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Integrated multi-omics analysis unveils metabolites-mediated microbial interactions: dynamic regulation and metabolic division of *A. flavus* SU-16 and *S. cerevisiae* HJ during co-fermentation

Shuangping Liu^{a,b,c,d,1}, Mingliang Li^{a,1}, Hailong Sun^c, Zhuoyue Zheng^a, Yuezheng Xu^{b,c},
Xiao Han^{a,d}, Zhilei Zhou^{a,b,c,d}, Jian Mao^{a,b,c,d,*}

^a National Engineering Research Center for Cereal Fermentation and Food Biomufacturing, State Key Laboratory of Food Science and Technology, School of Food Science and Technology, Jiangnan University, Wuxi 214122, China

^b Shaoxing Key Laboratory of Traditional Fermentation Food and Human Health, Jiangnan University (Shaoxing) Industrial Technology Research Institute, Shaoxing 312000, China

^c National Engineering Research Center of Huangjiu, Zhejiang Guyuelongshan Shaoxing Wine Co., Ltd., Shaoxing 312000, China

^d Jiangsu Provincial Engineering Research Center for Bioactive Product Processing, Jiangnan University, Wuxi 214122, China

^e School of Food Science and Technology, Shihezi University, Shihezi 832003, China

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ABSTRACT

This study aimed to gain insight into the interaction and metabolic mechanism between *Aspergillus flavus* U-16 and *Saccharomyces cerevisiae* HJ during co-fermentation. Despite the suppressed transcriptional activity of SU-16, its metabolites exerted a pivotal regulatory influence. The metabolites produced by SU-16 significantly increased the ethanol content to 17.0%. They also significantly increased the contents of crucial flavor substances, including amino acids (at least 5 times) and esters (up to 1 100 mg/L). SU-16 produced 130 metabolites that may affect flavor compounds, while HJ mainly controlled the synthesis of 161 compounds. Metabolites of SU-16 with different molecular weights were observed to exert differential regulatory effects on the fermentation process and the metabolic pathway of HJ. The small molecule metabolites of SU-16 promoted the growth of HJ significantly. In contrast, its macromolecular metabolites were found to mainly affect the expression of genes related to carbohydrate and amino acid metabolism in the HJ genome.

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1 Introduction

Microbial co-fermentation technology is a widely adopted key technology in fermented food production, including yogurt, soy sauce, vinegar, cheese, pickles, fermented meat products, various alcoholic beverages, etc. Microbial interactions involve active and

passive manifestations and encompass multiple types, including niche competition, functional complementarity, nutritional and metabolic dependence or competition, metabolite exchange, and quorum sensing. The complex interactions among co-fermenting microorganisms serve as the core driving force behind substrate consumption and the formation of flavors. These intricate interactions enhance flavor quality, promote process stability, and mitigate safety risks^[1-2].

In recent years, the analysis of microbial community structures in numerous fermented foods has identified core microorganisms involved in their production, drawing increased attention to microbial

¹ These authors contributed equally to this work

* Corresponding author at: National Engineering Research Center for Cereal Fermentation and Food Biomufacturing, Jiangnan University, Wuxi 214122, China.

E-mail address: maojian@jiangnan.edu.cn (J. Mao)

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interactions. Numerous studies have clarified that the composition of the core microorganisms within co-fermentation systems drives the entire fermentation process^[3]. Interactions between yeasts help improve specific characteristics of wine. The combination of *Torulaspora delbrueckii* and *Saccharomyces cerevisiae* significantly increases ethyl esters and volatile fatty acid levels^[4]. Co-fermentation of *Pichia fermentan* and *Oenococcus oeni* can enhance fruity aroma characteristics^[5]. In the fermentation of Baijiu, microbial interactions play a crucial role in shaping flavor. For instance, co-fermentation of various non-*Saccharomyces* yeast and *S. cerevisiae* contributes to the production of lipids and alcohols^[6]. Microbial interactions are integral to the cheese fermentation process. The co-fermentation of *Ligilactobacillus salivarius* and *Micrococcus fumeus* shows excellent potential in improving nutritional and flavor quality during cheese ripening^[7]. The co-fermentation of *Aspergillus* and *S. cerevisiae* is a crucial aspect of multi-strain co-fermentation technology widely employed in various fermented foods, such as soy sauce, Baijiu, and Huangjiu^[8]. Studies indicate that the interaction between *Aspergillus oryzae* and *S. cerevisiae* significantly influences the metabolism of flavor substances in Baijiu fermentation^[9]. In general, *Aspergillus* primarily contributes a diverse enzyme system, while *S. cerevisiae* is chiefly responsible for ethanol and flavor substance production^[10–11].

However, most research tends to concentrate on utilizing microbial co-fermentation to enhance the quality of the fermentation system, improve the synthesis of specific compounds, and optimize the combination of microorganisms. The understanding of comprehensive interaction mechanisms among key microorganisms during fermented food production remains significantly limited, hindering co-fermentation technology's further development and application. Furthermore, there is a shortage of in-depth understanding regarding the dynamic changes of microbial flora and their relationship with the fermentation process, particularly across various fermentation stages.

Huangjiu fermentation is a natural fermentation system that involves *Aspergillus flavus* SU-16 and *S. cerevisiae* HJ as core microorganisms. This time-honored process has been utilized and perfected in China for thousands of years. The *A. flavus* SU-16 has been used in Huangjiu fermentation due to its non-aflatoxin-producing properties^[12]. Through macro genome and triple amplicon sequencing, Hong^[13] and Liu et al.^[14] identified *A. flavus* and *S. cerevisiae* as the main microorganisms involved in the wheat Qu and fermentation processes, respectively. Moreover, Liu et al.^[14] investigated the fermentation performance of various strains by evaluating enzyme production and fermentation characteristics. The findings revealed that *A. flavus* SU-16 is indispensable in Huangjiu fermentation. In contemporary Huangjiu production, a simultaneous addition of pure *S. cerevisiae* HJ and cooked wheat Qu inoculated with *A. flavus* SU-16 is employed for fermentation. Using a semi-liquid fermentation method ensures the uniformity of the fermentation system, rendering it an ideal model for studying the interaction mechanisms between microorganisms in co-fermentation systems.

In previous investigations, we have already performed whole-genome sequencing on *S. cerevisiae* HJ (GenBank: GCA_015363515.1) and *A. flavus* SU-16 (GenBank: GCA_009856665.1), acquiring comprehensive genomic data for these microorganisms. In this study, using the Huangjiu fermentation system as a reference, we employed open fermentation by introducing pure cultures of *S. cerevisiae* HJ

and cooked wheat Qu inoculated with *A. flavus* SU-16. This method allowed *S. cerevisiae* HJ and *A. flavus* SU-16 to dominate the initial fermentation stage. We endeavored to utilize Dual RNA-seq to unravel the transcriptional regulatory patterns governing the interaction between *S. cerevisiae* HJ and *A. flavus* SU-16 during fermentation. Additionally, we conducted a comprehensive metabolomics analysis to elucidate the interactions, individual contributions, and metabolic division of *S. cerevisiae* HJ and *A. flavus* SU-16 within the co-fermentation system. We aimed to clarify the role and mechanism of microbial interactions in food fermentation, providing insights for a better understanding and application of co-fermentation systems.

2. Materials and methods

2.1 Materials

S. cerevisiae HJ was stored at 4 °C on a yeast extract peptone dextrose medium (YPD). The culture was transferred to a new slant of YPD and cultivated at 28 °C for 24 h before use. *A. flavus* SU-16 was inoculated in the potato dextrose agar (PDA) liquid medium and incubated in a shaker at 28 °C for 48 h. The resulting mycelial was washed thrice with sterile water, lyophilized, and stored at –80 °C until use. YPD and PDA culture media were purchased from Qingdao Hope Bio-Technology Co., Ltd. (Qingdao, China); wheat and glutinous rice were supplied by the Guyuelongshan Wine Company (Shaoxing, China); glucose, peptone, agarose, 3',5'-dinitrosalicylic acid (DNS), sodium hydroxide, formaldehyde, etc. were purchased from Sinopharm Reagent Co., Ltd. (Shanghai, China); flavour-related standards were chromatographically pure, which were purchased from Shanghai Anpu Experimental Technology Co., Ltd., (Shanghai, China) Sinopharm Chemical Reagent Co., Ltd., (Shanghai, China) and Sigma Company (St. Louis, Mo, USA); the primers used in the experiment were synthesized by Bioengineering (Shanghai) Co., Ltd; α -amylase and glucoamylase were obtained from Suzhou Hongda Enzyme Co., Ltd. (Suzhou, China).

2.2 Samples preparation and grouping

To prepare the cooked wheat Qu, crushed wheat was combined with 40% (m/m) water and sterilized at 121 °C for 30 min. Once the temperature of the wheat reached 30 °C, SU-16 spores were inoculated onto the wheat. The mixture was then fermented at 35 °C for 4 days until the surface of the wheat was covered with mycelium and spores^[15].

The cooked wheat Qu were weighed and crushed. Then, the crushed samples were mixed with 3 L deionized water and subjected to sonication at 30 °C for 45 min. The resulting mixture was centrifuged at 12 000 r/min for 12 min. The supernatant obtained post-centrifugation serves as the cooked wheat Qu extract. By employing an ultrafiltration device with membranes of different molecular weights, the supernatant undergoes ultrafiltration to isolate extracts containing SU-16 metabolites of different molecular weights (> 1 kDa and < 1 kDa).

For the Huangjiu fermentation, 1 000 g glutinous rice was soaked in water for 3 h. The soaked rice was then steamed. Once the steamed rice cooled to 28 °C, cooked wheat Qu, cultured HJ, and sterile water were added and thoroughly mixed. The mixture was pre-fermented

Table 1
Treatment and grouping of each fermentation system.

Group	Wheat Qu or its substitutes	Other components	Fermentation method
MQF	Cooked wheat Qu inoculated with <i>A. flavus</i> SU-16	Glutinous rice, <i>S. cerevisiae</i> HJ, Aseptic water	Open fermentation
HJF	Enzymic preparations with the same enzyme activity as cooked wheat Qu (α -amylase, glucoamylase)		
JTF	Extract of cooked wheat Qu		
JSY	Mycelium of <i>A. flavus</i> SU-16		
JS	Lyophilized mycelium of <i>A. flavus</i> SU-16	Glutinous rice, <i>S. cerevisiae</i> HJ,	
JSJT	Mycelium of <i>A. flavus</i> SU-16 + extract of cooked wheat Qu	Aseptic water,	
JSJTM	Mycelium of <i>A. flavus</i> SU-16 + sterilized extract of cooked wheat Qu	Sterilized wheat of equal weight to the MQF group	
EMJTM	Enzymic preparations + sterilized cooked wheat Qu extracts		
EM > 1K	Enzymic preparations + sterilized cooked wheat Qu extract with molecular weight > 1 kDa		
XJTF	Enzymic preparations + sterilized cooked wheat Qu extract with molecular weight < 1 kDa		
> 1K	Sterilized cooked wheat Qu extract with molecular weight > 1 kDa		
< 1K	Sterilized cooked wheat Qu extract with molecular weight < 1 kDa		

in an incubator at 28 °C for 120 h, followed by a post-fermentation phase at 16 °C for 240 h^[16]. The following Huangjiu simulation fermentation system was established to investigate the contribution of SU-16 or metabolites to the fermentation system during fermentation. Table 1 describes the addition of cooked wheat Qu or its substitutes to the fermentation system.

