differential compounds. The content of 17 compounds increased and the content of 8 compounds decreased after the thermophilic phase, suggesting that these microbial actions are more beneficial to the accumulation of metabolites in *Daqu*. 2,3-Butanediol was significantly negatively correlated with *Methylobacterium/Methylorubrum* and *Aquabacterium*. Tetramethylpyrazine, 2,3-dimethylpyrazine, and 2-ethyl-3,5,6-trimethylpyrazine were significantly positively correlated with *Glutamicibacter*. This is a genus of actinomycetes with the capacity to express D-amino acid oxidase, which oxidizes D-amino acids to the corresponding keto acids and produces

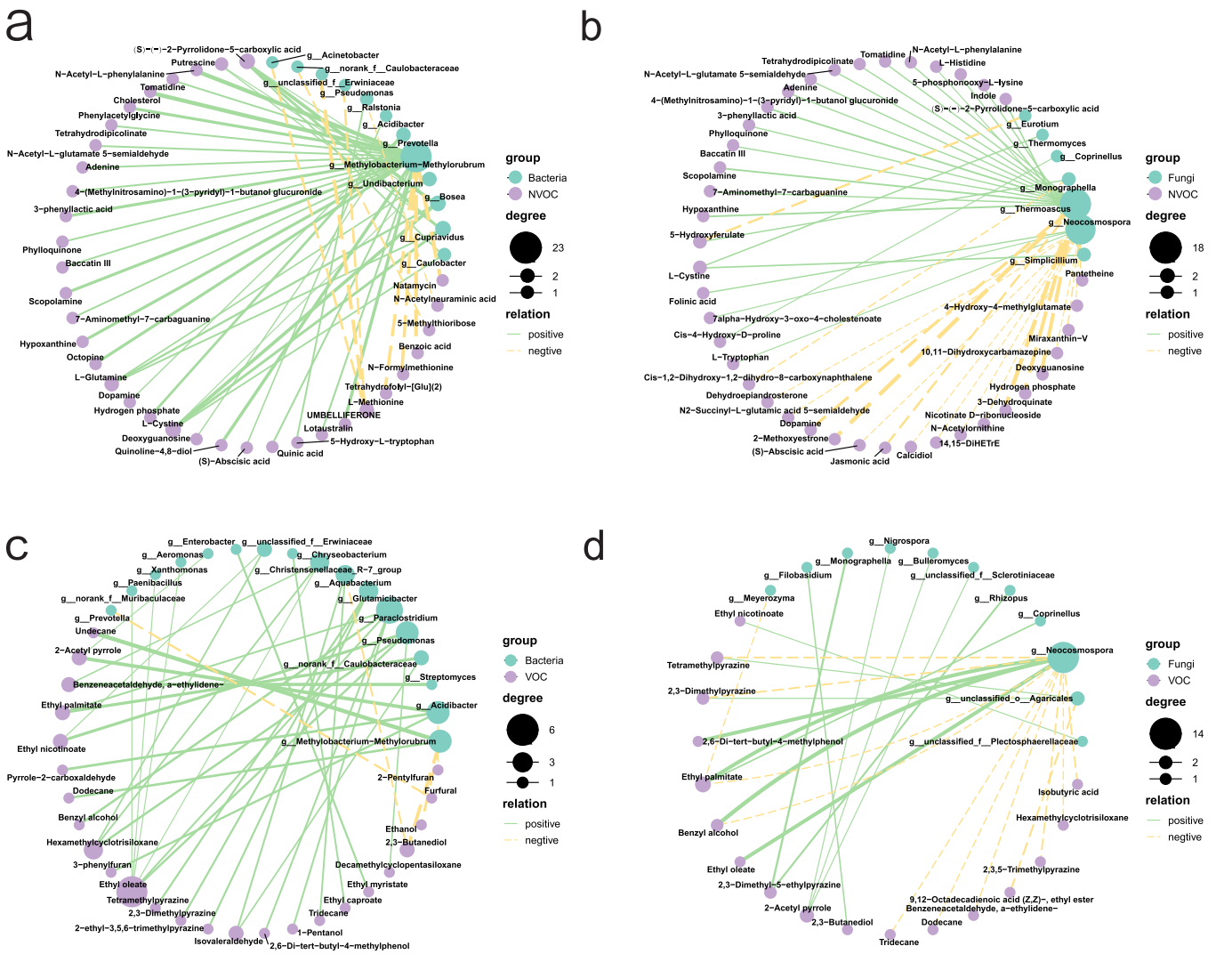

Fig. 5. Correlation analysis between differential compounds and microbial communities. Co-occurrence networks between NVOCs and bacteria (a) and fungi (b) and VOCs and bacteria (c) and fungi (d); r > 0.7, $p < 0.01$. Purple dots indicate compounds, green dots indicate microorganisms, the green solid line denotes a positive correlation, and the yellow dotted line denotes a negative correlation.

