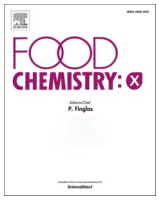
Food Chemistry: X 22 (2024) 101382

Contents lists available at ScienceDirect

Food Chemistry: X

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



Bioturbation analysis of microbial communities and flavor metabolism in a high-yielding cellulase *Bacillus subtilis* biofortified Daqu

Yanbo Liu [a,b,c,d,1], Haideng Li [a,e,1], Wenxi Liu [a,b,c,d], Kejin Ren [a,b,c,d], Xuehan Li [a,b,c,d], Zhenke Zhang [f], Runna Huang [f], Suna Han [f], Jianguang Hou [f], Chunmei Pan [a,b,c,d,*]

[a] College of Food and Biological Engineering (Liquor College), Henan University of Animal Husbandry and Economy, Zhengzhou 450046, China
[b] Henan Liquor Style Engineering Technology Research Center, Henan University of Animal Husbandry and Economy, Zhengzhou 450046, China
[c] Henan Province Brewing Special Grain Development and Application Engineering Research Center, Zhengzhou 450046, China
[d] Zhengzhou Key Laboratory of Liquor Brewing Microbial Technology, Henan University of Animal Husbandry and Economy, Zhengzhou 450046, China
[e] College of Biological Engineering, Henan University of Technology, Zhengzhou 450001, China
[f] Henan Yangshao Distillery Co., Ltd., Mianchi 472400, China

ARTICLE INFO

Keywords:
Daqu
Cellulase
Active microorganisms
Carbohydrate enzymes
Physical and chemical indicators
Flavor substances
Non-volatile metabolites

ABSTRACT

In this study, a fortified Daqu (FF Daqu) was prepared using high cellulase-producing *Bacillus subtilis*, and the effects of in situ fortification on the physicochemical properties, flavor, active microbial community and metabolism of Daqu were analyzed. The saccharification power, liquefaction power, and cellulase activity of the FF Daqu were significantly increased compared with that of the traditional Daqu (CT Daqu). The overall differences in flavor components and their contents were not significant, but the higher alcohols were lower in FF Daqu. The relative abundance of dominant active species in FF Daqu was 85.08% of the total active microbiota higher than 63.42% in CT Daqu, and the biomarkers were *Paecilomyces variotii* and *Aspergillus cristatus*, respectively. The enzymes related to starch and sucrose metabolic pathways were up-regulated and expressed in FF Daqu. In the laboratory level simulation of baijiu brewing, the yield of baijiu was increased by 3.36% using FF Daqu.

1. Introduction

The quality of Daqu plays a key role in the formation of the body and flavor of baijiu (Yang, Fan, & Xu, 2021) Daqu provides the driving force for fermentation, provides a large number of functional microorganisms and complex enzymes in baijiu-making, and uses raw materials for biochemical reactions such as alcoholic fermentation and aroma production, thereby giving baijiu a unique flavor (He, Huang, Wu, Jin, & Zhou, 2020). In addition, Daqu after fermentation and storage can provide a fermentation matrix for the brewing system as a raw material for brewing, and its own rich flavor substances and its precursors can also directly or indirectly enter the baijiu body during the brewing process (Xu et al., 2017). Therefore, it is particularly important to improve the microbial community structure, physical and chemical properties and the flavor substances and flavor precursors of Daqu to improve the baijiu yield and quality.

In the process of Daqu open fermentation, natural inoculation and entrapment of natural microorganisms rely on the accumulation of endogenous bioheat of microorganisms to promote microbial interaction and drive functional microbial community succession, so as to form a stable Daqu microecology (Liu et al., 2023; Yang et al., 2021). The interaction between microorganisms weaves a cooperative network of species working together for common goals for the evolution of Daqu microecosystem (He et al., 2020). However, the microbial succession of non-artificially controlled evolution limits the occurrence and effectiveness of synergistic effects between microbial communities, and it is difficult to ensure the unity of mass and functional expression of Daqu (Li et al., 2020). In recent years, the enhancement of functional microorganisms has been gradually applied to traditional fermented foods, such as baijiu, vinegar, soy sauce and kimchi, and the biodisturbance effect based on microbial enhancement has significantly changed the taxonomic composition, structure and interspecific interaction of

* Corresponding author at: College of Food and Biological Engineering (Liquor College), Henan University of Animal Husbandry and Economy, Zhengzhou 450046, China.
E-mail address: sige518888@163.com (C. Pan).
[1] Both authors contributed equally to the manuscript.

https://doi.org/10.1016/j.fochx.2024.101382
Received 5 February 2024; Received in revised form 8 April 2024; Accepted 9 April 2024
Available online 16 April 2024

microbial communities in traditional fermentation systems and the expression of enzyme-coding genes, which is conducive to the improvement of product quality and yield (He et al., 2020; He, Huang, Zhou, Wu, & Jin, 2019; Li et al., 2020). Therefore, targeted use of functional strains is one of the methods to improve the quality of Daqu.

Studies have shown that cellulase has certain activity in Daqu fermentation and fermented grains fermentation, and cooperates with amylase, glucoamylase, esterase and various microorganisms to ensure the smooth start and progress of fermentation (Shi et al., 2022). The use of cellulase can significantly improve the utilization rate of raw materials, reduce the fusel oil content and improve the aroma substances in baijiu (Zhuansun et al., 2022). In order to ensure the safety of baijiu, exogenous non-fermenting substances are prohibited from being added to the fermentation process, and functional microorganisms can be used as carriers to enter the fermentation system of fermented grains for functional expression (Li, Liu, Liu, Hui, & Pan, 2023). In addition, *Bacillus* bacteria are one of the important functional flora in the microecology of baijiu-brewing, which can secrete a variety of enzymes that hydrolyze raw material components, promote the smooth progress of fermentation process and the full use of biomass (Li, Fernandez, Vederas, & Gänzle, 2023; Tang et al., 2023; Tong et al., 2022). However, due to the little understanding of the biological disturbance mechanism of functional strain enhancement in Daqu, the progress of the enhancement application of functional microorganisms in Daqu is greatly limited.

At present, the study on the use of high-cellulase strains for the fortification of Daqu has not been reported, and there is less understanding of the differences in the active microbiota of Daqu before and after fortification. To this end, this study used metatranscriptomics, metabolomics, physicochemical factors and enzyme activity determination and HS-SPME-GC–MS to analyze the bioturbation effect of high-yield cellulase *Bacillus subtilis* on Daqu.

2. Materials and methods

2.1. Production and sampling of Daqu

The high-yielding cellulase *Bacillus subtilis* was screened from fermented grains, and this strain was screened from our previous work (Liu et al., 2020). Fortified Daqu (experimental group FF Daqu) and traditional Daqu (control group CT Daqu) were produced from Daqu-making workshop of Henan Yangshao Distillery Co., Ltd., and the fortification of functional strains was based on the production process of traditional Daqu, which was carried out by uniform spraying of bacterial liquid onto the surface of the pressed and molded Daqu, with the concentration of bacterial liquid of 10^6 cells/mL (Fig. S1A). Mature Daqu was collected from the upper, middle and lower layers of the stack and the left and right sides of the middle layer (Fig. S1B). Daqu was broken from the middle, the section was observed, the aroma was smelled and the quality was judged (Fig. S1C). Crush each qualified daqu and mix evenly. The samples are packed and sealed in a sterile sampling bag numbered FF-1, FF-2, FF-3, FF-4, FF-5, CT-1, CT-2, CT-3, CT-4, CT-5. Transport to laboratory on dry ice and store at −80 °C. Among them, samples numbered 1, 2, and 3 were used for physicochemical index determination, volatile compound analysis, and metatranscriptome extraction. All samples were analyzed for non-volatile metabolites.

2.2. Analysis of physical and chemical factors and enzyme active substances of Daqu

The moisture content of Daqu was determined by oven-drying constant weight method, that is, dried at 101–105 °C, and the weight change between the last two measurements was <0.002 g. The total titration acidity was determined by acid-base neutralization method (In short, 0.1 mol/L NaOH was used to titrate the sample to pH 8.2, so as to calculate the acidity of Daqu). Starch was hydrolyzed by hydrochloric acid, and the amount of glucose was determined by Fehling method to reflect starch content (Liu, Li, et al., 2023). The fermentation power was determined by carbon dioxide weight loss method (the weight of carbon dioxide produced by liquid fermentation at 35 °C for 72 h with sorghum juice as starter). Under the condition of 35 °C, pH 4.6, the time spent on the disappearance of the blue-violet reaction of the Daqu hydrolysis soluble starch solution to iodine was used to reflect the liquefaction power. The saccharification power was determined by Fehling method, the esterification power was determined by saponification method, and the amino acid nitrogen was determined by potentiometric titration method (Yang, Fan, & Xu, 2021; Yang, Wang, et al., 2021). Cellulase enzyme activity was measured using the CMCA-DNS method according to the standard QB 2583–2003 (Cellulases), which was determined by the quantity of reducing sugar that was hydrolyzed from the substrate sodium carboxymethyl cellulose by 0.5 mL of Daqu filtrate in 30 min at 50 °C (U, mg/g·min) (Shi et al., 2022). The linear equation of glucose obtained in this experiment was $y = 0.1342\times$ - 0.1619 , $R^2 = 0.991$.

2.3. Analysis of volatile compounds in Daqu

Sample preparation: 1 g of Daqu was taken into a headspace bottle, 2 g of NaCl, 5 mL of distilled water were added, the stopper was screwed tightly and shaken well. The headspace vial was preheated in a water bath at 60 °C for 10 min, and a solid-phase CAR/PDMS (75 μm CAR/PDMS, carbon molecular sieve/polydimethylsilane) extraction fibre tip was inserted into the silicone stopper of the headspace vial, and was inserted into the sample for headspace adsorption for 30 min. GC conditions: the chromatographic column was HP-FFAP (30 m × 0.32 mm × 0.25 μm); no shunt, flow rate 1.21 mL/min; inlet temperature: 250 °C; heating procedure: 40 °C was held for 3 min, 5 °C/min to 60 °C without holding, 8 °C/min to 230 °C and held for 7 min; mass spectrometry (MS) conditions: the interface temperature was 220 °C, and the ionization mode was an electron ionization source with an electron energy of 70 eV, and the ion source temperature was 200 °C (Kang, Chen, Han, & Xue, 2022). The compounds were quantified by calculating the percentages of GC peak areas. Then analysis of relative odor activity value (ROAV) based on methods reported in the literature (Mu et al., 2023).