2.3 RNA-extraction and RNA-sequencing

Samples were collected at specific fermentation time points (0, 24, 48, 72, and 120 h), and total RNA was extracted using the Trizol method. RNA sequencing libraries were constructed and sequenced on the Illumina HiSeq platform. Quality control of clean data was conducted using FastQC. The data were then aligned to the genomes of HJ and SU-16 using HISAT2, and gene models were used for genome positioning analysis. The featureCounts tool was employed for quantitative transcript analysis, calculating gene expression in HJ and SU-16 genomes regarding Fragments Per Kilobase of exon model Per Million mapped Fragments (FPKM). Genes were categorized into low, medium, and high expression levels based on FPKM values using thresholds of 0.1, 3.75, and 15. DESeq2 was utilized for read normalization, and differentially expressed genes (DEGs) between samples were identified using a negative binomial distribution model with screening conditions of fold change (FC) ≥ 2 and a *P*-value after multiple hypothesis testing < 0.05. Functional annotation using Gene Ontology (GO) and metabolic pathway enrichment analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) were performed on DEGs. Pathways with a $P_{adj} < 0.05$ were considered significantly enriched.

2.4 Biomass detection

Fermentation samples were taken every 24 h during the fermentation process to determine *S. cerevisiae* HJ, *A. flavus* SU-16, and total fungi biomass. Weigh 5 g of fermentation samples, grind in liquid nitrogen, add 10 mL of sterile water, vortex, and shake for 10 min. Centrifuge at 1 000 r/min for 8 min, collect the supernatant, repeat twice, mix the resulting supernatant and centrifuge at 12 000 r/min for 8 min, extract DNA in the sediment using the CTAB method, and perform q-PCR on the fermentation samples using the primers in Table 2 to detect. The different concentrations of microbial genomes as templates, the Ct value after q-PCR amplification was used as the

horizontal coordinate, and the logarithmic value of the corresponding microbial biomass was used as the vertical coordinate to draw the standard curve. The biomass of total fungi, *S. cerevisiae* HJ, and *A. flavus* SU-16 during fermentation was calculated separately.

Table 2
Primers used for q-PCR detection.

Projects	Primer	Sequences (5' to 3')	Amplified region	Product
Total fungi	F R	GAGTCGAGTTGTTTGGGAATGC TCTCTTTCCAAAGTCTTTTCATCTTT	28S	123 bp
<i>S. cerevisiae</i> HJ	F R	CCCCTCAAGAACGAAATGGTG CTCAAAGGCAGAAGCAGAATCG	MEX67	132 bp
<i>A. flavus</i> SU-16	F R	GTCGTCCCTCTCCGG CTGGAAAAAGATTGATTGCG	ITS1	135 bp

2.5 Determination of chemical indexes

The contents of total acid, amino nitrogen, and ethanol were measured according to the technique described in GB/T 13662-2018 (national standards for Huangjiu); the reducing sugar content was measured using the 3,5-dinitro-salicylic acid colorimetric technique^[17].

2.6 Determination of non-volatile and volatile compounds

The contents of the amino acid and organic acid were determined using high-performance liquid chromatography (HPLC)^[18]. The higher alcohol content was determined by dispersive liquid-liquid microextraction coupled with gas chromatography-mass spectrometry (GC-MS)^[19]. Headspace solid phase microextraction (HS-SPME) combined with GC-MS was used to determine the content of other volatile compounds^[20]. The processing and detection methods of samples are shown in the supplementary material.

2.7 Untargeted metabolomics analysis

Untargeted metabolomics was employed to examine the impact of SU-16 and HJ on the formation of flavor substances at various fermentation stages, specifically in the MQF, JTF, HJF, and XJTF group. The samples fermented for 0, 24, 48, 72, and 120 h were centrifuged at 12 000 r/min for 8 min. The supernatant was collected and stored at -80 °C until use. The processing and detection methods of samples are shown in the supplementary material.

2.8 Statistical analysis

The GraphPad Prism 9.0 and Origin 2022 software were used for statistical analysis of significant differences and data visualization. All experiments are in triplicate. All data were expressed as means $\pm$ SD. Orthogonal partial least squares-discriminant analysis (OPLS-DA) was performed using SIMCA-P software (version 14.1, Umetrics, Umea, Sweden), and differential metabolites were screened. The screening conditions for significantly differential metabolites were VIP value ≥ 1 , $P < 0.05$, and FC ≥ 2 . The screened critical differential metabolites were annotated based on the KEGG compound database, and the annotated metabolites were mapped to the corresponding KEGG pathway.

3. Results and discussion

3.1 Transcriptional activity of SU-16 undergoes inhibition during the co-fermentation process

Dual RNA-seq and transcriptome analyses were conducted on samples from the co-fermentation system. The results in Figs. 1A-C demonstrate the distribution and expression levels of reads associated with HJ and SU-16 genomes. The “others” should be related to other bacteria and fungi during open fermentation; most reads (88.14%–97.40%) were related to the HJ genome; in contrast, only a tiny fraction of reads (0.97%–2.75%) were related to the SU-16 genome (Fig. 1A). Over 99.82% of the genes in the HJ genome were transcribed during fermentation. Among these genes, 11.55%–21.44% had FPKM ≥ 3.75 . A more significant proportion of genes (76.48%–87.48%) had FPKM ≥ 15 (Fig. 1B). In contrast to the HJ genome, most genes in the SU-16 genome (66.36%–90.83%) had FPKM < 0.1 , suggesting that these genes were not actively transcribed throughout the fermentation process (Fig. 1C).

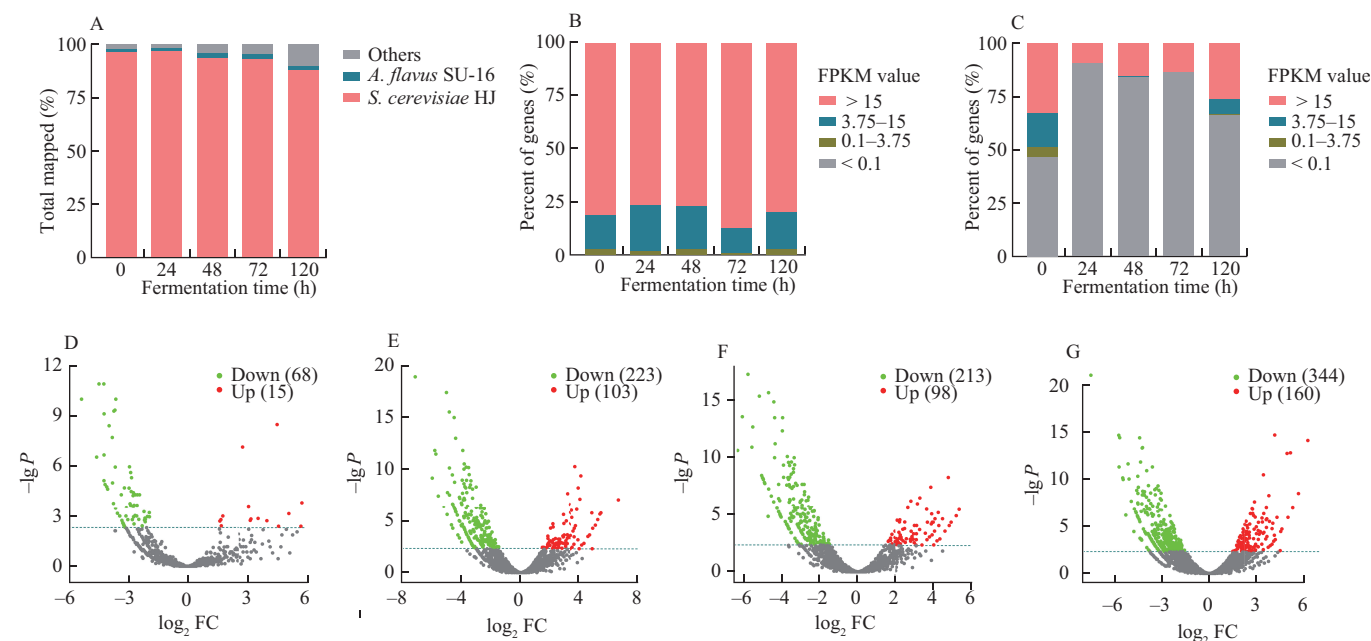


Fig. 1 (A) Ratio of the clean reads mapped to *S. cerevisiae* HJ and *A. flavus* SU-16 genome. Analysis of the gene expression levels in (B) *S. cerevisiae* HJ and (C) *A. flavus* SU-16 genome. Differentially expressed genes in *A. flavus* SU-16 genome during fermentation: (D) 24 h; (E) 48 h; (F) 72 h; (G) 120 h; samples at 0 h were used as control. Functional annotation of differentially expressed genes of *A. flavus* SU-16 genome: (H) GO annotation; (I) KEGG annotation.

Figs. 1D-G show the number and functional characteristics of DEGs in the SU-16 genome at different time points compared to the initial fermentation stage. At different fermentation time points, many genes exhibited differential expression in the SU-16 genome, with 68, 223, 213, and 344 genes showing significant down-regulation at 24, 48, 72, and 120 h, respectively. Conversely, a smaller number of genes (15, 103, 98, and 160) were found to be up-regulated at the corresponding time points. The percentage of DEGs relative to the total genes in the SU-16 genome ranged from 0.6% to 4.0%. Functional annotation analysis of the DEGs showed that most down-regulated genes primarily involve signaling pathways, membrane transport, DNA processing, and other essential life activities associated with basal metabolism (Figs. 1H-I).

The findings suggest that during co-fermentation, the growth and transcriptional activity of SU-16 were suppressed, restricting its capacity to execute functions encoded in its genome.

3.2 Metabolites of SU-16 exert regulatory effects on the co-fermentation system

We tested the physicochemical indicators and microbial biomass of each fermentation system. The ethanol content of the MQF, JTF, and JSJT group exhibited rapid increases during the initial fermentation stage, whereas the JSY and HJF group showed a slower rate of increase. By the end of fermentation, the ethanol content of the MQF, JTF, and HJF group had reached approximately 17.0% (V/V), whereas the JSJT and JSY group reached only 15.7% and 13.0%, respectively. The JS and JSJTM group did not achieve successful fermentation (Fig. 2A). The MQF and JTF group exhibited total acid levels of 5.5 and 4.5 g/L, respectively. Conversely, the acid content in the HJF group and groups with added mycelium was generally low (Fig. 2B). The addition of cooked wheat Qu or its extract enhanced the efficiency of reducing sugar utilization during fermentation (Fig. 2C). The amino nitrogen contents of the MQF and JTF group reached 0.7 and 0.8 g/L, respectively, which were significantly higher than those of other groups. Additionally, the amino nitrogen content of the JSJT

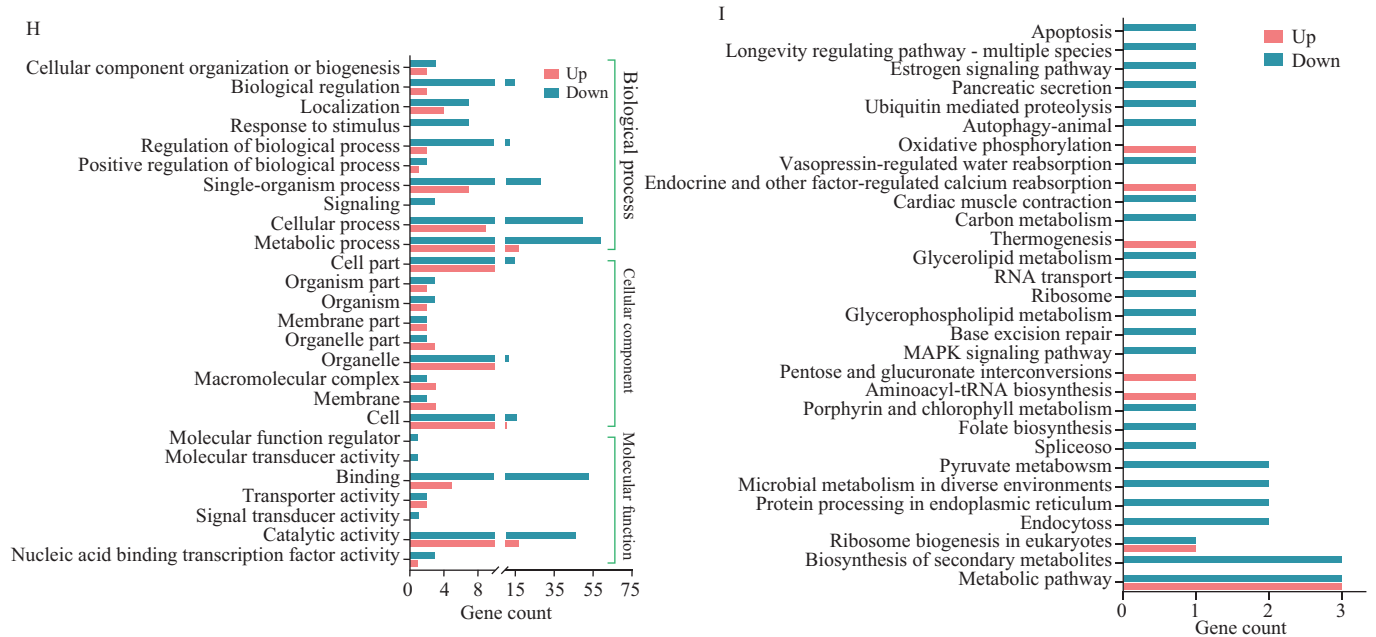


Fig. 1 (Continued)

group was significantly higher than that of the JS and JSJTM group (Fig. 2D).