ammonia (Xu et al., 2023), a key precursor for pyrazine synthesis. Furfural was significantly negatively correlated with *Prevotella.* As shown in Fig. 5d, 11 species of fungi in the fungus–VOCs network were significantly correlated with 17 differential compounds, 14 of which increased in content and 3 of which decreased in content after the thermophilic phase, suggesting that these microbial actions are more beneficial to the accumulation of metabolites in *Daqu. Neocosmospora* was significantly correlated with 14 VOCs; four of these correlations were positive, including two esters, one phenolic compound, and one alcoholic compound, and ten were negative, including a variety of alkanes and pyrazines. Ethyl palmitate was significantly positively correlated with *Neocosmospora* and *Coprinellus* and significantly negatively correlated with *Neocosmospora* and *Meyerozyma*. Tetramethylpyrazine and 2,3-dimethylpyrazine were significantly positively correlated with *unclassified_o_Agaricales*; 2,3-dimethyl-5-ethylpyrazine was significantly positively correlated with *Rhizopus* and *Monographella*; and 2,3-butanediol was significantly positively correlated with *Filobasidium*.

In addition, as shown in Fig. S3ab, a total of 23 bacterial and 15 fungal genera were involved in differential substance metabolism during the thermophilic phase. Six of these bacterial genera (*Methylobacterium/Methylorubrum, norank_f_Caulobacteraceae, Acidibacter, Prevotella, Pseudomonas,* and *unclassified_f_Erwiniaceae*) and three of the fungal genera (*Monographella, Coprinellus,* and *Neocosmospora*) have been implicated in the formation of NVOCs and VOCs. In summary, 38 microorganisms were involved in the metabolism of substances during the thermophilic phase, and their activity facilitated the assimilation of nonvolatile substances and accelerated the accumulation of flavor substances.

3.4. *Metabolic mapping of microbial communities during the thermophilic phase*

To further clarify the metabolic relationship between the microorganisms and differential metabolites, we constructed a microbial metabolic map during the thermophilic phase. The thermophilic phase pathway map (Fig. 6) was mainly composed of glycolysis, the tricarboxylic acid (TCA) cycle, butyric acid metabolism, and five amino acid metabolism pathways (phenylalanine metabolism, tryptophan metabolism, cysteine and methionine metabolism, D-glutamine and D-glutamate metabolism, and arginine and proline metabolism). The levels of the four most common esters (ethyl caproate, ethyl laurate, ethyl myristate, and ethyl oleate), which are composed of ethanol and fatty acids, decreased during fermentation; this was probably due to the decomposition of these esters by the sustained high temperatures. Microorganisms produce acetoin via the butyric acid metabolic pathway, and this