2.4. Metatranscriptomic analysis of Daqu

2.4.1. RNA extraction and shotgun sequencing

Total RNA was extracted using the RNA Power Soil® Total RNA Isolation Kit (12866–25) (MoBio, USA) according to the manufacturer's instructions. The extracted RNA was completed using 1.5% agarose gel electrophoresis and UV spectrophotometer for quality determination and quantification. Meanwhile, RNA integrity (RIN ≥ 5.5) was detected using an Agilent 2100 (Agilent, USA). After removal of ribosomal RNA for microbial RNA, Illumina's TruSeq Stranded mRNA LT Sample Prep Kit (Illuminia, USA) was used for reverse transcription as well as macrotranscriptome birdshot sequencing library construction. Each library was sequenced by Illumina NovaSeq platform (Illumina, USA) with PE150 strategy at Personal Biotechnology Co., Ltd. (Shanghai, China).

2.4.2. Metatranscriptomics analysis

Raw sequencing reads were processed to obtain quality-filtered reads for further analysis. First, sequencing adapters were removed from sequencing reads using Cutadapt (v1.17). Secondly, low quality reads were trimmed using a sliding-window algorithm in fastp (v0.20.0). Then, Ribosomal RNA was removed by SortMeRNA (v4.2.0) using its default rRNA reference database.

Once quality-filtered reads were obtained, taxonomical classifications of metatranscriptomics sequencing reads from each sample were performed using Kaiju with greedy-5 mode against an nr-derived database, which included proteins from archaea, bacteria, viruses, fungi and microbial eukaryotes. All raw sequences were deposited in the NCBI Sequence Read Archive under accession number PRJNA1087545.

2.5. Analysis of non-volatile compounds

Sample preparation: 1). Accurately weigh an appropriate amount of sample into a 2 mL centrifuge tube, add 100 mg glass bead. 2). Add 1000 μL 50% methanol water (stored at 4 °C), vortex for 30 s. 3). Put the centrifuge tube containing the sample into the 2 mL adapter matched with the instrument, immerse it in liquid nitrogen for rapid freezing for 5 min, take out the centrifuge tube and thaw at room temperature, put the centrifuge tube into the 2 mL adapter again, install it into the tissue grinder and grind it at 55 Hz for 2 min. 4). Repeat step 3 twice. 5). Take out the centrifuge tube, centrifuge for 10 min at 12,000 rpm and 4 °C, take all the supernatant, transfer it to a new 2 mL centrifuge tube, concentrate and dry it. 6). Accurately add 300 μL of 2-Amino-3-(2-chloro-phenyl)-propionic acid (4 ppm) solution prepared with 50% methanol water (stored at 4 °C) to redissolve the sample, filter the supernatant by 0.22 μm membrane and transfer into the detection bottle for LC-MS detection. Liquid chromatography condition: The LC analysis was performed on a Vanquish UHPLC System (Thermo Fisher Scientific, USA). Chromatography was carried out with an ACQUITY UPLC ® HSS T3 (150 × 2.1 mm, 1.8 μm) (Waters, Milford, MA, USA). The column maintained at 40 °C. The flow rate and injection volume were set at 0.25 mL/min and 2 μL, respectively. For LC-ESI (+)-MS analysis, the mobile phases consisted of (B2) 0.1% formic acid in acetonitrile (v/v) and (A2) 0.1% formic acid in water (v/v). Separation was conducted under the following gradient: 0–1 min, 2% B2. 1–9 min, 2% ~ 50% B2. 9–12 min, 50% ~ 98% B2. 12–13.5 min, 98% B2. 13.5–14 min, 98% ~ 2% B2. 14–20 min, 2% B2. For LC-ESI (−)-MS analysis, the analytes were carried out with (B3) acetonitrile and (A3) ammonium formate (5 mM). Separation was conducted under the following gradient: 0–1 min, 2% B3. 1–9 min, 2% ~ 50% B3. 9–12 min, 50% ~ 98% B3. 12–13.5 min, 98% B3. 13.5–14 min, 98% ~ 2% B3.14–17 min, 2% B3. Mass spectrum conditions: Mass spectrometric detection of metabolites was performed on Q Exactive (Thermo Fisher Scientific, USA) with ESI ion source. Simultaneous MS1 and MS/MS (Full MS-ddMS2 mode, data-dependent MS/MS) acquisition was used. The parameters were as follows: sheath gas pressure, 30 arbs. Aux gas flow, 10 arbs. Spray voltage, 3.50 kV and − 2.50 kV for ESI (+) and ESI (−), respectively. Capillary temperature, 325 °C. MS1 range, *m/z* 100–1000. MS1 resolving power, 70,000 FWHM. number of data dependant scans per cycle, 10. MS/MS resolving power, 17,500 FWHM. normalized collision energy, 30 eV. dynamic exclusion time, automatic.

2.6. Analysis of cellulose content and baijiu yield of fermented grains

Take 2.5 kg of sorghum, crush the sorghum granules into 4–5 petals by crusher, add 75% 90 °C hot water with sorghum quality, mix well and soak for 12 h, evenly spread on the upper layer of grate with gauze, put it in a boiling water steamer, steam the grain for 40 min, immediately take out the grain, add 20% warm water of sorghum quality, and add 0.5 kg of rice husk steamed for 30 min to mix well, spread and cool. When the grain temperature was lower than 25 °C, 0.75 kg of Daqu powder was added to the control group (CT Daqu) and the experimental group (FF Daqu). After mixing, it is packed in a ceramic jar and subjected to solid fermentation at room temperature. The cellulose content was determined by fermentation for 35 days, and the colorimetric method was used: cellulose was hydrolyzed to β-glucose by heating under acidic conditions. Then, under the action of concentrated sulfuric acid, the monosaccharides are dehydrated to form furfural compounds. Colorimetric assays can be performed by reacting anthrone reagents with the blue-green color of furfural compounds (Xu et al., 2023). The linear equation of cellulose is $y = 0.0023\times$ - 0.0164, $R^2 = 0.977$.

After 35 days of solid-state fermentation, the fermented grains were distilled (the steamed rice husk was added according to the moisture content of fermented grains), and the baijiu head (rich in higher alcohols) was removed after 20 mL. The yield of base baijiu (kg) = the conversion coefficient of base baijiu sample alcohol content converted to 65%vol base baijiu × the quality of base baijiu before conversion. Raw material baijiu yield (%) = converted to 65%vol base baijiu yield / raw material consumption × 100.

2.7. Data processing

Use Excel 2019 for raw data collation (Microsoft Corp., Redmond, WA, USA), Origin 2023 for histogram graphing (OrginLab., Northampton, MA, USA), SAS 9.2 for data statistics and analysis (SAS Inc., Cary, NC, USA). Multivariate statistical analysis and mapping were performed using R language vegan package, ggplot2 package, ropls package, etc.

3. Results

3.1. Sensory evaluation and physical and chemical property analysis of Daqu

Sensory evaluation showed that the surface of FF Daqu was yellowish brown, with a few cracks, neat cross-section, abundant hyphae, a little flocculent color, and pure aroma. The appearance of CT Daqu was yellowish brown with a few cracks. The section has rich hyphae, single color and no variegated color, pure aroma, both Daqu reached the excellent level (Fig. S1C). The physical and chemical properties of Daqu are direct indicators of whether Daqu fermentation is mature and the quality of Daqu (Deng et al., 2020). As shown in Fig. S2, the cellulase activity, acidity, amino acid nitrogen content, saccharifying power and liquefaction power of FF Daqu were significantly higher than those of CT Daqu. The cellulase activity, saccharification power and liquefaction power in the enzyme activity increased by 8.97%, 122.67% and 157.57%, respectively. The acidity and amino acid nitrogen content in the physical and chemical factors increased by 18.18% and 95.52%, respectively. There was no significant difference in moisture content, starch content, fermentation ability and esterification ability between two kinds of Daqu.

3.2. HS-SPME-GC–MS analysis of Daqu volatile flavor compounds

3.2.1. Composition and difference analysis of Daqu flavor substances

A total of 60 volatile flavor compounds were identified in these two kinds of Daqu, including 16 acids, 13 esters, 10 aldehydes, 7 alcohols, 4 ketones, 4 phenols, 3 aromatics and 3 others. Among them, 44 and 50 flavor compounds were identified in FF and CT Daqu, respectively (Table S1). The relative content of acid compounds in FF Daqu was significantly higher than that in CT Daqu. In addition, the ketone compounds in FF Daqu were significantly lower than those in CT Daqu, while there was no significant difference in alcohols, esters, phenols, aldehydes and aromatic compounds (Fig. 1A). PCA analysis showed that the confidence ellipse was not separated within the 95% confidence interval, and the flavor composition of the two Daqu was similar (the overall difference explained rate was 66.4%). The bioturbation of high-yield cellulase strains to strengthen Daqu had little effect on the flavor composition of Daqu (PERMANOVA: $R^2 = 0.39$, $p = 0.1$) (Fig. 1B). In order to further analyze the difference in the contribution of flavor substances between the two Daqu samples, the variable importance projection (VIP) of OPLS-DA was used to determine the candidate differential flavor compounds in Daqu samples. It can be seen that Q^2 was 0.4, R^2Y was 0.7, and R^2X was 0.6, indicating that the model fitting accuracy was good (Fig. 1C). With VIP value >1 as the threshold, as shown in Fig. 1D, 29 flavor substances are the key substances for the difference in Daqu flavor.