The biomass of SU-16 in the groups supplemented with mycelium displayed a significant increase within the first 48 h (Fig. 2E). In the JS group, the presence of SU-16 mycelium and the low ethanol content sustained a high biomass of SU-16 throughout the fermentation process. In the JSJTM group, the addition of sterilized cooked wheat Qu extract and SU-16 mycelium likely created a favorable environment for the growth of SU-16, explaining the substantial SU-16 biomass observed. The JSJT and JSY group exhibited only a slight increase in SU-16 biomass. There was a decrease in the biomass of SU-16 after 48 h in all groups except the JS group. In contrast, the groups supplemented with cooked

wheat Qu showed no signs of SU-16 growth. The biomass of SU-16 consistently declined, and by the end of fermentation, the MQF group's SU-16 biomass was less than 20% of the initial fermentation level.

As the ethanol and liquid content of the fermented mash increased, the biomass of HJ in the JS and JSY group decreased after 24 h. The growth rate of HJ in the JSJTM group was slightly slower, and the biomass continued to increase until 48 h, after which it started to decline. The biomass of HJ in the JSJT and HJF group remained relatively constant from 24 h until the end of pre-fermentation. Conversely, the biomass of HJ in the MQF and JTF group showed continuous growth from the start of fermentation until the end of pre-fermentation (Fig. 2F). The MQF, JTF, and JSJT group had higher

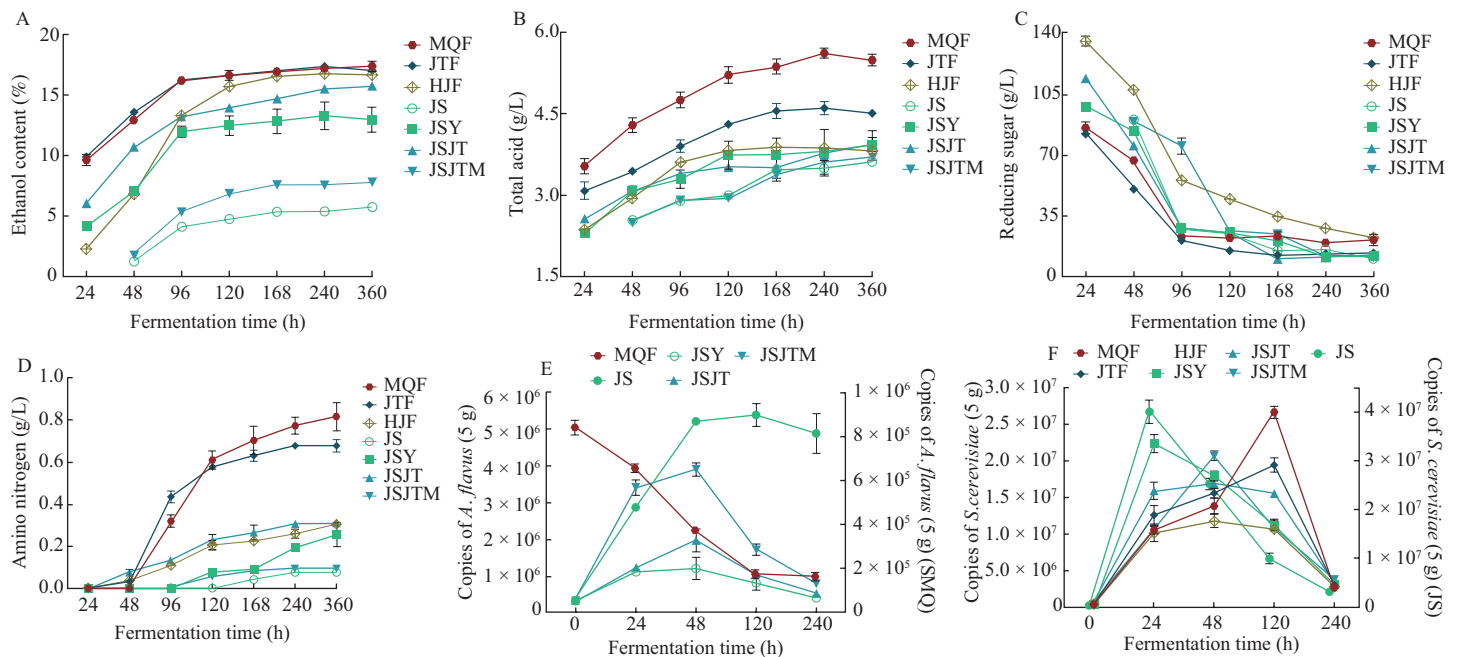


Fig. 2 Changes in biomass and physicochemical indexes during Huangjiu fermentation. (A) Alcohol content; (B) Total acid; (C) Reducing sugar; (D) Amino nitrogen; (E) Biomass of *A. flavus* SU-16; (F) Biomass of *S. cerevisiae* HJ.

biomass of HJ at the end of pre-fermentation compared to the HJF group.

The results above demonstrate that adding cooked wheat Qu or its extract facilitates normal Huangjiu fermentation, whereas the addition of SU-16 mycelium alone fails to meet the requirements of Huangjiu fermentation. Consequently, we infer that the metabolites of SU-16 in cooked wheat Qu play a crucial role in the co-fermentation process, as opposed to the SU-16 strain itself. Additionally, it was observed that the biomass of HJ in groups supplemented with mycelium, cooked wheat Qu, or its extract consistently exceeded that of the HJF group throughout the entire fermentation duration, so we infer that the metabolites produced by SU-16 can stimulate the growth of HJ during fermentation.

3.3 Regulatory properties of SU-16 metabolites with different molecular weights on fermentation proceeds and HJ growth

Based on these results, we further designed to add different molecular weights of cooked wheat Qu extract to the fermentation system to observe the effects of different molecular weights of SU-16 metabolites on the fermentation process and HJ growth. According to the results presented in Figs. 3A-D, all groups except the < 1K group met the fermentation requirements and completed the process. The ethanol content in all groups reached a level greater than 16.5% (V/V). The total acid content was significantly higher in the MQF group than in the HJF group. Although there was no significant difference among groups, the total acid content in all groups was higher than in the HJF group. Interestingly, the MQF, JTF, and > 1K group showed a significantly higher rate of reducing sugar consumption than the other groups. Furthermore, these groups' final amino nitrogen content was significantly higher than the HJF group's.

The biomass of HJ during fermentation was detected (Fig. 3E). Results indicated that the biomass of HJ in groups supplemented with cooked wheat Qu or its extract was notably higher than in the HJF group, particularly in the < 1K and EMJTM group. Although the < 1K group cannot meet the needs of Huangjiu fermentation, at 24 h and 48 h, the biomass of HJ in the < 1K and EMJTM group was significantly higher than that of other groups. However, at 120 h, the biomass of HJ in these 2 groups was already the lowest among all fermentation groups. In addition, at 120 h, except for the < 1K and the EMJTM group, the biomass of HJ in all other groups exceeded that of the HJF group.

These findings indicate that the metabolites of SU-16, especially the small molecule metabolites, can promote the propagation of HJ in the early stage of pre-fermented (before 48 h) but are not sufficient to maintain the growth of HJ throughout the entire pre-fermentation process. However, its macromolecular metabolites sustained the growth of HJ in the late fermentation period (after 48 h).

3.4 Regulatory properties of SU-16 metabolites with different molecular weights on the synthesis of flavor substances

We examined the content of several flavor substances in each group to determine the effect of different molecular weight SU-16 metabolites on the formation of flavour substances in the fermentation system. Amino acids are crucial flavor constituents in Huangjiu and are essential precursors for synthesizing various flavor substances during fermentation. The total amount of amino acids in groups supplemented with cooked wheat Qu, its extract, or mycelium was significantly higher compared to the HJF group. In the HJF group, the primary amino acids detected were alanine, arginine, proline, glycine, phenylalanine, aspartic acid, and leucine, with a total content

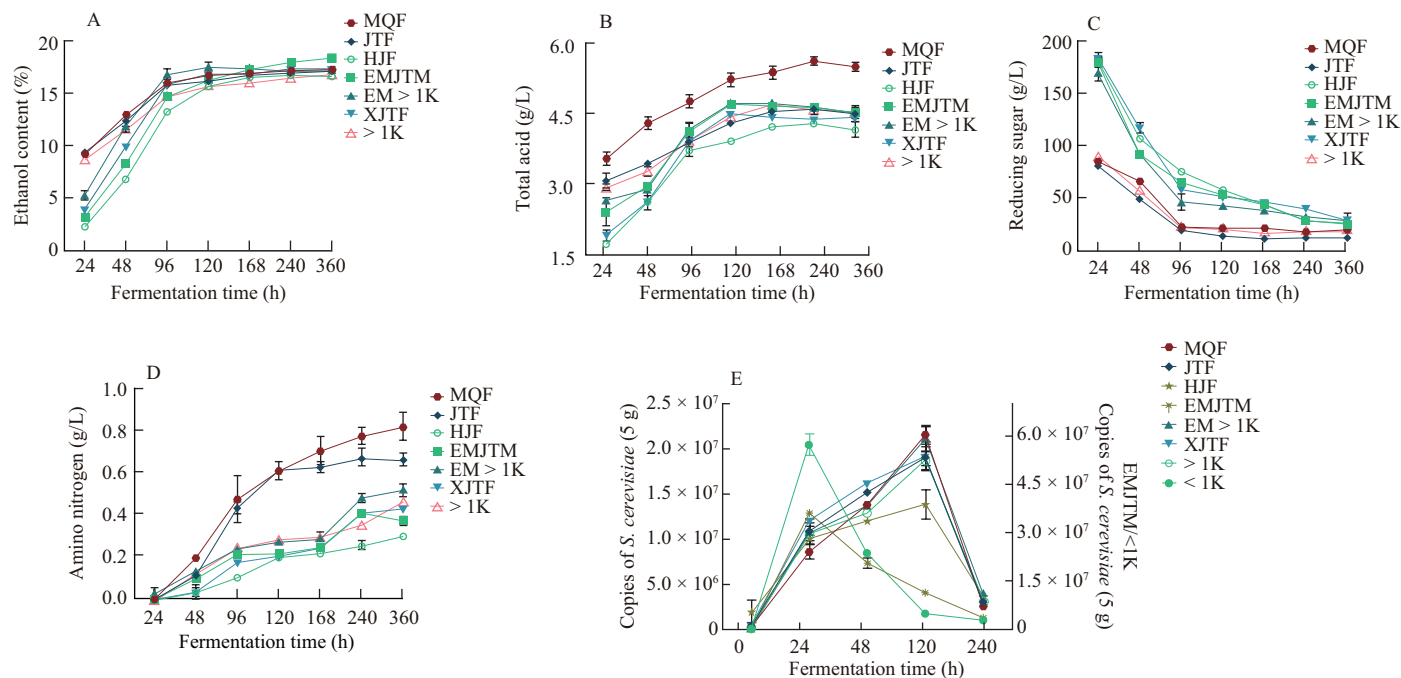


Fig. 3 Changes of the physicochemical indexes during fermentation: (A) Alcohol content; (B) Total acid; (C) Reducing sugar; (D) Amino nitrogen. (E) The biomass of *S. cerevisiae* HJ during fermentation. The content of (F) amino acids, (G) organic acids, and (H) higher alcohol in the fermentation samples. The content of volatile compounds in the fermentation samples: (I) Esters; (J) Aldehydes; (K) Phenolics; (L) Acids.