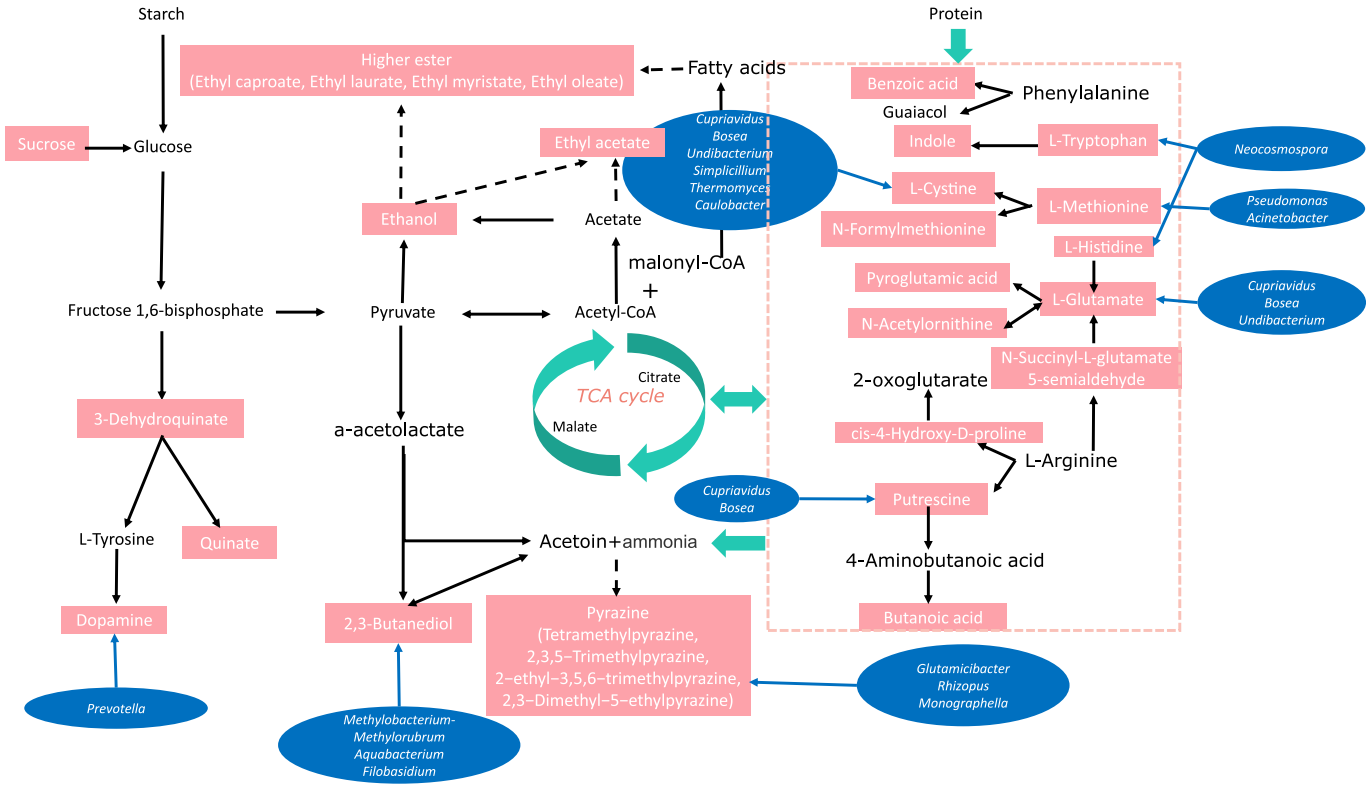

Fig. 6. Microbial metabolic mapping during the thermophilic phase. Pink boxes represent differential compounds; blue ovals represent correlative microbes.

compound can be combined with ammonia (which is produced by microorganisms via amino acid metabolism) to form pyrazines through a non-enzymatic reaction (X. Shi, et al., 2022). The levels of tetramethylpyrazine, 2,3,5-trimethylpyrazine, 2-ethyl-3,5,6-trimethylpyrazine, and 2,3-dimethyl-5-ethylpyrazine all increased to varying degrees during the thermophilic phase, and the microorganism genera involved were mainly *Glutamicibacter*, *Rhizopus*, and *Monographella*. *Rhizopus* secretes proteases that degrade proteins into amino acids (Zheng, Tabrizi, Nout, & Han, 2011), and *Glutamicibacter* breaks down amino acids into ammonia and corresponding keto acids. Putrescine, a biogenic amine with an unpleasant aroma (Gao et al., 2023), can be produced by *Cupriavidus* and *Bosea* through arginine and proline metabolism, but microorganisms can further convert it to butanoic acid. L-tryptophan and L-histidine generation are linked to *Neocosmospora*. L-glutamate and L-cystine generation are linked to *Cupriavidus*, *Bosea*, and *Undibacterium*, among others. L-tryptophan can be converted to L-cystine, *N*-formylmethionine, and L-histidine can be converted to L-glutamate. L-glutamate is further converted to pyroglutamic acid and *N*-acetylornithine, which can be further converted to α-ketoglutaric acid and pyruvate. These compounds then enter the TCA cycle, activating the metabolic network.

4. Conclusion

This study utilized metabolomics and high-throughput sequencing to provide a comprehensive understanding of the substance changes and microbial communities in *Daqu* during the thermophilic phase. Pathways related to amino acid and carbohydrate metabolism were very active during the thermophilic phase. In addition, the thermophilic phase favored the production of pyrazines, whereas the ester production was minimized. The results suggest that prolonging the thermophilic phase would favor the microbial expression of amino acid-related metabolic pathways but not tetramethylpyrazine accumulation. Thirty-eight microbe species were involved in the metabolism of substances during the thermophilic phase, and their actions facilitated the assimilation of NVOCs and accelerated the production of VOCs. These results suggest that an appropriate temperature and duration in the thermophilic phase could facilitate the accumulation of flavor substances and their precursors in *Daqu* fermentation.

CRediT authorship contribution statement

Yong Du: Conceptualization, Data curation, Visualization, Writing – original draft, Writing – review & editing. **Jie Tang:** Conceptualization, Writing – review & editing. **Dan Liu:** Conceptualization, Data curation. **Nian Liu:** Investigation, Resources. **Kui Peng:** Investigation, Resources. **Chaokai Wang:** Investigation, Resources. **Dan Huang:** Conceptualization, Data curation. **Huibo Luo:** Project administration, Supervision, Writing – review & editing.

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

Data will be made available on request.

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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.2023.101044.

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