3.2.2. Analysis of flavor contribution compounds of Daqu

The relative odor activity value (ROAV) was calculated according to the relative abundance of the odor threshold binding assay of the compound, and the main flavor substance (ROAV ≥1) common to CT

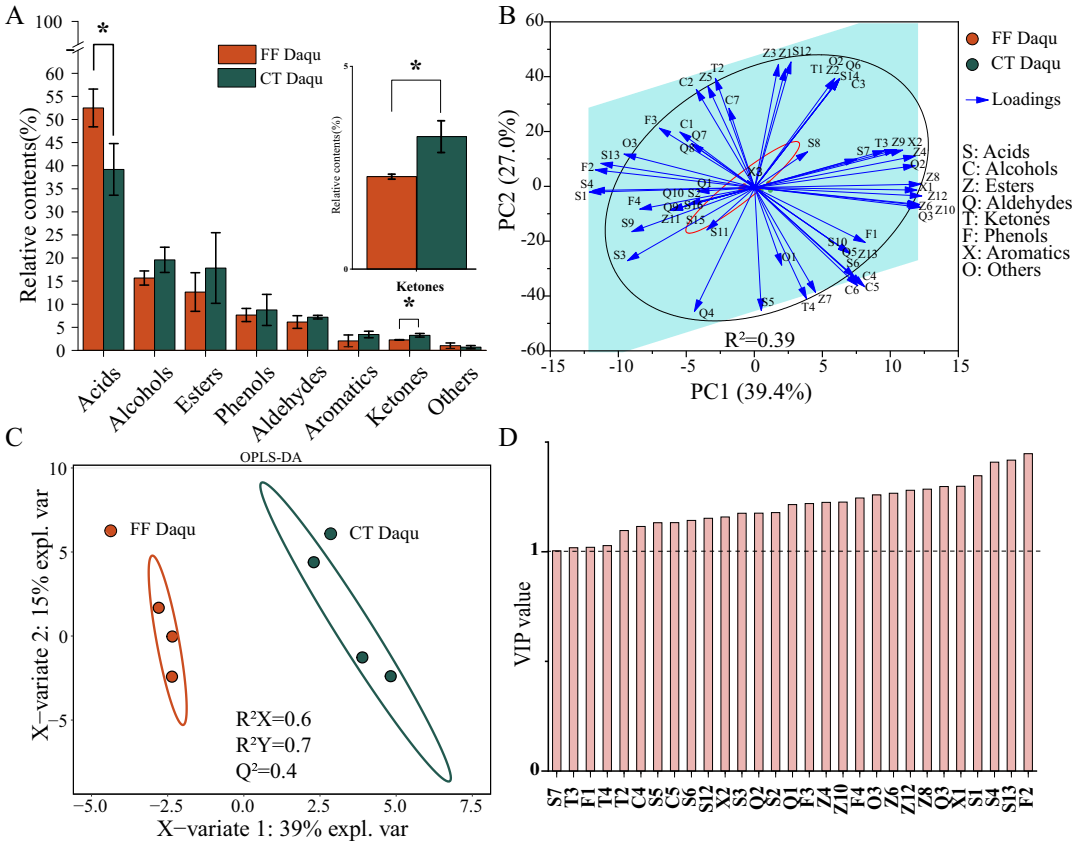


Fig. 1. CT and FF Daqu volatile flavor composition classification histogram (A), principal component analysis (B), OPLS-DA score diagram (C) and volatile flavor substance VIP value diagram (D).

and FF Daqu was 3-Methylbutanoic acid, Hexanoic acid, Phenylethyl alcohol, Hexanoic acid ethyl ester and Acetophenone, there was no significant difference in the relative content of the above main flavor substances ($p > 0.05$) (Table S1). In addition, the unique main flavor substance of CT Daqu is 1-Octen-3-ol, Octanoic acid ethyl ester, and the unique main flavor substance of FF Daqu is Nonanal (Table S1). Octanoic acid, 9-Octadecenoic acid ethyl ester, Benzaldehyde are modified compounds for the overall flavor of CT Daqu ($0.1 < ROAV < 1$). 2-Methylpropanoic acid, Octanoic acid, Benzaldehyde, and Vanillin are modified compounds for the overall flavor of FF Daqu (Table S1). The composition and content of the main body and modified flavor compounds of the two Daqu were not significantly different ($p > 0.05$).

3.3. Analysis of the composition and differences of active microbiota of the Daqu

Metatranscriptomics was utilized to explore the active microorganisms of mature Daqu, and 43.53 Gbp of raw data were obtained from 288,295,034 reads, in which the percentage of ambiguous bases was low (0.002%), the GC accounted for about 45% of the total, and the percentage of bases with base identification accuracy of 99% or more (Q20) were higher than 92%, and both the amount of sequencing and the high quality base percentage indicated that the overall quality of sequencing was high (Table S2). Sequences that were too short in length, contained too many ambiguous bases and adulterated with adapters (Adapter) were further screened and filtered to obtain 88.34% of valid reads yielding 38.17 Gbp of clean data for subsequent analysis (Table S2). In addition, splicing was performed with MEGAHIT, 559630 non-redundant contigs with an N50 length of 642 bp were obtained, which were then predicted to 10,089 non-redundant proteins (Table S3).

Using Kaiju annotated to 151 phylums, 233 orders, 607 orders, 1350 families, 4124 genera, and 15,042 species. The relative abundance of dominant phyla (relative abundance >1%), dominant genera, and dominant species of the FF Daqu and CT Daqu accounted for 98.43%, 97.34%, 95.79%, 91.50%, and 85.08%, 63.42%, respectively, of all active microorganisms. As shown in Fig. 2A, fungi predominate in the active microbial community. The dominant phylum of FF Daqu is Ascomycota and Pisuviricota (Fig. 2B), the dominant genus is *Aspergillus*, *Paecilomyces*, *Penicillium* (Fig. 2C), and the dominant species are *Paecilomyces variotii*, *Aspergillus chevalieri*, *Aspergillus cristatus*, *Aspergillus glaucus*, *Aspergillus ruber*, *Aspergillus wentii*, *Penicillium steckii*, *Penicillium decumbens* (Fig. 2D). The dominant phylum of CT Daqu is Ascomycota (Fig. 2B), and the dominant genus is *Aspergillus*, *Paecilomyces*, *Penicillium*, *Talaromyces* and *Rasamsonia* (Fig. 2C), the dominant species are *Paecilomyces* variotii, *Aspergillus chevalieri*, *Aspergillus cristatus*, *Aspergillus glaucus*, *Aspergillus ruber*, *Aspergillus wentii*, *Penicillium steckii*, *Penicillium decumbens*, *Rasamsonia emersonii*, *Aspergillus terreus*, *Ophiocordyceps lanpingensis*, *Aspergillus sclerotialis*, *Penicillium brasilianum*, *Aspergillus* sp. *HF37, Aspergillus pseudoglaucus, Metarhizium majus partitivirus 1* (Fig. 2D). As shown in Fig. 2E, the principal coordinate analysis explained 99.8% of the differences in the original data on the PCoA1 axis, indicating that the species composition spectra of FF and CT Daqu were different, and UPGMA hierarchical clustering analysis expressed that the two Daqu sample groups had a high degree of similarity. By analyzing the difference of ANOSIM (Analysis of similarities) at the species level of the two Daqu samples, the R value was 1, indicating that the difference between the groups was large and the difference within the group was small. In order to test whether the difference was statistically significant, 999 substitution tests were performed based on PERMANOVA, and the *P* value was $0.1 > 0.05$, and the difference between FF and CT Daqu groups was not significant, indicating that functional strain strengthening did not significantly change the structure

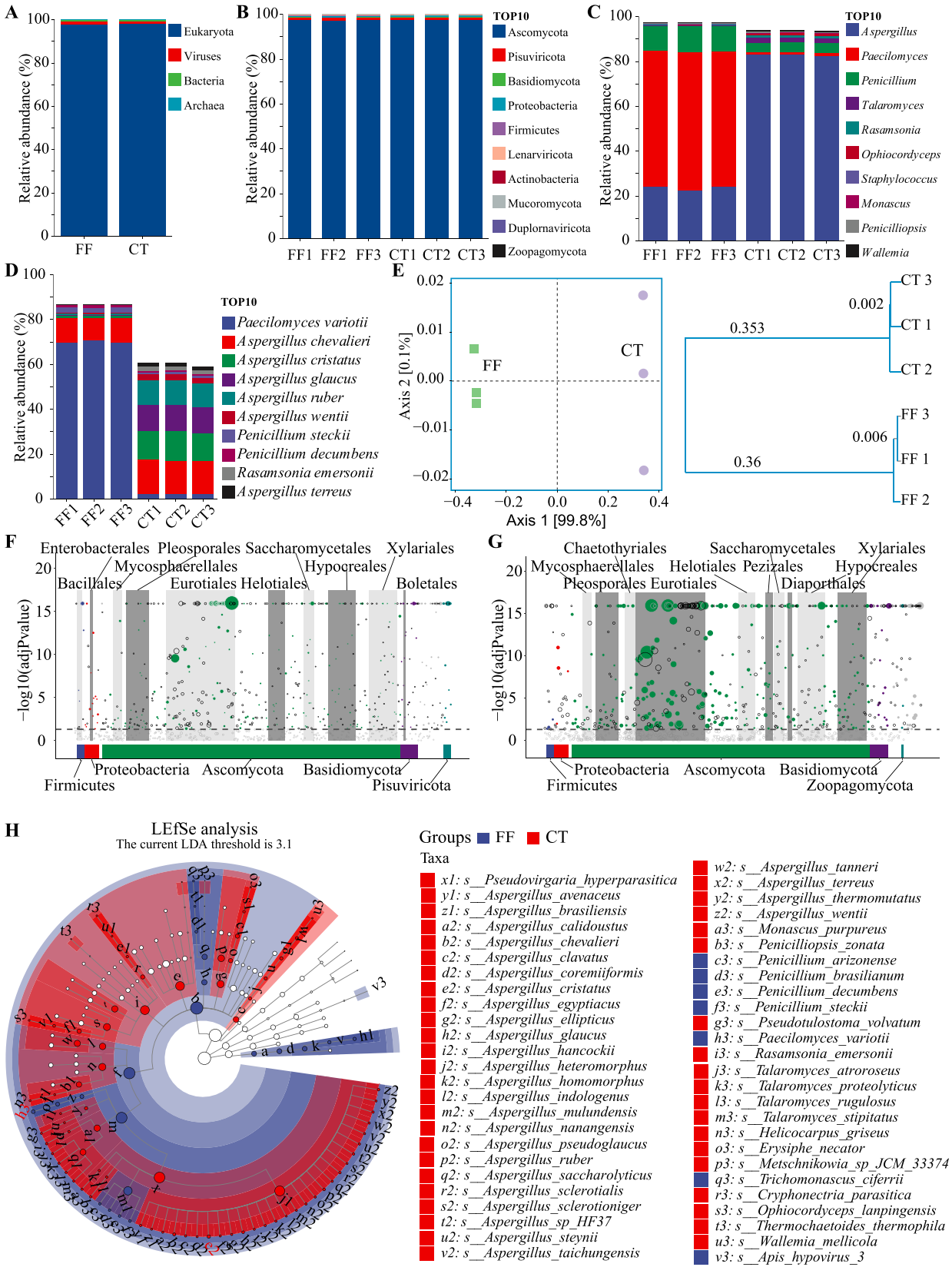


Fig. 2. Composition of active microbial communities of CT and FF Daqu (A: boundary level, B: phylum level, C: genus level, D: species level), species composition PCA and HCA analysis (E), microbiota composition difference analysis (F, G) and LEfSe analysis (H).

of Daqu active microbiota.