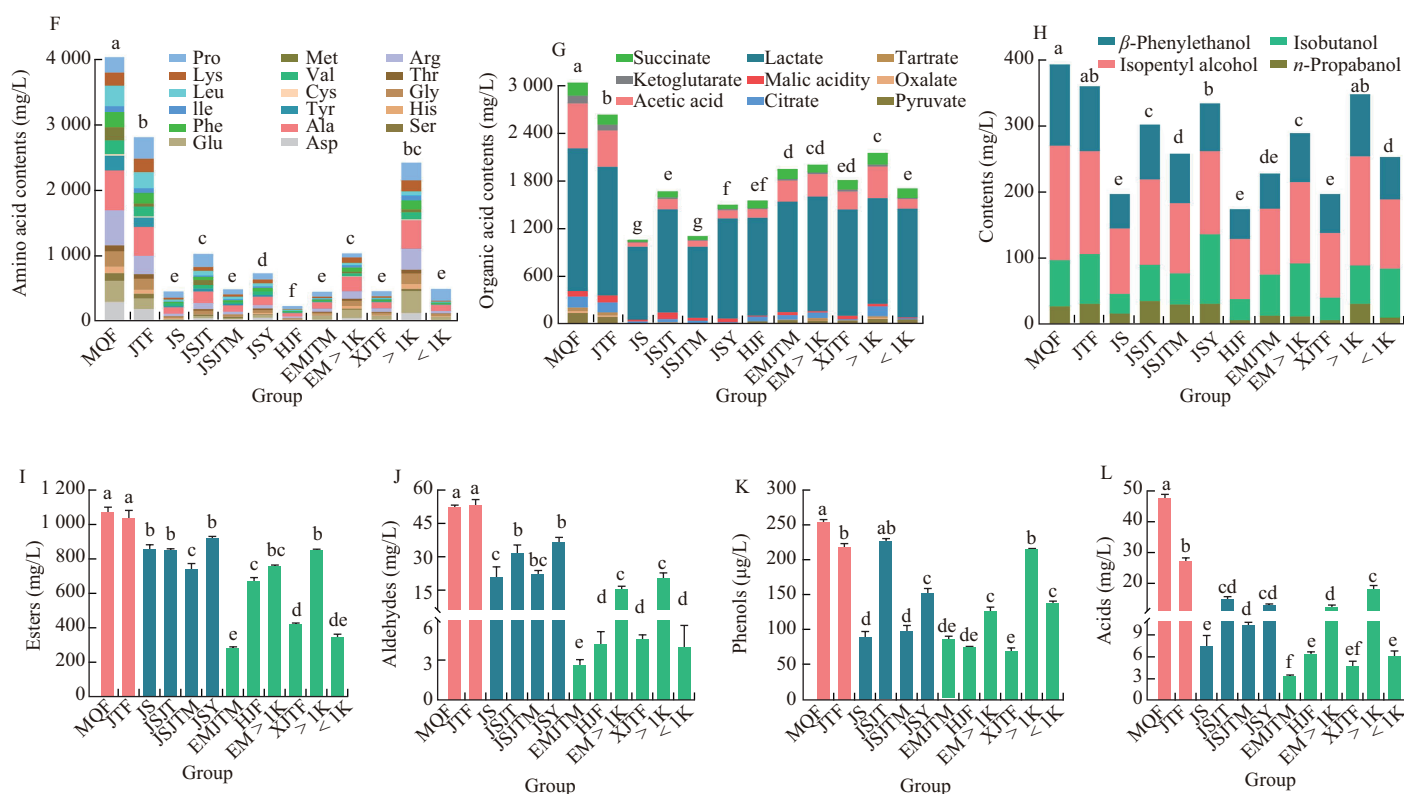


Fig. 3 (Continued)

of approximately 245 mg/L. In the EMJTM, XJTf, JS, < 1K, and JSJTM group, the total amount of amino acids was almost twice that of the HJF group, with a significant increase observed for arginine, proline, glycine, and leucine. The MQF, JTF, > 1K, JSJT, and EM > 1K group exhibited a similar amino acid composition ratio, with more than 5 times higher content than the HJF group (Fig. 3F).

Lactate and acetic acid were the primary organic acids detected in Huangjiu. The proportion of organic acids did not show significant differences between groups. However, the organic acid content of the JS, JSY, and JSJTM group was lower than that of the HJF group. Conversely, the > 1K, EMJTM, EM > 1K, JTF, and MQF group had significantly higher organic acid content than the HJF group (Fig. 3G).

Among the critical flavor components in Huangjiu, higher alcohol, including β -phenyl ethanol, isobutanol, *n*-propyl alcohol, and isoamyl alcohol, play a significant role. Proper higher alcohol contributes to a harmonious taste in Huangjiu. The test results for the higher alcohol are depicted in Fig. 3H. In all fermentation groups with cooked wheat Qu or its extract added, the higher alcohol content was higher than that of the HJF group. Among them, the higher alcohol content of the JS, XJTf, and EMJTM group showed no significant difference compared to the HJF group. The MQF, JTF, and > 1K group showed similar higher alcohol content, approximately twice that of the HJF group. Notably, isoamyl alcohol and β -phenyl ethanol reached levels exceeding 160 and 120 mg/L, respectively.

A quantitative analysis of 15 esters, 7 aldehydes, 5 polyphenols, and 4 volatile acid components was conducted. The predominant

ester compounds in Huangjiu include ethyl lactate, ethyl acetate, isopentyl acetate, and ethyl isovalerate. Among the primary aldehydes are acetaldehyde, benzaldehyde, isovaleraldehyde, and furfural, with furfural and 5-methyl furfural being the primary contributors to the burnt sweetness in food. Volatile phenolic compounds such as guaiacol and 4-ethyl guaiacol impart a smoky and spicy taste, playing a pivotal role in the flavor of Huangjiu. It is noteworthy that excessive volatile acidity can introduce off-flavors in Huangjiu and potentially lead to rancidity development. The content of esters in groups supplemented with cooked wheat Qu or its extract ranged from 650 to 1 100 mg/L, which was more than 2.5 times higher than the HJF group (Fig. 3I). The content of aldehydes was significantly higher in all groups supplemented with cooked wheat Qu, its extract, or mycelium compared to the HJF group (Fig. 3J). The results of phenols and volatile acids showed a similar trend (Figs. 3K-L). Except for the JS, JSJTM, EMJTM, and XJTf group, the content of phenols and volatile acids was significantly higher in other groups compared to the HJF group.

Based on the above results, the addition of cooked wheat Qu or its extract significantly increased the levels of amino acids, organic acids, higher alcohol, esters, aldehydes, polyphenols, and volatile acids in the fermentation system compared to the addition of SU-16 mycelium, revealing the ability of SU-16 metabolites in cooked wheat Qu to regulate the synthesis of flavor substances. Additionally, the macromolecular metabolites of SU-16 exhibited a more pronounced promoting effect on the synthesis of flavor substances than the small molecule metabolites.

3.5 Macromolecular SU-16 metabolites exert a more profound regulatory effect on the metabolic pathways of HJ

Untargeted metabolomics was employed to identify changes in HJ metabolism within the MQF, JTF, XJTF, and HJF group. We observed 143 differential metabolites between the MQF and JTF group, as illustrated in Fig. 4A. Furthermore, a comparison between the JTF and HJF group revealed 516 differential metabolites (Fig. 4B). The XJTF group exhibited 315 differential metabolites compared to the HJF group (Fig. 4C). We found 472 differential metabolites in the JTF group compared to the XJTF group (Fig. 4D).

The differential metabolites observed between the groups were subjected to enrichment analysis in the KEGG pathway database. Under the influence of SU-16 metabolites, the differential metabolites exhibited significant enrichment in several key pathways, including synthesizing secondary metabolites, ABC transporter proteins, amino acid and synthesis, carbohydrate metabolism, and nitrogen

metabolism. Specifically, only 30 differential metabolites between the MQF and JTF group could be annotated to the 13 metabolic pathways of *S. cerevisiae* (Fig. 4E). And, 143 differential metabolites between the JTF and HJF group were annotated to 58 metabolic pathways of *S. cerevisiae* (Fig. 4F). Additionally, 98 differential metabolites between the XJTF and HJF group were enriched in 37 metabolic pathways of *S. cerevisiae* (Fig. 4G). Moreover, when comparing the differential effects of total SU-16 metabolites and small molecule metabolites on HJ metabolism, we found that 124 differential metabolites between the JTF and XJTF group were enriched in 53 pathways of *S. cerevisiae* (Fig. 4H).

The findings above indicate that small molecular metabolites within SU-16 metabolites during fermentation have a relatively minor impact on the metabolism of HJ. In contrast, the macromolecular metabolites secreted by SU-16 during the Qu-making stage substantially affect the metabolism of HJ during the fermentation process.

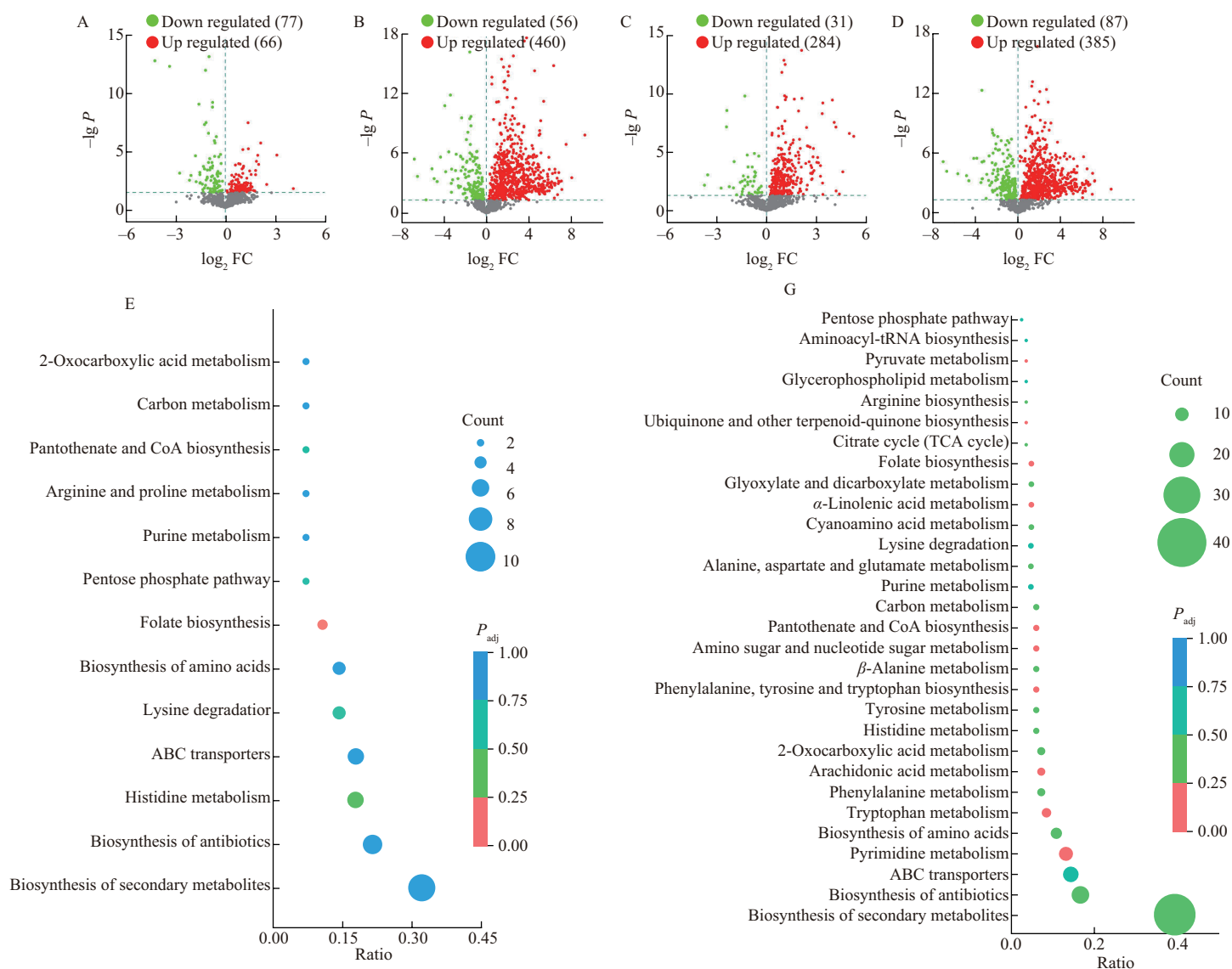


Fig. 4 Differential metabolites among different groups: (A) MQF vs. JTF; (B) JTF vs. HJF; (C) XJTF vs. HJF; (D) JTF vs. XJTF. KEGG pathway enrichment of differential metabolites: (E) MQF vs. JTF; (F) JTF vs. HJF; (G) XJTF vs. HJF; (H) JTF vs. XJTF.

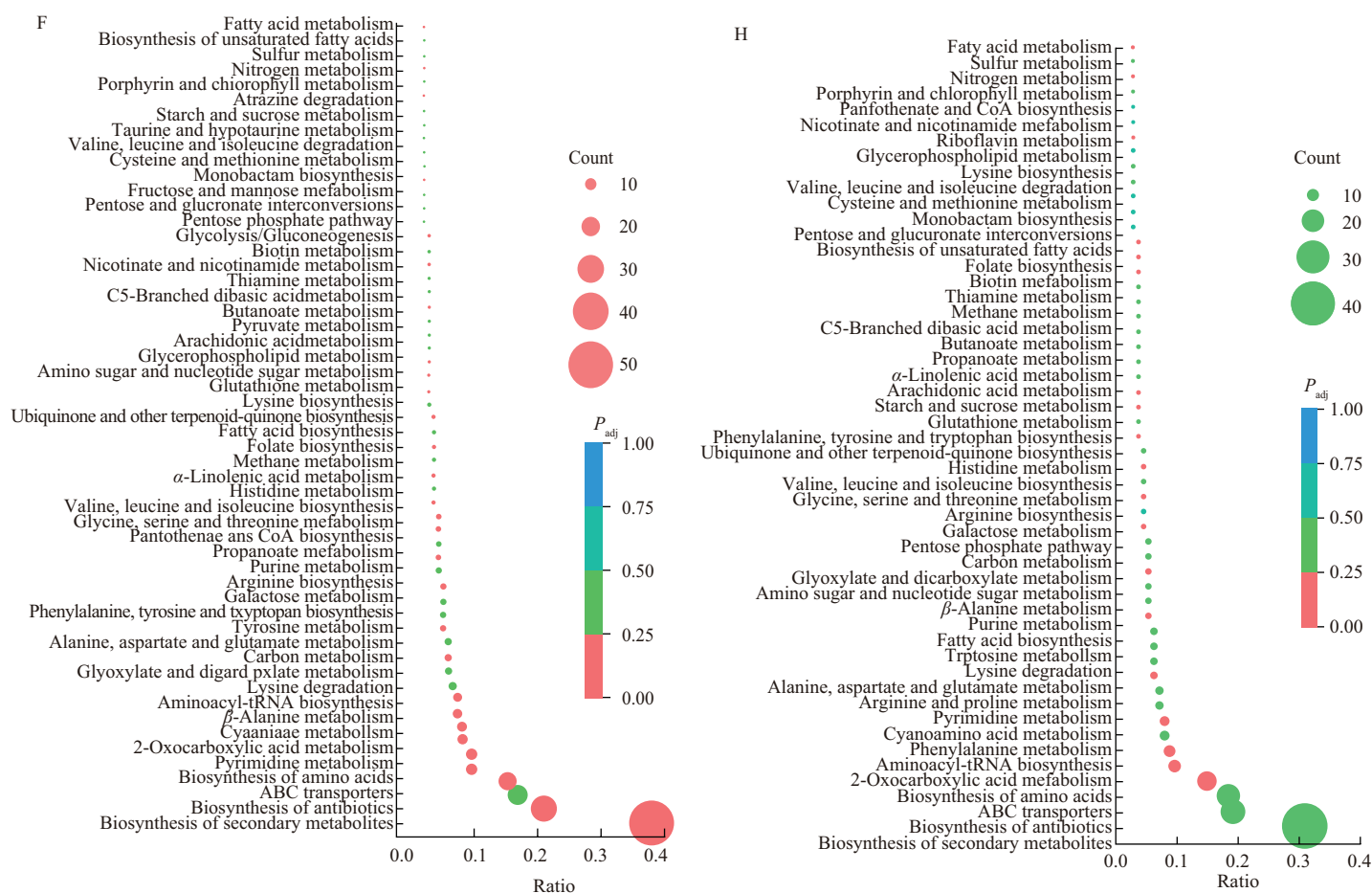


Fig. 4 (Continued)

3.6 Effect of SU-16 metabolites on the formation of flavour substances

Differential metabolites between the JTF, XJTF, and HJF group were identified and analyzed, employing selection criteria of $VIP \geq 1$, $P < 0.05$, and $FC \geq 2$ (Fig. 5).

Under the influence of SU-16 metabolites, most differential metabolites associated with Huangjiu flavor exhibited significant up-regulation; the most important changes were amino acids and their downstream metabolites. Notably, in the groups supplemented with SU-16 metabolites, phenylacetaldehyde, phenylacetic acid, and phenyl lactic acid showed significant up-regulation. Furthermore, the macromolecule metabolites derived from SU-16 facilitated the significant up-regulation of benzoic acid, benzaldehyde, 2-methyl furan, furfural, pyruvic acid, and vanillic acid during fermentation. SU-16 small molecule metabolites had a more significant influence on phenylacetic acid and phenyl lactic acid, while the macromolecular metabolism of SU-16 primarily affected the formation of β -phenyl ethanol, β -phenylacetamide, and phenyl pyruvic acid. Various amino acids, including tyrosine, phenylalanine, valine, leucine, isoleucine, and threonine, were found to be closely associated with the production of flavor-related compounds in Huangjiu. Both macromolecules and small molecule metabolites of SU-16 regulate the content of most amino acids. The ornithine, *D*-ornithine, *L*-serine, *L*-phenylalanine, *L*-valine, and *L*-tyrosine content were significantly up-regulated in fermentation groups supplemented with SU-16 metabolites.

Additionally, changes in *D*-glutamine, *D*-proline, *L*-histidine, *L*-glutamic acid, *L*-aspartic acid, leucine, *L*-norleucine, and *L*-lysine were more influenced by SU-16 macromolecular metabolites. However, compounds such as *L*-lactic acid, isobutyric acid, fumaric acid, ethyl butyrate, and tryptamine are predominantly metabolized by HJ and exhibit lesser susceptibility to the influence of SU-16 metabolites.

3.7 Metabolic division between SU-16 and HJ in co-fermentation system

We identified 528 differential metabolites between SU-16-inoculated cooked wheat Qu and wheat alone. Subsequently, these differential metabolites were mapped to the corresponding metabolic pathways in the genome of *A. flavus*, as depicted in Fig. 6A. Among these metabolites, 179 matched 56 pathways in the *A. flavus* genome. The metabolites were primarily concentrated in pathways associated with secondary metabolite synthesis, ABC transporters, antibiotic synthesis, amino acid biosynthesis, etc.

The compounds annotated to the metabolic pathway of the *A. flavus* genome are entirely associated with the substance metabolism of SU-16. The 130 significantly up-regulated differential metabolites are produced through the metabolism of SU-16 in cooked wheat Qu. These metabolites encompass various categories, including 26 amino acids, peptides and their derivatives, 5 benzaldehyde and its

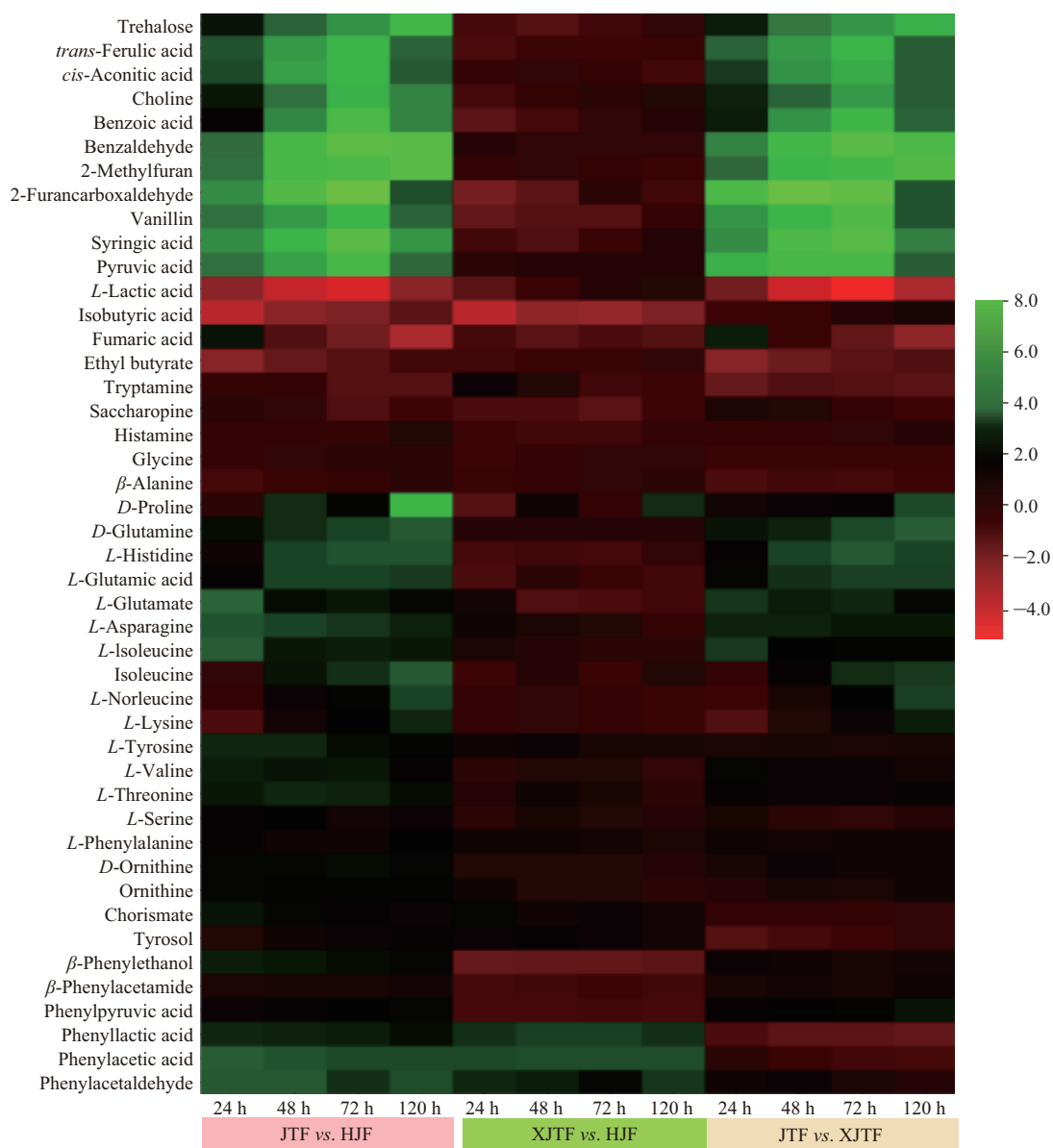


Fig. 5 Changes between the JTF, HJF, and XJTF group in differential metabolites during fermentation.

derivatives, 17 carbohydrates and their derivatives, 11 fatty acids and complexes, etc. Among these metabolites, several showed particularly significant up-regulation by more than 10-fold, including β -phenyl ethanol, phenyl-ethyl aldehyde, furfural, histamine, pure phenolic acid, ornithine, threonine, tyrosine, and pyroglutamic acid, etc. (Fig. 6B).