By normalizing the composition abundance of each classification level of each sample, the abundance difference of each classification unit of active microorganisms between sample groups was carried out. As shown in Fig. 2F and G, the classification of FF and CT Daqu samples was highly consistent at the phylum level, and the different species were mainly classified into Eurotiales, Mycosphaerellales, Pleosporales, Helotiales, Saccharomycetales, Hypocreales, and Xylariales. Compared with CT Daqu, FF Daqu was significantly upregulated in Bacillales, Enterobacterales, Boletales, and Chaetothyriales, Pezizales, and Diaporthales species were significantly down-regulated. The nonparametric Kruskal-Wallis and Wilcoxon rank sum tests were used to perform simultaneous differential analysis for all classification levels combined with Linear discriminant analysis (LDA) effect size. h3 (*Paecilomyces variotii*) and e2 *(Aspergillus cristatus)* are the taxonomic units with the largest horizontal nodes of the FF and CT Daqu taxonomic clades, respectively, and are biomarkers reflecting the differences between the two large curves (Fig. 2H).

3.4. Carbohydrate-active enzyme (CAZy) composition and its contributing microbial analysis

The search module using MMseqs2 was used to compare and annotate 10,089 non-redundant proteins with commonly used protein databases. The abundance of each protein corresponding to the CAZy enzyme family was statistically annotated to different modules. The results showed that the abundance of glycoside hydrolase (GH) and oxidoreductase (AA) was significantly higher in FF Daqu than CT Daqu, and the abundance of polysaccharide cleavage enzyme (PL) and carbohydrate-binding module (CBM) was significantly lower than that of CT Daqu. glycosyltransferases (GTs) and glycoesterases (CEs) did not differ significantly between the two types of Daqu (Fig. 3A). A total of 9 enzymes were annotated in the PL family, and PL3_2 and PL1_1 were the most abundant enzymes in FF Daqu and CT Daqu, respectively (Fig. 3B). A total of 11 enzymes were annotated in the CE family, and CE5 and CE8 were the enzymes with the largest proportion of FF Daqu and CT Daqu, respectively (Fig. 3C). A total of 85 enzymes were annotated in the GH family, and the enzymes with the highest abundance in FF Daqu and CT Daqu were GH19 and GH18, respectively (Fig. 3D). A total of 51 enzymes were annotated in the GT family, and the most abundant enzyme in the two Daqu was GT4 (Fig. 3E). There were 26 annotations in the CBM family, and CBM13 accounted for the highest proportion of the two large curved species (Fig. 3F). There were 16 enzymes in the AA family, AA1 accounted for the highest proportion in FF Daqu and AA10 accounted for the highest proportion in CT Daqu (Fig. 3G).

Among them, the GH enzyme family is classified by function as

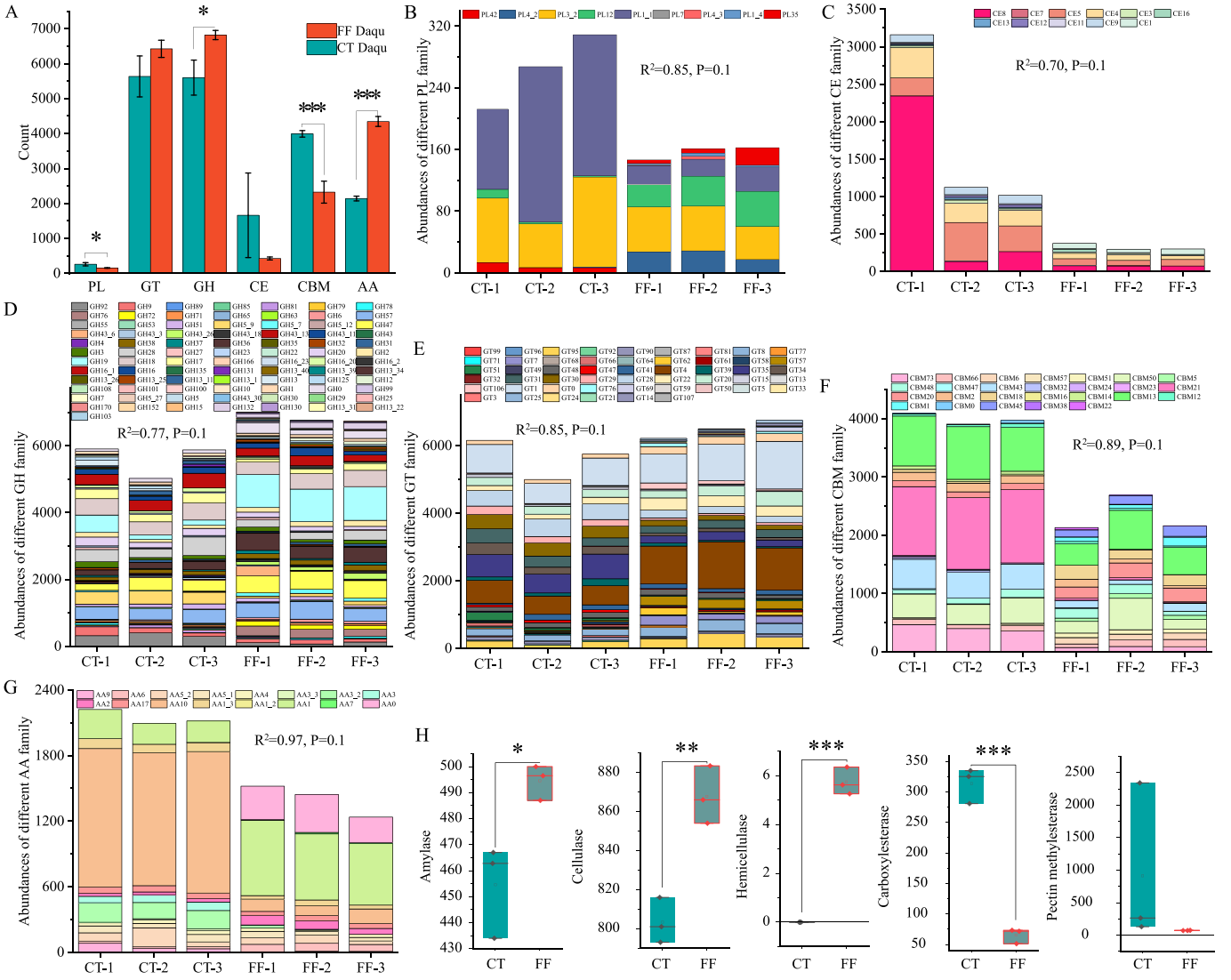


Fig. 3. Carbohydrate active enzyme (CAZy) family abundance comparison (A) and composition (B: PL, C: CE, D: GH, E: GT, F: CBM, G: AA family) and some enzyme comparison (H) analysis.

amylase (GH13, GH13_1, GH15, GH57), cellulase (GH1, GH2, GH3, GH5, GH6, GH7, GH9, GH12, GH16, GH30, GH51, GH131), and hemicellulase (GH5). And the CE enzyme family includes carboxylesterase (CE4, CE12), pectin methyl esterase (CE8). Further comparing the abundance of the above enzymes in the two Daqu samples, the abundance of amylase, cellulase and hemicellulase in FF Daqu was significantly higher than that in CT Daqu, and carboxylesterase and pectin methyl esterase were lower than that in CT Daqu (Fig. 3H). These results were more in line with the results of the analysis of the Daqu's physicochemical properties.

Amylase producers differed between FF and CT Daqu, with the main sources in FF Daqu being *Aspergillus chevalieri*, *Trichomonascus ciferrii*, *Byssochlamys spectabilis*, *Penicillium decumbens*, *Penicillium steckii*, and in the CT Daqu mainly from *Aspergillus cristatus*, *Aspergillus ruber*, *Macrophomina phaseolina* (Table S4). Cellulase, carboxylesterase and pectin methyl esterase contributing microorganisms were more widespread, and overall, the source of the above enzymes in FF Daqu was mainly *Penicillium*, while CT Daqu was mainly biased towards *Aspergillus* (Table S4). In addition, hemicellulase was only derived from *Byssochlamys* spectabilis in FF Daqu (Table S4).

3.5. Daqu non-volatile metabolite metabolomic analysis

As shown in Figs. 4A and B, PCA based on reliable metabolites explained 48.8% and 46.2% of the total variation in ESI+ and ESI- mode, respectively. FF Daqu and CT Daqu had obvious separation in both modes, and the cumulative explanatory rates (R^2X) of the PC1 axis direction model were 0.569 and 0.551, respectively, indicating that the goodness of fit of the model was good. The within-group error is further ignored and the random error irrelevant to the research purpose is eliminated, and a supervised orthogonal-partial least squares discriminant analysis (OPLS-DA) model is constructed, so as to maximize the intergroup differences. The OPLS-DA model had good reliability and predictability in the ESI+ model: $R^2Y = 1$, $Q^2 = 0.976$ and in the ESI- model: $R^2Y = 1$, $Q^2 = 0.939$, and the results showed significant differences between the two Daqu metabolites (PERMANOVA: $R^2 = 0.89$, $p = 0.014$). At the same time, the displacement test shows that the OPLS-DA model does not have overfitting and can be used for subsequent screening of characteristic metabolites between different taxa.

Hierarchical cluster analysis showed that 12 classes of metabolites were classified according to chemical structure and secondary classification, which dominated the difference between two kinds of Daqu. In FF Daqu, Alcohols and polyols, Amino acids, peptides, and analogues, Purines and purine derivatives, Lineolic acids and derivatives, Indoles, Fatty acids and conjugates were higher than CT Daqu, Pyridinecarboxylic acids and derivatives, Methoxyphenols, Lineolic acids and derivatives, Indoles, Carbonyl compounds, Benzoic acids and derivatives, Amines, Carbohydrates and carbohydrate conjugates were lower than CT Daqu (Fig. 4C). Projected with the importance value of the first principal component variable of OPLS-DA: VIP >1 and statistical significance ($p < 0.05$), FF Daqu and CT Daqu obtained 250 and 108 differential metabolites from ESI+ and ESI- modes, respectively, and the differential volcano plot showed that FF compared with CT upregulated metabolites 184 and downregulated metabolites 174, and the first 5 metabolites with the smallest *P* value were upregulated as 1,7-Dimethyluric acid, L-threo-3-Phenylserine, N-Acetyl-*L*-phenylalanine, downgraded to Miglitol, Glucose 6-phosphate (Fig. 4D).

KEGG PATHWAY enrichment analysis was performed on KEGG (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg/) by differential metabolite comparison, and a total of 209 pathways were enriched (Fig. 4E).

3.6. Correlation analysis of dominant active microbiota with physicochemical properties, flavor contributing compounds (ROAV>0.1) and differential metabolites

The difference in physicochemical properties leads to changes in the microbiota, which will change the enzymatic properties of Daqu. For the entire fermentation system, changes in enzymatic properties lead to differences in metabolic levels, which in turn affect flavor and change physicochemical properties. Correlations between 14 active microorganisms and physicochemical properties, 23 flavor-contributing compounds and 50 differential metabolites were analyzed using Spearman's correlation test. For physicochemical properties, the correlation network showed that the active microorganisms were significantly correlated with Amino nitrogen, Cellulose activity, Acidity, Liquefying power, and Saccharifying power ($r^2 > 0.5$, $p < 0.05$). Of these, there were 10 degrees between Acidity and the active microorganisms, of which significant positive correlations were found between *Metarhizium majus partitivirus 1*, *Penicillium steckii*, *Penicillium decumbens*, *Paecilomyces variotii*. Saccharifying power and Liquefying power showed significant positive correlation with *Penicillium steckii* and *Penicillium decumbens*, respectively, while other physicochemical properties showed significant negative correlation with active microorganisms (Fig. 5A). For flavor-contributing compounds, only 9 compounds were significantly correlated with active microorganisms. Hexadecanoic acid, ethyl ester is the largest node in the flavor network, and is significantly positively correlated with 8 active microorganisms such as *Metarhizium majus partitivirus 1*, *Aspergillus chevalieri*, *Aspergillus terreus*, and *Aspergillus sclerotialis*. Acetophenone, 9-Octadecenoic acid ethyl ester, Benzyl alcohol, Phenylethyl alcohol, Benzaldehyde and microbiota were significantly positively correlated, while Acetic acid and Phenol were mainly negatively correlated (Fig. 5A).

For non-volatile metabolites, L-threo-3-Phenylserine, Pyridoxal 5′-phosphate, Aspartame, D-Octopine, L-Arginine, N-Acetyl-*L*-phenylalanine, 5'-Deoxyadenosine, Glucose 6-phosphate, Uridine, and Styrene Oxide showed significant correlation with the active microorganisms. *Metarhizium majus partitivirus 1*, *Aspergillus terreus* and *Aspergillus sclerotialis* had a high degree of connection with metabolites, accounting for 32.57% of the total. 3-Methyl-2-oxovaleric acid, 4-Methylbenzaldehyde, Capsaicin, Digalacturonate, Fructoselysine 6-phosphate, gamma- Aminobutyric acid, and Tryptophanol were significantly negatively correlated with active microorganisms only (Fig. 5B).

3.7. Comprehensive analysis of metabolites and enzymes

In order to reconstruct the active microbial metabolic network of mature Daqu, a metabolic map covering non-volatile metabolites and active microbial enzymes was constructed based on the public database (KEGG) and the results of pathway analysis and enrichment analysis of differential metabolites (Fig. 6). Based on the actual production of Daqu and the needs of brewing, the conversion of carbohydrates and amino acids was comprehensively analyzed. In Starch and sucrose metabolism, amylose generates starch under the action of 1,4-α-glucan branching enzyme [EC: 2.4.1.18], and then forms dextrin under the action of α-amylase [EC: 3.2.1.1], and finally generates D-Glucose under enzymatic action. The source of D-Glucose was also hydrolyzed by beta-fructofuranosidase [EC: 3.2.1.26] to hydrolyze sucrose and cellulose. Endoglucanase [EC: 3.2.1.4], cellulose 1,4-β-cellobiase [EC: 3.2.1.91] and β-glucosidase [EC: 3.2.1.21] were up-regulated in FF Daqu, and the content of cellobiose was lower than that in CT Daqu, indicating that the ability of cellulose hydrolysis in fortified Daqu was higher. Various intermediates of the glycolysis process are crosslinked with other metabolic pathways, such as: β-D-Fructose-6P, Glycerate-3P, Phosphoenolpyruvate. The first key enzyme of glycolysis, hexokinase [EC: 2.7.1.1], was highly expressed in fortified Daqu, catalyzing glucose to form α-D-Glucose-6P, and then formed more β-D-Fructose-6P under the action of highly expressed phosphohexose isomerase [EC: 2.7.1.1].

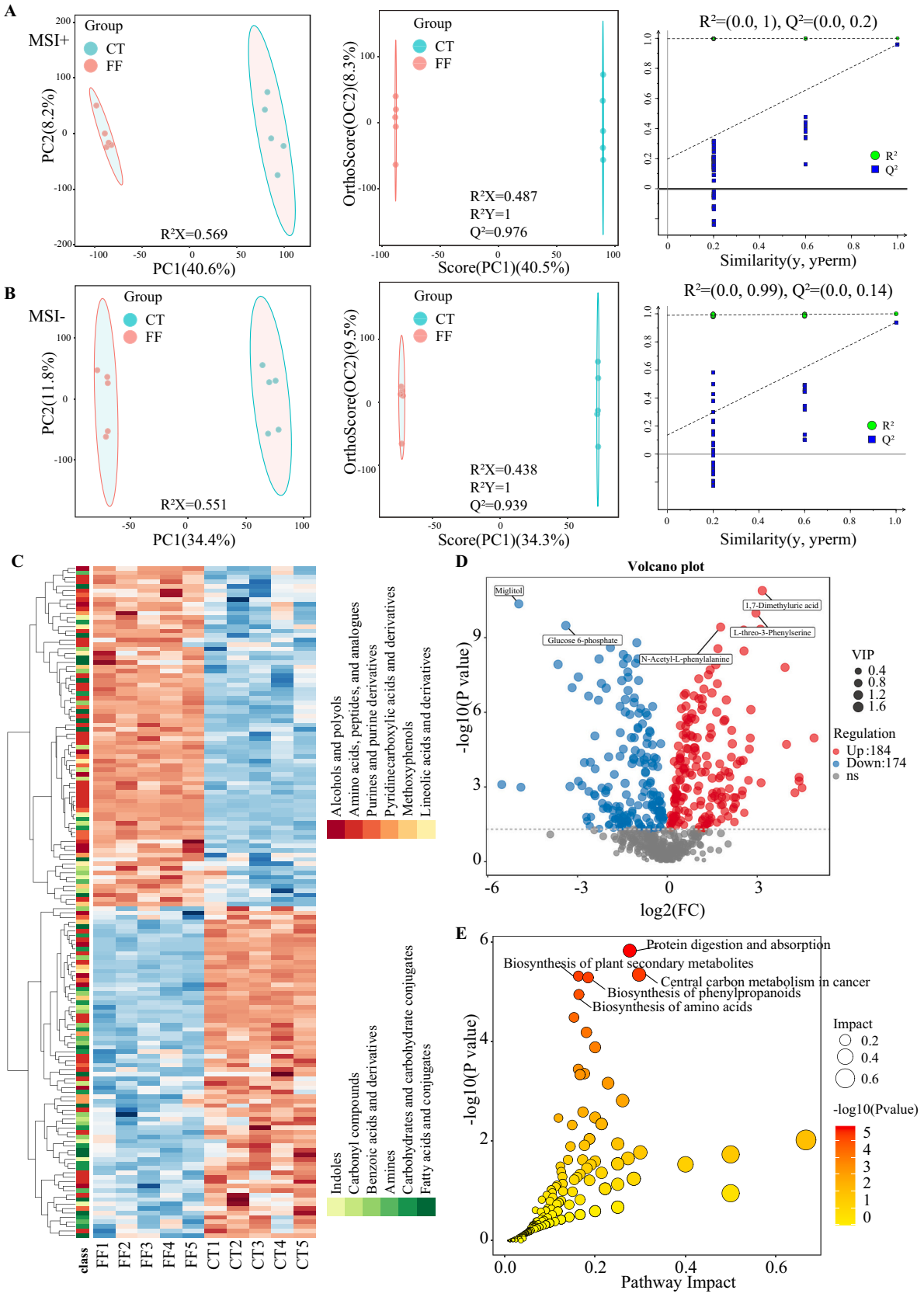


Fig. 4. PCA, OPLS-DA and their model permutation test analyses of metabolite composition of FF Daqu and CT Daqu (A, B), hierarchical cluster analysis (C), volcano plot of differential metabolites (D) and metabolic pathway enrichment bubble plot (E).

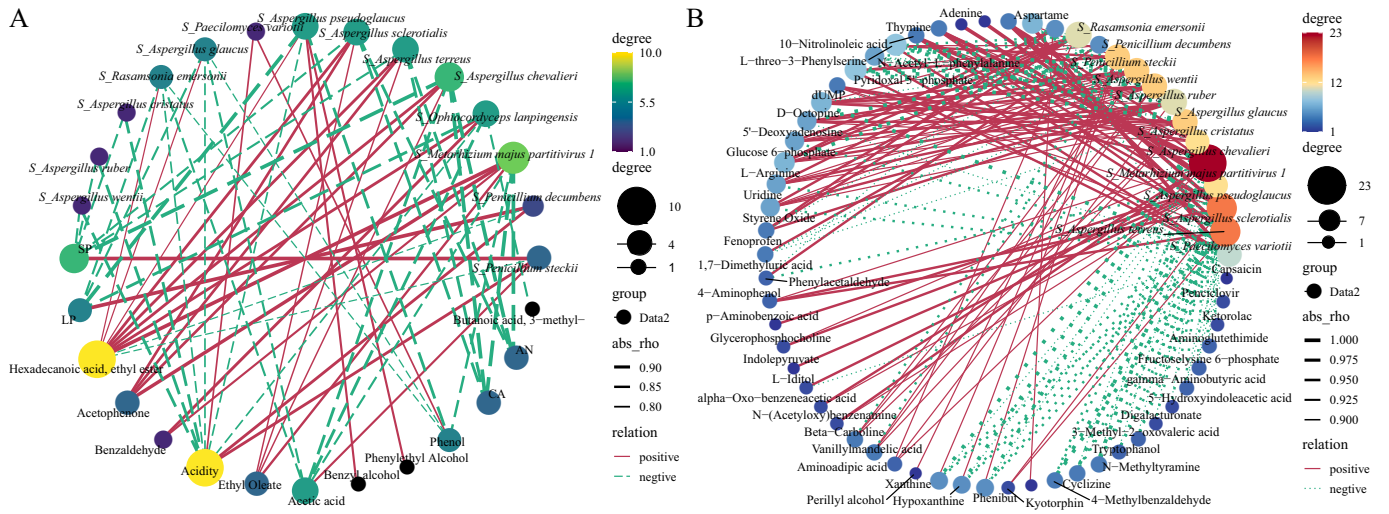


Fig. 5. Correlation analysis of species-level active microorganisms with physicochemical properties, flavor substances (A), and nonvolatile metabolites (B). AN: Amino nitrogen, CA: Cellulose activity, LP: Liquefying power, SP: Saccharifying power.