The role of HJ in the formation of flavor substances was investigated in a fermentation system using pure HJ as the sole starter and supplemented with enzyme preparations. Metabolites in samples at various fermentation stages were detected, and metabolic differences were analyzed using the samples throughout the fermentation process and the samples at the initiation of fermentation. A total of 646 differential metabolites were obtained. The identified differential metabolites were annotated with metabolic pathways and mapped to the *S. cerevisiae* genome metabolic pathways. As depicted in Fig. 6C, the results revealed that 221 metabolites were matched to 51 metabolic pathways of *S. cerevisiae*. These metabolites were predominantly involved in various pathways, including synthesizing secondary metabolites, ABC transporters, antibiotic synthesis,

phenylalanine metabolism, and synthesis, as well as various other amino acid metabolic pathways.

The compounds annotated to the metabolic pathway of the *S. cerevisiae* genome are entirely associated with the substance metabolism of HJ. HJ produces 161 significantly up-regulated differential metabolites during fermentation. These differential metabolites encompass various categories, including 19 amino acids, peptides and their derivatives, 18 fatty acids and their complexes, 13 carbohydrates, and their products, as well as polyols, aldehydes, amines, and other compounds such as β -phenyl ethanol, tryptophol, tyrosol, phenylethylamine, and furfural. Notably, amino acids like valine, threonine, and leucine, involved in the Ehrlich pathway related to the production of heterocyclic alcohols, did not exhibit significant changes during fermentation with the addition of HJ alone. On the contrary, compounds such as phenyl pyruvic acid, phenylethylamine, and β -phenyl ethanol, which are associated with the drinking comfort of Huangjiu, showed significant up-regulation (Fig. 6D).

3.8 DEGs of HJ related to amino acid transport and metabolism during co-fermentation

Under the influence of SU-16 metabolites, HJ exhibits 77 DEGs associated with amino acid transport and metabolism. Among these, 22 genes encoding amino acid transport proteins have been identified, while 55 genes are involved in amino acid metabolism (Fig. 7A). Specific genes, namely *GAP1*, *AGP1*, *BAP2*, *BAP3*, and *TAT2*, are likely involved in phenylalanine transport. Furthermore, *GAP1*, *PUT4*, and *AGP1* have been identified as highly expressed genes when amino acids act as the primary nitrogen source^[21]. *GAP1* plays a critical role in amino acid transport, and the invasive growth of yeast, and *GAT1* positively influences its functionality. Additionally, phenylpyruvate decarboxylase activities are positively affected by *GAT1*^[21]. During fermentation, genes such as *GAT1*, *AGP1*, *BAP2*, *GAP1*, and *PUT4* were significantly up-regulated, while *AVT5*, *BAP3*, and *CAN1* were significantly down-regulated.

Most genes within the pool of 55 DEGs associated with amino acid biosynthesis and catabolic pathways exhibited significant down-regulation in their expression levels. Nevertheless, 17 genes within this group demonstrated significant up-regulation, emphasizing the intricate regulatory dynamics governing amino acid metabolism in

response to the fermentation environment. During the co-fermentation process, HJ's metabolism of amino acids may have been affected by nitrogen catabolite repression (NCR)^[21]. The expression of genes related to preferred amino acid metabolism (such as *AGP1*, *GAP1*, *GPX1*, *HOM3*, *IDH2*, and *GOT2*) was significantly up-regulated. In contrast, genes related to non-preferred amino acid metabolism (such as *CAN1*, *ARG1*, *ARG2*, *ARGH*, *ARGJ*, *HISC*, *LYS1*, *LYS9*, and *LYS12*) were significantly down-regulated. However, interestingly, some DEGs (*ARGF*, *AMD1*, *CARNMT1*, *METC*, *PDHD*, *PUT4*) involved in the catabolism of non-preferred amino acids such as carnosine, cysteine, lysine, methionine, and proline were also significantly expressed, this may be related to the significant up-regulation of *GAT1*.

3.9 DEGs of HJ related to carbohydrate during co-fermentation

The sample fermented for 0 h was analyzed as a control, and 113 DEGs associated with carbohydrate metabolism were identified in the HJ genome across various fermentation stages (Fig. 7B). Among these, 15 glycosyltransferase-encoding genes involved in the biosynthesis of mannan, chitin, glycogen, glucan, alginate,

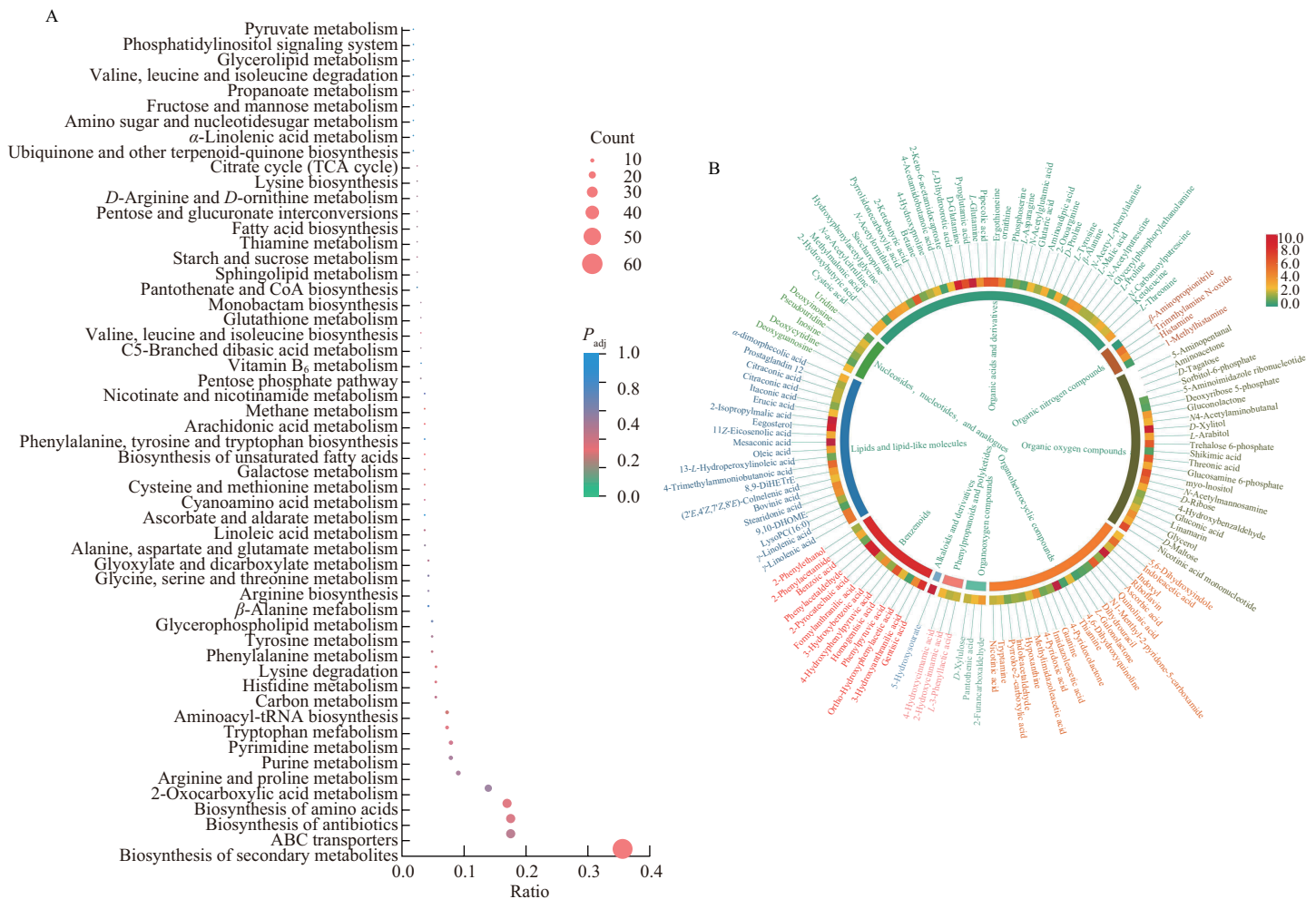


Fig. 6 (A) Number of differential metabolites mapping to the metabolic pathway of the *A. flavus* genome. (B) Potential contributions of *A. flavus* SU-16 metabolites in cooked wheat Qu to Huangjiu flavour. (C) Number of differential metabolites mapping to the metabolic pathway of the *S. cerevisiae* genome. (D) Potential contributions of *S. cerevisiae* HJ metabolites to Huangjiu flavour during fermentation.

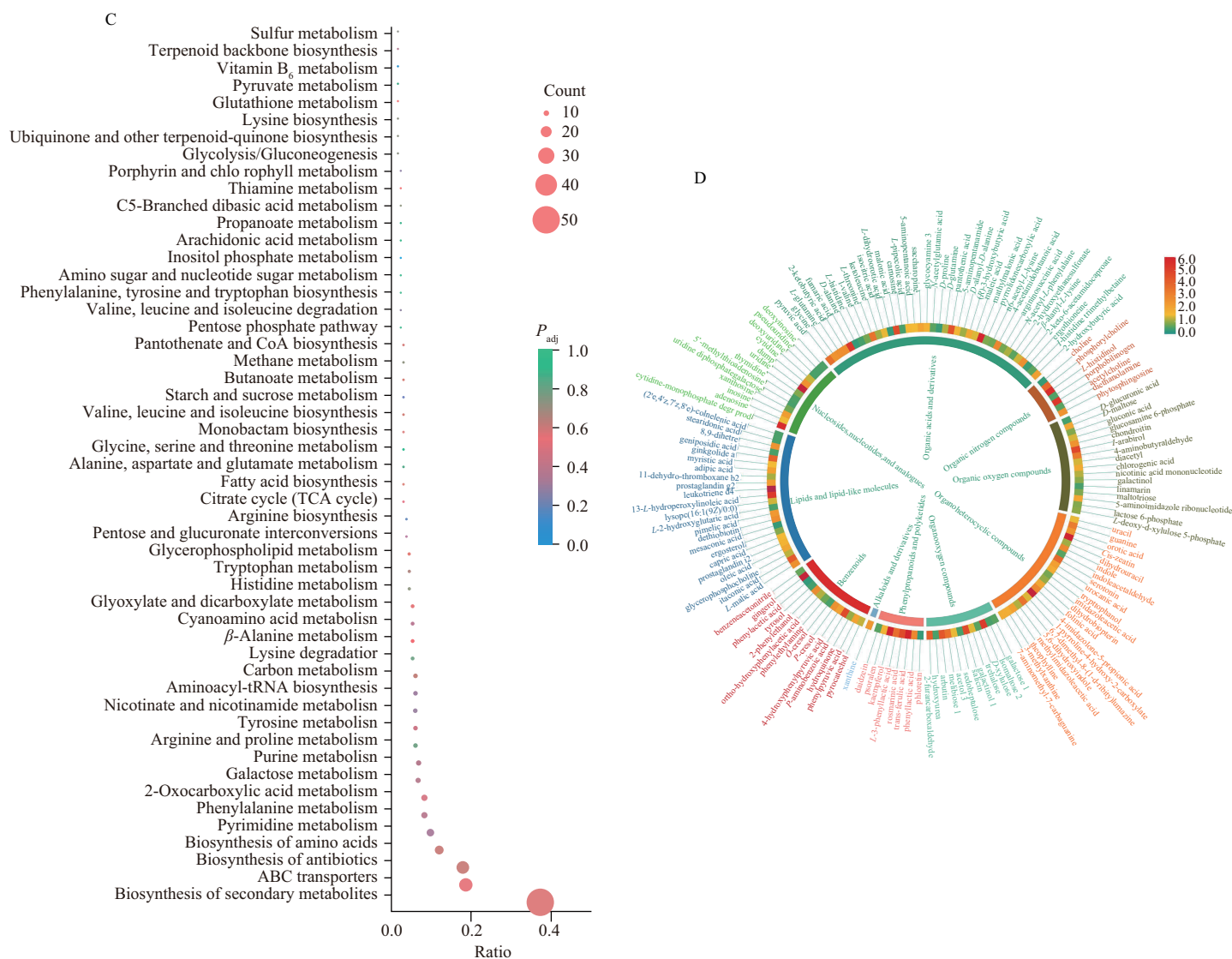


Fig. 6 (Continued)