In pectin metabolism, the degradation of D-galacturonic acid mediated by fungi in CT Daqu was higher, and endopolygalacturonase [EC: 3.2.1.15] catalyzed the formation of Digalacturonate. Digalacturonate is hydrolyzed to D-Galacturonate by galactouronic acid 1,4-α-galacturonase [EC: 3.2.1.67], and the methoxy group is removed to form methanol. In terms of alcohol metabolism, the expression levels of alcohol dehydrogenase EutG, [EC: 1.1.1.2] and [EC: 1.1.2.8] in FF Daqu were higher than those in CT Daqu. In fatty acid metabolism, the expression of long-chain acyl-CoA synthetase [EC: 6.2.1.3] in CT Daqu was higher than that in FF Daqu, which catalyzed Hexadecanoate to form Hexadecanoyl-CoA. Therefore, the content of Hexadecanoate in FF Daqu was higher than that in CT Daqu.

In the glycolysis pathway, β-D-Fructose-6P and Phosphoenolpyruvate are involved in Phenylalanine, tyrosine and tryptophan biosynthesis, which are converted to Chorismate by 3-deoxy-7-phosphate heptanoate synthase [EC: 2.5.1.54], and participate in phenylalanine metabolism. In the branched-chain amino acid metabolic pathway, the branched-chain amino acid transaminase [EC: 2.6.1.42] activity of CT Daqu was higher, and valine, leucine and isoleucine were degraded to the corresponding α-keto acids. Pyruvate metabolism, TCA cycle and amino acid metabolism were closely related. α-ketoglutarate and oxaloacetate were up-regulated in FF Daqu, and succinyl-CoA and fumaric acid were up-regulated in CT Daqu. In terms of Alanine, aspartate and glutamate metabolism, α-ketoglutarate was converted to glutamic acid by glutamate synthase [EC: 1.4.1.14], and then decarboxylated by glutamate decarboxylase [EC: 4.1.1.15] to generate GABA (γ-aminobutyric acid). Both enzymes were expressed in FF Daqu, and the expression level was higher than that of CT Daqu. L-Argininosuccinate and Adenylosuccinate were formed by aspartic acid under the action of arginine succinate synthase [EC: 6.3.4.5] and adenosine succinate synthase [EC: 6.3.4.4], respectively. Subsequently, Adenylosuccinate was cleaved by enzyme to form fumaric acid and entered the TCA cycle. The more active proline iminopeptidase [EC: 3.4.11.5] in FF Daqu hydrolyzes peptides to produce proline, which is then converted into arginine and enters pyruvate metabolism.

3.8. Baijiu-making experiments

The solid-state fermentation of baijiu was simulated at the laboratory level. The control group obtained 0.865 kg of 70%vol base baijiu on average, and the experimental group obtained 0.943 kg of 70%vol base baijiu on average (Table S5). The yield of base baijiu converted into 65% vol baijiu was 0.932 kg in the control group and 1.016 kg in the experimental group. The baijiu yield of the experimental group (40.64%) was significantly higher than that of the control group (37.28%) (Table S5). The use of fortified Daqu could increase the baijiu yield by 3.36%.

4. Discussion

The results of physicochemical property analysis and sensory evaluation showed that functional strains of cellulase fortified with Daqu improved the quality of Daqu, and significantly increased the cellulase activity, liquefaction power, saccharification power, acidity and amino acid nitrogen content. The process of saccharification is the premise of baijiu production, and the cellulase system contains endocellulase, exocellulase and β-glucosidase three enzymes, under its synergistic action to hydrolyze cellulose into oligosaccharides, disaccharides, and finally hydrolysis into glucose (Bayer, Belaich, Shoham, & Lamed, 2004). In addition, the importance of β-glucosidases in baijiu-making lies in their potential to release flavor compounds from glycosidically bound and nonvolatile flavorless compounds(James, Yao, Ke, & Wang, 2023). The increase of liquefaction and saccharification power showed that the amylase system (alpha-amylase, glucoamylase and alpha-glucosidase) could efficiently degrade starch to generate oligosaccharides and glucose (Xia et al., 2022). Acidity is one of the main factors promoting the succession of Daqu microbial community, and acid-producing microorganisms such as lactic acid bacteria produce organic acids in the early fermentation stage, which inhibits the growth of miscellaneous bacteria and affects the assembly of Daqu microecology (Ma et al., 2022). Under the action of proteases, proteins are broken down into small peptides and amino acids, which provide nutrients for microbial growth and reproduction (Zhuansun et al., 2022). Amino acids are precursors of organic acids, so the increase of amino acid nitrogen content may be another reason for the increase of acidity. The above results showed that the strengthening of functional strains improved the quality of Daqu from the aspect of physical and chemical properties.

PERMANOVA analysis showed that there was no significant difference in flavor composition between fortified Daqu and traditional Daqu. The flavor substances in Daqu can be transmitted to baijiu in the subsequent fermentation and distillation of fermented grains, so the flavor of Daqu will also affect the quality of baijiu (Xu et al., 2017). In this study, the overall relative content of acids in fortified Daqu was significantly higher than that in traditional Daqu. Acetic acid, 2-Methylpropanoic acid, 3-Methylbutanoic acid, Hexanoic acid, Heptanoic acid, and Nonanoic acid were considered to be the key aroma compounds in baijiu. These acids can give baijiu a pleasant and soft aroma and taste

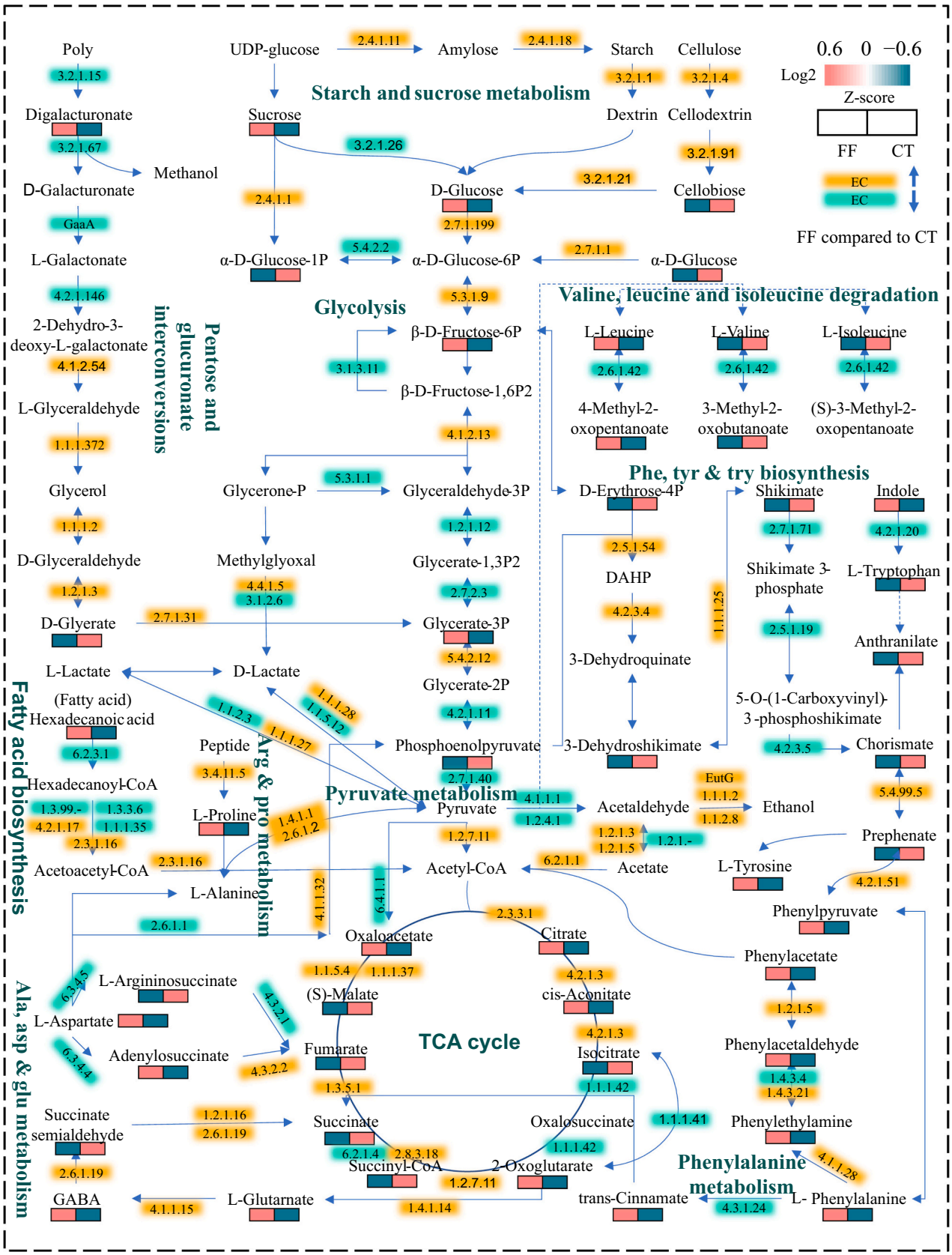


Fig. 6. Comprehensive metabolic pathways of non-volatile metabolites and enzymes. The left side of the small rectangle of the black border represents the content of the metabolite in FF Daqu, and the right side represents the content of the metabolite in CT Daqu. Red represents the higher content of the substance in the Daqu. The yellow rectangle and the blue rectangle represent the up-regulated expression and down-regulated expression of the enzyme, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Chen et al., 2022; Gao, Fan, & Xu, 2014). And these acids are precursors for the synthesis of the corresponding ethyl esters, such as: ethyl acetate, ethyl isovalerate, ethyl hexanoate, ethyl caprylate, esters provide baijiu fruity, floral, sweet and so on (Xu et al., 2022). Hexanoic acid ethyl ester (Ethyl hexanoate) accounted for >10% in both Daqu, which was the main aroma substance in Luzhou-flavor baijiu, and also existed in Maotai-flavor baijiu and Fen-flavor baijiu, with fruit aroma such as pineapple and banana (Yan, Zhang, Zou, & Hou, 2021). The content of higher alcohols (3-Methyl-1-butanol, 1-Hexanol, 1-Octen-3-ol, Benzyl alcohol, Phenylethyl alcohol) in fortified Daqu was lower than that in traditional Daqu. The low concentration of higher alcohols had a positive contribution to the balance of baijiu and could increase the mellow sweetness of baijiu (Hu et al., 2021). Studies have reported that the formation of higher alcohols is related to various enzyme activities and fermentation conditions during fermentation. The hydrolysis rate of polysaccharides and the release rate of fermentable sugars significantly affect the concentration of higher alcohols (Klosowski et al., 2015). In this study, the use of high-yield cellulase strains significantly increased the activity and acidity of these enzymes, which explained the reason for the low content of higher alcohols in Daqu. However, the potential response mechanism between higher alcohol formation and various enzymes still needs further study.