and *N*-glycans exhibited significant up-regulation in response to SU-16 metabolites. Notably, the overexpression of *GSY1* and *GSY2* can enhance the survival and fermentation ability of *S. cerevisiae* under glucose deficiency conditions^[22]. Conversely, 7 genes linked to the mannan and *N*-glycan biosynthesis pathways were notably down-regulated (*ALG5*, *ALG8*, *ALG11*, *ALG12*, *PMT3*, *PMT4*, and *PMT6*). Genes such as *EXG2*, *GLC3*, *SGA1*, *UTH1*, *CRR1*, *SCW10*, and *PGU13*, involved in the metabolism of glucan, starch, oligosaccharides, *N*-glycans, and pectin, displayed significant up-regulation. Furthermore, 4 genes involved in the degradation and biosynthesis of α,α -alginate (*ATH1*, *NTH1*, *NTH2*, and *TPS2*) exhibited significant up-regulation. Simultaneously, the expression levels of 15 genes associated with the hydrolysis of chitin (*CTS2*), dextran (*EXG1*, *SCW4*, *SCW10*, *SCW11*), and oligosaccharides (*GANAB*, *CWH41*, *IMA1-5*, *MAL12*, *MAL32*) were markedly down-regulated. Notably, *IMA1-5*, *MAL12*, and *MAL32* demonstrated hydrolysis activity on dextrans and isomaltulose, leading to the production of *D*-glucose.

S. cerevisiae utilizes *D*-glucose in alcoholic fermentation, employing glycolysis, the pentose phosphate pathway, and the

TCA cycle to yield flavor substances. Only approximately 1/3 of the DEGs demonstrated up-regulated expression levels throughout fermentation. During glycolysis, the expression levels of *PGM1*, *GAL10*, *FBP1*, *TDH1*, *ERR1-3*, *TDH1*, *ERR1*, *ERR2*, *THI3*, *PDC6*, *ACS1*, *SFA1*, *ALD2* and *ALD3* were significantly increased. The up-regulation of *ACS1* potentially contributes to heightened ethanol tolerance in yeast cells. Pyruvate decarboxylase (*PDC1*, *PDC5*, and *PDC6*), a pivotal enzyme in alcoholic fermentation, demonstrated notable expression changes during fermentation. Specifically, *PDC6* experienced significant up-regulation, while the expression of *PDC1* and *PDC5* was down-regulated. Notably, among the genes associated with the pentose phosphate pathway, only the expression levels of *ZWF1*, *SOL1*, *SOL4*, and *GND2* demonstrated a significant increase. Acetyl coenzyme A generated through glycolysis can combine with oxaloacetate, forming citric acid and entering the TCA cycle. Interestingly, most genes in the TCA cycle displayed significant down-regulation. Only *CIT2*, *IDH1*, *SDH4*, and *MDH2* expression levels were up-regulated. *CIT2* is responsible for the condensation of succinyl-CoA and oxaloacetate to form citric acid. Oxaloacetate within

the TCA cycle can convert to phosphoenolpyruvate through the action of phosphoenolpyruvate carboxykinase, enabling its re-entry into the glycolysis and gluconeogenesis pathway. The gene encoding this enzyme, *PCK1*, exhibited significant up-regulation in this process. Notably, there

are interconversions between glycolysis and the pentose phosphate pathway, involving *D*-glucose-6-P and *D*-glucono-1,5-lactone-6P, *D*-fructose-6-P and glyceraldehyde-3P, as well as *D*-xylulose-5P, and glyceraldehyde-3P, along with *D*-erythrose-4P (Fig. 8).

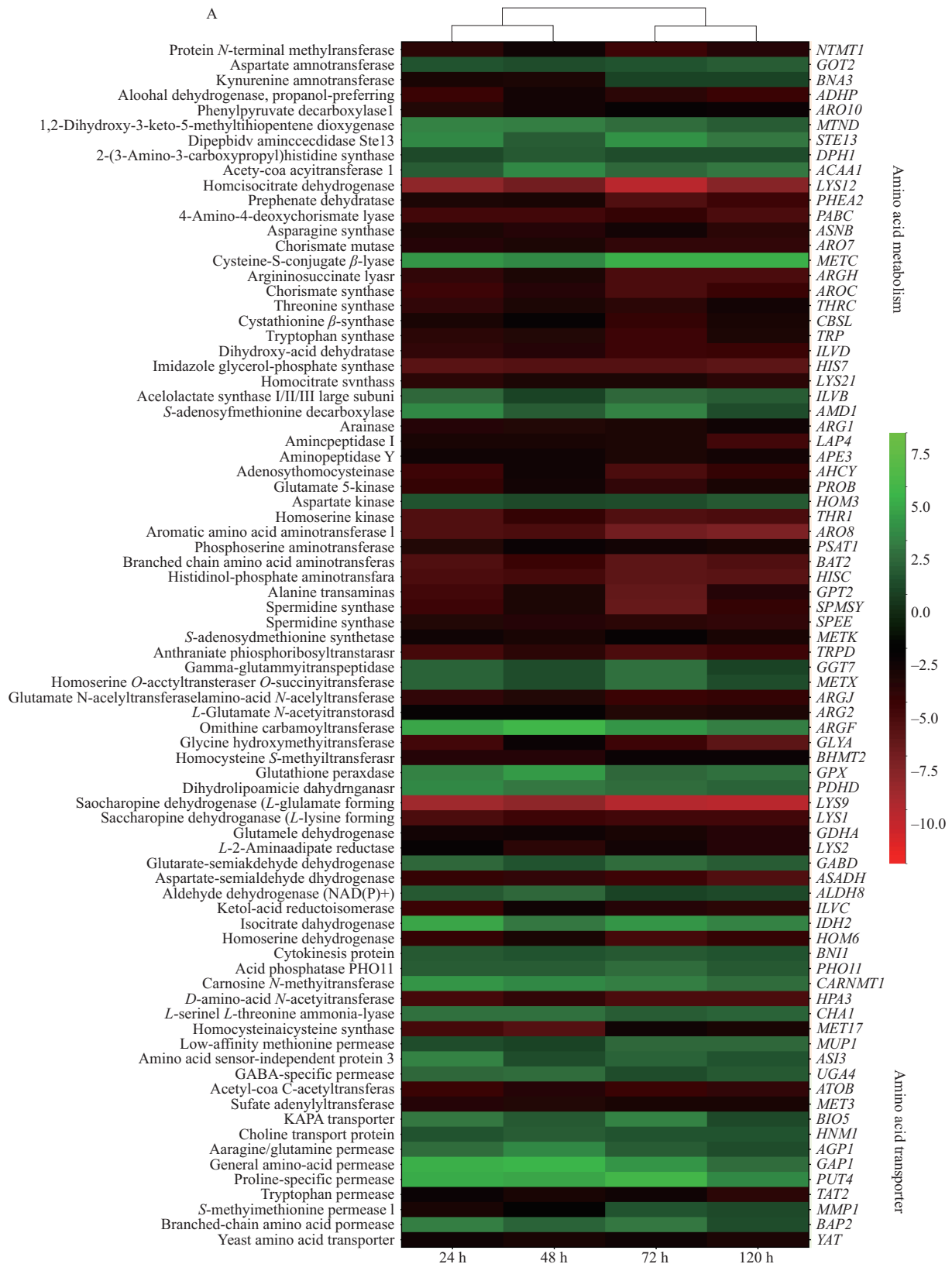


Fig. 7 (A) Differentially expressed genes related to amino acid transport and metabolism. (B) Differentially expressed genes related to carbohydrate metabolism. 24 h, 24 h vs. 0 h; 48 h, 48 h vs. 0 h; 72 h, 72 h vs. 0 h; 120 h, 120 h vs. 0 h.

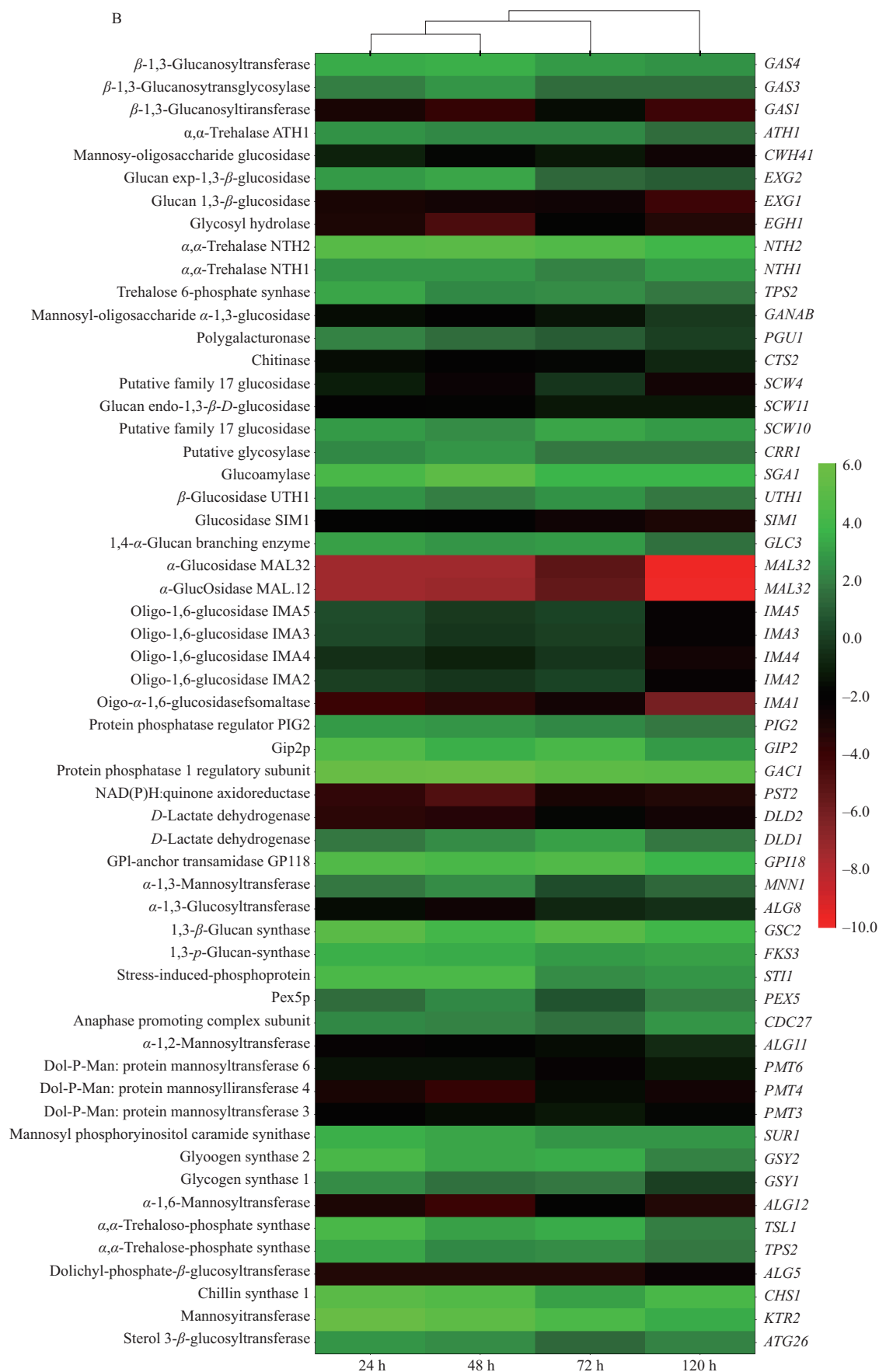


Fig. 7 (Continued)

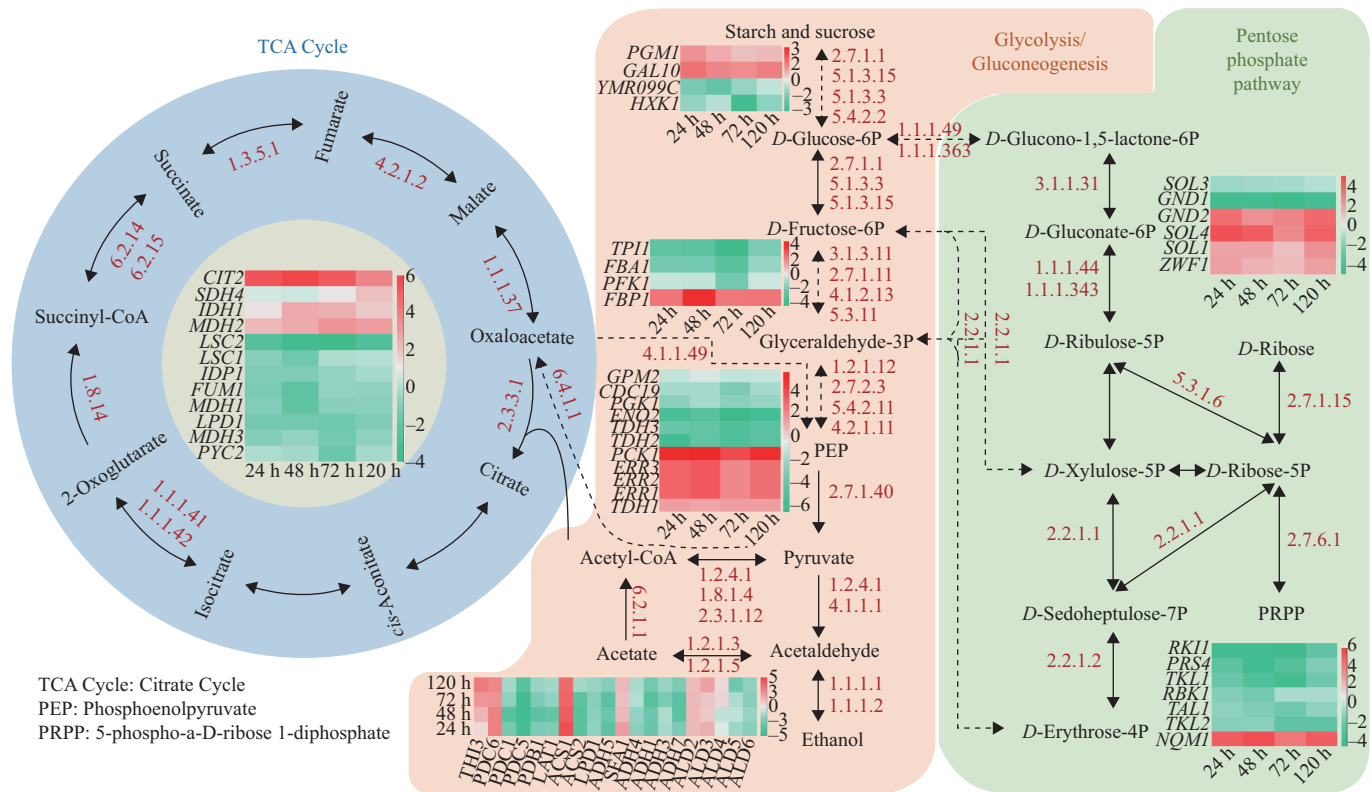


Fig. 8 Differentially expressed genes involved in glycolysis/gluconeogenesis, the TCA cycle, and the pentose phosphate pathway. 24 h, 24 h vs. 0 h; 48 h, 48 h vs. 0 h; 72 h, 72 h vs. 0 h; 120 h, 120 h vs. 0 h.

4. Discussion

In recent years, the application of omics technology in microbial fermentation research has prompted people to shift their focus to understanding the mechanisms of microbial interactions^[23-24]. Dual RNA-seq offers an approach for the simultaneous detection of multiple microbial transcripts in mixed systems, providing a comprehensive understanding of microbial interactions at the transcriptional level^[25-26]. In this investigation, the application of dual RNA-seq and transcriptome analysis revealed substantial inhibition of the growth and transcriptional activity of SU-16 during fermentation. Due to the significant difference in the number of reads between the 2 genomes, dual RNA-seq analysis cannot be effectively conducted. The low transcript levels of the *SU-16* gene suggest minimal growth and metabolic activities during the fermentation process, which underscore the active gene expression of HJ in the fermentation system and its predominant role in the fermentation process. Previous studies have shown that typical metabolites of *S. cerevisiae* significantly inhibit the growth of *L. plantarum*^[27]. It is speculated that continuous changes in environmental factors, such as an increase in total acid, ethanol, and a decrease in oxygen content, along with the production of signaling molecules like β -phenyl ethanol and tryptophol, inhibited the growth of SU-16, led to a loss of transcriptional activity in most of its genes^[28]. Hence, the transcriptional and metabolic activities of the SU-16 strain during fermentation have a relatively limited impact on the fermentation system.

Subsequent investigations concentrated on analyzing the role of SU-16 metabolites within the co-fermentation system. In the context

of multi-strain co-fermentation, the metabolites produced by certain microorganisms serve dual roles as both nutrients and signaling molecules^[29]. Indirect interactions due to extracellular metabolites were demonstrated in wine fermentation^[30]. In this study, through the assessment of fundamental physicochemical indicators of Huangjiu fermentation and the growth status of microorganisms, it was discerned that the metabolites of SU-16 in cooked wheat Qu serve as pivotal factors propelling Huangjiu fermentation. These metabolites play a crucial role in ensuring the production of ethanol, total acid, and amino nitrogen, along with optimizing the utilization efficiency of reducing sugars. Simultaneously, these metabolites contribute to the promotion of the growth of HJ, emphasizing their essential role in shaping the overall fermentation outcomes.

Furthermore, the findings indicated that SU-16 metabolites with different molecular weights exert distinct regulatory effects on the fermentation system. Small molecule metabolites, mainly including amino acids, peptides, carbohydrates, and fatty acids, primarily function as nutrients to support the growth of HJ in the early stage of fermentation. These small molecules can also serve as precursors for the biosynthesis processes within HJ. Nevertheless, the contribution of these small molecular substances alone falls short of meeting the demands of the fermentation process. By comparing fermentation samples with SU-16 metabolites of different molecular weights added, it was found that the macromolecular metabolites of SU-16 significantly increased the amino acids, organic acids, higher alcohol, esters, aldehydes, phenols, and volatile acids more than the small molecule metabolites. These substances constitute the primary components of the flavor profile in Huangjiu, underscoring the

pivotal role of macromolecular metabolites in shaping the sensory characteristics of the final product.

The non-targeted metabolomic analysis further highlighted the substantial influence of SU-16's macromolecular metabolites on the metabolic pathways of HJ. Metaproteomic characterization analysis of wheat Qu indicates that microbial-derived proteins cover a variety of functions, such as energy metabolism, hydrolysis reactions, and signal transduction^[31]. *A. flavus* is the species that contains the most significant number of enzymes related to material metabolism during Huangjiu fermentation^[32], serving as the primary source of highly active glucoamylase, liquefaction enzyme, and protease activities^[14]. The macromolecular metabolites generated by SU-16 in cooked wheat Qu may contain diverse enzyme systems. These enzymes, such as α -amylase, protease, and lipase, play a crucial role in the breakdown of raw materials. The small molecular substances obtained by breaking down the raw materials further serve as nutrients for the growth of *S. cerevisiae*. In addition, genomic analysis of SU-16 indicated an abundance of carbohydrate-active enzymes^[12]. As *S. cerevisiae* lacks several enzymes related to primary carbon metabolism^[33], these macromolecular metabolites may provide functional supplementation for the metabolic activities of HJ during fermentation.

The metabolites found in cooked wheat Qu are not exclusively produced by SU-16 alone. They comprise a diverse range of compounds derived from wheat and metabolites generated by microorganisms in the surrounding environment. In order to ascertain the provenance of these compounds, this study employed a combination of metabolomic and transcriptomic techniques, thereby overcoming the limitations of conventional methods. Through differential metabolite analysis between cooked wheat Qu and wheat, we identified 130 significantly up-regulated substances produced by SU-16 in cooked wheat Qu. These substances mainly include amino acids, peptides, carbohydrates, fatty acids, etc. When incorporated into the Huangjiu fermentation process with wheat Qu, these metabolites contribute to the formation of flavor substances. Analyzing the fermentation system using pure HJ as the sole starter demonstrated that HJ produces 161 significantly up-regulated substances during fermentation, primarily comprising amino acids, peptides, fatty acids, carbohydrates, polyols, aldehydes, etc. In addition, the analysis of SU-16 metabolites on flavor substance formation in Huangjiu revealed differing sensitivities in flavor substance synthesis to SU-16 metabolite regulation. Therefore, 2 primary sources of flavor substances exist in the co-fermentation process: the SU-16 metabolite in cooked wheat Qu and the others produced through HJ metabolism under the influence of SU-16 metabolites.

Metabolomic analysis has affirmed that microbial interactions impact the ultimate metabolite profile by inducing changes across various metabolic pathways^[34-35]. The metabolism of carbohydrates and amino acids plays a crucial role in the efficiency of alcoholic fermentation and the formation of key flavor compounds. *S. cerevisiae* can produce higher alcohol levels through the amino acid catabolism pathway^[36]. Carbohydrates are the primary carbon source for alcoholic fermentation in *S. cerevisiae*^[37-38]. Previous reports have shown a close relationship between the interactions between *S. cerevisiae* and *S. pombe* and the carbohydrate and amino acid metabolism pathways^[35]. In this study, analysis of DEGs during the fermentation process revealed significant differences in the expression of genes related to carbohydrate and

amino acid metabolism in the HJ genome during co-fermentation with SU-16. These results underscore the active involvement and significant role of carbohydrate and amino acid metabolism pathways in co-fermentation.

Wheat Qu is rich in amino acids, polypeptides, oligosaccharides, phenols, and various enzymes, such as liquefaction enzyme, glucoamylase, protease, and esterase^[39-40], which collectively play a crucial role in flavor development. Traditionally, the perception of Qu's role has centered on enriching environmental microorganisms, providing various enzymes for the fermentation system, and contributing small molecule metabolites directly employed as flavor substances^[39]. Our findings further emphasize that in co-fermentation systems with *S. cerevisiae*, metabolites from *Aspergillus* in wheat Qu are more influential driving factors than those of the *Aspergillus* strain. In addition to macromolecular metabolites significantly influencing *S. cerevisiae* metabolism, including various enzyme systems supporting raw material decomposition and substance synthesis, small molecule metabolites produced by *Aspergillus* also play a crucial role in flavor formation and microbial growth. These small molecule metabolites serve as nutrients for microbial growth and precursors for synthesizing flavor substances and have a certain regulatory effect on *S. cerevisiae* metabolism. This interaction and metabolic division of labor between metabolites and microorganisms maintains a stable and efficient fermentation system, which jointly shapes the flavor characteristics of the product. This metabolites-mediated interaction ensures that *Aspergillus* remains central to the fermentation system despite experiencing inhibited growth and transcriptional activity during co-fermentation with *S. cerevisiae*.

In summary, the study, with Huangjiu fermentation as a reference, provides insights into both the fermentation mechanism of Huangjiu and the dynamic regulation and metabolic division between *A. flavus* and *S. cerevisiae* during co-fermentation. The understanding gained from this research enhances the knowledge of microbial interactions in co-fermentation systems.

Competing interests

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://doi.org/10.26599/FSHW.2024.9250445>.

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