The dominant species of mature fortified Daqu and traditional Daqu were *Paecilomyces variotii*, *Aspergillus chevalieri*, *Aspergillus cristatus*, *Aspergillus glaucus*, *Aspergillus ruber*, *Aspergillus wentii*, *Penicillium steckii*, *Penicillium decumbens*. In previous studies, bacteria (*Lactobacillus*, *Bacillus*, *Acinetobacter*, *Leuconostoc*, and *Weissella*) were considered to be the dominant genus in the fermentation process of Daqu at the DNA level, and fungi were active in the early stage, while bacteria were more active in the middle and late stages (Zhao, Su, Mu, Mu, & Jiang, 2021; Zhu et al., 2022). Obviously, this result needs to be redefined at some stage of fermentation or based on microbial activity. A recent study also showed that *Limosilactobacillus*, *Staphylococcus*, *Pichia*, *Rhizopus*, and *Lichtheimia* were metabolically active in the early stage of fermentation, and a variety of heat-resistant filamentous fungi became transcriptionally active groups during the high temperature period and the end of fermentation, indicating that they contributed significantly to the enzyme activity and aroma of mature Daqu (Liu et al., 2023). The results of this study are consistent with its. In the later stage of fermentation, the temperature of Daqu decreased and the moisture content decreased (Liu, Li, et al., 2023). The decrease of temperature led to the decrease of abundance of *Bacillus* and *Thermoactinomyces* (Xiao et al., 2017). In addition moisture content is an important driver of microbiota evolution, strongly shaping microbial lifestyles by altering nutrient access, controlling oxygen diffusion and regulating mobility potentials (Greenlon et al., 2022). Viruses have been detected in both fortified and traditional Daqu, with Pisuviricota being one of the active dominant phyla in fortified dacquoise. Viruses are widely present in dacquoise and are involved in the decomposition of complex polysaccharides during fermentation, with critical effects on microbial community and metabolism (Du et al., 2023; Kang et al., 2022).

Annotation of carbohydrate-active enzymes based on transcription levels showed that there were significant differences in amylase and cellulase abundance between fortified and traditional Daqu, which was consistent with the results of physicochemical property analyses. Enzymatic activity of Daqu is improved through the fortification of functional strains, leading to changes in the functional properties of Daqu. Fermentation of Daqu is a microbial assembly under natural conditions, while adjusting the initial microbial inoculation ratio can reconstruct the fermentation microbiota to produce the target product efficiently. Thus in situ enhancement of functional microorganisms can alter the taxonomic composition, structure and interspecies interactions of microbial communities in fermentation systems and intervene in enzyme-encoding gene expression (He et al., 2019). In addition, the fermentation process of Daqu is not only microbial enrichment and succession, but also includes primary and secondary metabolite metabolism processes, endogenous bioheat-dominated community microbial metabolism (Liu, Li, et al., 2023; Luo et al., 2022). The differences in microbial community composition of Daqu will lead to changes in the overall metabolic pathways, which are embodied in the flavor formation (Mu et al., 2023), functional characteristics (Shi et al., 2022) and color (Gan et al., 2019) of Daqu. On the whole, the carbohydrate enzyme family expression genus of the two Daqu has changed. After *Bacillus subtilis* strengthening, *Penicillium* and *Byssochlamys* are the main expression of GH family enzymes. The difference is that *Aspergillus* is the main expression genus of GH family in traditional Daqu. *Byssochlamys spectabilis* is one of the main sources of glycoside hydrolase in Daqu (He et al., 2023).

Correlation analysis showed that acetic acid and phenol in volatile compounds were negatively correlated with active microbial flora. Acetic acid, Furfural and Phenol are considered to be inhibitive compounds derived from cellulose hydrolysis and have antifungal properties (Ayepa et al., 2024). In this study, FF Daqu volatile compounds Acetic acid, Furfural and Phenol with high cellulase activity were also higher than CT Daqu (Table S1). There was a significant negative relationship between Capsaicin and active microorganisms. Capsaicin has been reported to have an antibacterial effect, which binds to complex I of the electron transport chain, thereby inhibiting oxidative phosphorylation and subsequent ATP production, thereby inhibiting the ability to produce energy (Periferakis et al., 2023). It has also been shown that Capsaicin can inhibit the biosynthesis of ergosterol in the cell wall, thereby damaging the structure and integrity of the cell (Behbehani, Irshad, Shreaz, & Karched, 2023).

Significant difference in non-volatile metabolites between fortified and traditional Daqu. Metabolic profiles were constructed based on carbohydrate active enzymes and non-volatile metabolites to reflect the overall metabolic impact of functional microbial fortification. Enzymes related to starch and cellulose metabolic processes were up-regulated in fortified Daqu, and hydrolysis released more glucose to participate in glycolysis and the TCA cycle, thus linking fat and amino acid metabolism. In the baijiu production process using wheat as the fermentation substrate and sorghum as the substrate, starch is difficult to be degraded fully due to cell wall encapsulation, which reduces the yield and leads to residual available biomass in the by-products (Ye et al., 2018). In this study, the pectin degradation capacity of fortified Daqu was reduced compared to traditional Daqu, and the expression of polygalacturonase was down-regulated in fortified Daqu. The decomposition of pectin in cereal grains can lead to the formation of methanol, but the intake of beverages containing a certain amount of methanol poses a health risk (Góes, Fabris, Muller, & Fabris, 2016). The use of high-temperature cooking of raw materials and fractional distillation in the production of baijiu reduces the methanol content, but this does not completely guarantee that it is at a safe level. And the control of pectinase activity can further control methanol content (Han & Du, 2022). The activities of the enzymes involved in the synthesis pathway of higher alcohols in fortified Daqu are lower than those of traditional Daqu. The formation of higher alcohols (isobutanol, isoamyl alcohol, and reactive pentanol) is related to the metabolism of branched-chain amino acids, which are decarboxylated and reduced to higher alcohols by branched-chain amino acid aminotransferase [EC: 2.6.1.42], which generates the corresponding α-keto acids (Koonthongkaew, Ploysongsri, Toyokawa, Ruangpornvisuti, & Takagi, 2022). The higher alcohol synthesis pathways are mainly summarised as the Ehrlich pathway and the Harris pathway, but in general they are caused by differences in the content of various amino acids (Wang, Wei, Guo, & Xiao, 2020), and the free amino acid content of fortified Daqu is significantly higher than that of traditional Daqu, which may be one of the reasons leading to the decrease in the higher alcohol content of fortified Daqu.

5. Conclusion

In this study, the bioturbation effects of functional microorganisms

were deeply understood based on metatranscriptomics, metabolomics, flavoromics, physicochemical property analysis and multivariate statistical analysis. *Penicillium* and *Byssochlamys* were the main contributors of carbohydrate active enzymes in fortified Daqu, and *Aspergillus* was the main contributor of carbohydrate active enzymes in traditional Daqu. The enzymatic properties of Daqu changed with the change of microbiome, and the saccharification power, liquefaction power and cellulase activity in fortified Daqu were significantly improved. The differences in enzymatic properties changed the metabolic characteristics, and 358 differential metabolites were identified from 716 non-volatile metabolites. The main flavor substances (ROAV $\geq$1) shared by fortified Daqu and traditional Daqu: 3-Methylbutanoic acid, Hexanoic acid, Phenylethyl alcohol, Hexanoic acid ethyl ester and Acetophenone had no significant difference ($p > 0.05$). These results are helpful to promote the application of functional microorganisms in Daqu and baijiu fermentation and provide ideas for the utilization of biomass resources.

CRediT authorship contribution statement

Yanbo Liu: Supervision, Resources, Methodology. **Haideng Li:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation. **Wenxi Liu:** Data curation. **Kejin Ren:** Software. **Xuehan Li:** Formal analysis. **Zhenke Zhang:** Supervision. **Runna Huang:** Writing – review & editing. **Suna Han:** Resources. **Jianguang Hou:** Supervision, Resources. **Chunmei Pan:** Writing – review & editing, Supervision, Resources, Funding acquisition.

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.

Acknowledgments

This work supported by the key research and development project of Henan Province (231111112000) and the key discipline construction project of food science and engineering of Henan University of Animal Husbandry and Economy (XJXK202203).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101382.

References

Ayepa, E., Li, Q., Zhang, Z., Wang, H., Herman, R. A., Ouyang, Y., … Ma, M. (2024). Molecular mechanism of tolerance evolution in *Candida tropicalis* to inhibitory compounds derived from corn Stover hydrolysis. *Biomass & Bioenergy, 181*, Article 107031.

Bayer, E. A., Belaich, J.-P., Shoham, Y., & Lamed, R. (2004). The cellulosomes: Multienzyme machines for degradation of plant cell wall polysaccharides. *Annual Review of Microbiology, 58*, 521–554.

Behbehani, J. M. M., Irshad, M., Shreaz, S., & Karched, M. (2023). Anticandidal activity of capsaicin and its effect on Ergosterol biosynthesis and membrane integrity of Candida albicans. *International Journal of Molecular Sciences, 24*(2).

Chen, P., Liu, Y., Wu, J., Yu, B., Zhao, H., Huang, M., & Zheng, F. (2022). Sensory-directed decoding of key aroma compounds from Jiugui-series baijiu, the representative of Fuyu-flavor-type baijiu (FFTB). *Journal of Food Composition and Analysis, 114*, Article 104799.

Deng, L., Mao, X., Liu, D., Ning, X. Q., Shen, Y., Chen, B., … Luo, H. B. (2020). Comparative analysis of physicochemical properties and microbial composition in high-temperature Daqu with different colors. *Frontiers in Microbiology, 11*.

Du, H., Chen, B. W., Fu, W. B., Yang, F., Lv, X. B., Tan, Y. W., … Xu, Y. (2023). Composition and function of viruses in sauce-flavor baijiu fermentation. *International Journal of Food Microbiology, 387*, Article 110055.

Gan, S. H., Yang, F., Sahu, S. K., Luo, R. Y., Liao, S. L., Wang, H. Y., … Liu, H. (2019). Deciphering the composition and functional profile of the microbial communities in Chinese Moutai liquor starters. *Frontiers in Microbiology, 10*, Article 01540.

Gao, W., Fan, W., & Xu, Y. (2014). Characterization of the key odorants in light aroma type Chinese liquor by gas chromatography-Olfactometry, quantitative measurements, aroma recombination, and omission studies. *Journal of Agricultural and Food Chemistry, 62*(25), 5796–5804.

Góes, R. E.d., Fabris, L. V. M., Muller, M., & Fabris, J. L. (2016). Light-assisted detection of methanol in contaminated spirits. *Journal of Lightwave Technology, 34*(19), 4499–4505.

Greenlon, A., Sieradzki, E., Zablocki, O., Koch, B. J., Foley, M. M., Kimbrel, J. A., … Banfield, J. F. (2022). Quantitative stable-isotope probing (qSIP) with metagenomics links microbial physiology and activity to soil moisture in Mediterranean-climate grassland ecosystems. *mSystems, 7*(6). e00417-00422.

Han, Y., & Du, J. (2022). Relationship of the methanol production, pectin and pectinase activity during apple wine fermentation and aging. *Food Research International, 159*, Article 111645.

He, G., Huang, J., Wu, C., Jin, Y., & Zhou, R. (2020). Bioturbation effect of fortified Daqu on microbial community and flavor metabolite in Chinese strong-flavor liquor brewing microecosystem. *Food Research International, 129*, Article 108851.

He, G., Huang, J., Zhou, R., Wu, C., & Jin, Y. (2019). Effect of fortified Daqu on the microbial community and flavor in Chinese strong-flavor liquor brewing process. *Frontiers in Microbiology, 10*.

He, M., Jin, Y., Liu, M., Yang, G., Zhou, R., Zhao, J., & Wu, C. (2023). Metaproteomic investigation of enzyme profile in daqu used for the production of Nongxiangxing baijiu. *International Journal of Food Microbiology, 400*, Article 110250.

Hu, Y. L., Yang, Q., Chen, D., Fu, B. A., Zhang, Y., Zhang, Y., … Zhao, S. M. (2021). Study on microbial communities and higher alcohol formations in the fermentation of Chinese Xiaoqu baijiu produced by traditional and new mechanical technologies. *Food Research International, 140*, Article 109876.

James, A., Yao, T., Ke, H. M., & Wang, Y. S. (2023). Microbiota for production of wine with enhanced functional components. *Food Science and Human Wellness, 12*(5), 1481–1492.

Kang, J. M., Chen, X. X., Han, B. Z., & Xue, Y. S. (2022). Insights into the bacterial, fungal, and phage communities and volatile profiles in different types of Daqu. *Food Research International, 158*, Article 111488.

Klosowski, G., Mikulski, D., Macko, D., Miklaszewska, B., Kotarska, K., & Czuprynski, B. (2015). Influence of various yeast strains and selected starchy raw materials on production of higher alcohols during the alcoholic fermentation process. *European Food Research and Technology, 240*(1), 233–242.

Koonthongkaew, J., Ploysongsri, N., Toyokawa, Y., Ruangpornvisuti, V., & Takagi, H. (2022). Improvement of fusel alcohol production by engineering of the yeast branched-chain amino acid Aminotransaminase. *Applied and Environmental Microbiology, 88*(13). e00557–00522.

Li, H. D., Liu, S. Y., Liu, Y. B., Hui, M., & Pan, C. M. (2023). Functional microorganisms in baijiu Daqu: Research progress and fortification strategy for application. *Frontiers in Microbiology, 14*, Article 1119675.

Li, W. W., Fan, G. S., Fu, Z. L., Wang, W. H., Xu, Y. Q., Teng, C., … Li, X. T. (2020). Effects of fortification of Daqu with various yeasts on microbial community structure and flavor metabolism. *Food Research International, 129*, Article 108837.

Li, Z., Fernandez, K. X., Vederas, J. C., & Gänzle, M. G. (2023). Composition and activity of antifungal lipopeptides produced by *Bacillus* spp. in daqu fermentation. *Food Microbiology, 111*, Article 104211.

Liu, W. H., Chai, L. J., Wang, H. M., Lu, Z. M., Zhang, X. J., Xiao, C., … Xu, Z. H. (2023). Bacteria and filamentous fungi running a relay race in Daqu fermentation enable macromolecular degradation and flavor substance formation. *International Journal of Food Microbiology, 390*, Article 110118.

Liu, Y., Liu, Y., Zhao, Z., Liu, R., Pan, C., & Sun, X. (2020). Isolation and ldentification of high-yield Cellulase strains from ZhangGongLaoJiu Daqu. *Genomics and Applied Biology, 39*(09), 4031–4040.

Liu, Y. B., Li, H. D., Dong, S. M., Zhou, Z., Zhang, Z. K., Huang, R. N., … Pan, C. M. (2023). Dynamic changes and correlations of microbial communities, physicochemical properties, and volatile metabolites during Daqu fermentation of Taorong-type baijiu. *LWT- Food Science and Technology, 173*, Article 114290.

Luo, S., Zhang, Q., Yang, F., Lu, J., Peng, Z., Pu, X., … Wang, L. (2022). Analysis of the formation of sauce-flavored Daqu using non-targeted metabolomics. *Frontiers in Microbiology, 13*, Article 857966.

Ma, S., Luo, H., Zhao, D., Qiao, Z., Zheng, J., An, M., & Huang, D. (2022). Environmental factors and interactions among microorganisms drive microbial community succession during fermentation of Nongxiangxing daqu. *Bioresource Technology, 345*, Article 126549.

Mu, Y., Huang, J., Zhou, R. Q., Zhang, S. Y., Qin, H., Dong, Y., … Tang, H. F. (2023). Comprehensive analysis for the bioturbation effect of space mutation and biofortification on strong-flavor Daqu by high-throughput sequencing, volatile analysis and metabolomics. *Food Chemistry, 403*, Article 134440.

Periferakis, A.-T., Periferakis, A., Periferakis, K., Caruntu, A., Badarau, I. A., Savulescu-Fiedler, I., … Caruntu, C. (2023). Antimicrobial properties of capsaicin: Available data and future research perspectives. *Nutrients, 15*(19).

Shi, W., Chai, L. J., Fang, G. Y., Mei, J. L., Lu, Z. M., Zhang, X. J., … Xu, Z. H. (2022). Spatial heterogeneity of the microbiome and metabolome profiles of high-temperature Daqu in the same workshop. *Food Research International, 156*, Article 111298.

Tang, Q. X., Chen, X. R., Huang, J., Zhang, S. Y., Qin, H., Dong, Y., … Zhou, R. Q. (2023). Mechanism of enhancing pyrazines in Daqu via inoculating *Bacillus licheniformis* with strains specificity. *Foods, 12*(2), 304.

Tong, W., He, P., Yang, Y., Qiao, Z., Huang, D., Luo, H., & Feng, X. (2022). Occurrence, diversity, and character of Bacillaceae in the solid fermentation process of strong aromatic liquors. *Frontiers in Microbiology, 12*, Article 811788.

Wang, Y. P., Wei, X. Q., Guo, X. W., & Xiao, D. G. (2020). Effect of the deletion of genes related to amino acid metabolism on the production of higher alcohols by *Saccharomyces cerevisiae*. *BioMed Research International, 2020*, Article 6802512.

Xia, Y., Zhu, M., Du, Y. K., Wu, Z. Y., Gomi, K., & Zhang, W. X. (2022). Metaproteomics reveals protein composition of multiple saccharifying enzymes in nongxiangxing daqu and jiangxiangxing daqu under different thermophilic temperatures. *International Journal of Food Science and Technology, 57*(8), 5102–5113.

Xiao, C., Lu, Z. M., Zhang, X. J., Wang, S. T., Ao, L., Shen, C. H., … Xu, Z. H. (2017). Bio-heat is a key environmental driver shaping the microbial Community of Medium-Temperature Daqu. *Applied and Environmental Microbiology, 83*(23). e01550–01517.

Xu, R., Feng, N., Li, Q., Wang, H., Su, Y., & Zhu, W. (2023). Short-term supplementation of pectin alters substrate dynamics and modulates microbial carbohydrate metabolism in the gut of a pig model. *Journal of Agricultural and Food Chemistry, 71*(27), 10470–10482.

Xu, Y. Q., Sun, B. G., Fan, G. S., Teng, C., Xiong, K., Zhu, Y. P., … Li, X. T. (2017). The brewing process and microbial diversity of strong flavour Chinese spirits: A review. *Journal of the Institute of Brewing, 123*(1), 5–12.

Xu, Y. Q., Zhao, J. R., Liu, X., Zhang, C. S., Zhao, Z. G., Li, X. T., & Sun, B. G. (2022). Flavor mystery of Chinese traditional fermented baijiu: The great contribution of ester compounds. *Food Chemistry, 369*, Article 130920.

Yan, Q., Zhang, K. Z., Zou, W., & Hou, Y. C. (2021). Three main flavour types of Chinese baijiu: Characteristics, research, and perspectives. *Journal of the Institute of Brewing, 127*(4), 317–326.

Yang, L., Fan, W., & Xu, Y. (2021). GC × GC-TOF/MS and UPLC-Q-TOF/MS based untargeted metabolomics coupled with physicochemical properties to reveal the characteristics of different type daqus for making soy sauce aroma and flavor type baijiu. *LWT- Food Science and Technology, 146*, Article 111416.

Yang, Y., Wang, S. T., Lu, Z. M., Zhang, X. J., Chai, L. J., Shen, C. H., … Xu, Z. H. (2021). Metagenomics unveils microbial roles involved in metabolic network of flavor development in medium-temperature daqu starter. *Food Research International, 140*, Article 110037.

Ye, G., Luo, H., Ren, Z., Ahmad, M. S., Liu, C.-G., Tawab, A., … Mehmood, M. A. (2018). Evaluating the bioenergy potential of Chinese liquor-industry waste through pyrolysis, thermogravimetric, kinetics and evolved gas analyses. *Energy Conversion and Management, 163*, 13–21.

Zhao, C., Su, W., Mu, Y., Mu, Y., & Jiang, L. (2021). Integrative metagenomics-metabolomics for analyzing the relationship between microorganisms and non-volatile profiles of traditional Xiaoqu. *Frontiers in Microbiology, 11*, Article 617030.

Zhu, M., Zheng, J., Xie, J., Zhao, D., Qiao, Z. W., Huang, D., & Luo, H. B. (2022). Effects of environmental factors on the microbial community changes during medium-high temperature Daqu manufacturing. *Food Research International, 153*, Article 110955.

Zhuansun, W. W., Xu, J., Li, Z. Q., Liu, H. Z., Zhao, Y., Chen, L. L., … Xu, Q. X. (2022). Dynamic changes in the microbial community, flavour components in jiupei of a novel Maotai-Luzhou-flavoured liquor under various daqu blending modes and their correlation analysis. *LWT- Food Science and Technology, 172*, Article 114167.