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Metagenomics-based insights into the microbial community profiling and flavor development potentiality of *baijiu Daqu* and *huangjiu* wheat *Qu*

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ABSTRACT

Daqu and wheat *Qu* are saccharification and fermenting agents in Chinese *huangjiu* and *baijiu* production. This study aimed to investigate the difference between *Daqu* and wheat *Qu* in physicochemical indices, microbial communities, functional genes, and the metabolic network of key microbes responsible for flavor synthesis by whole-metagenome sequencing and metabolite analysis. Herein, physicochemical indices indicated that compared with wheat *Qu*, *Daqu* exhibited higher protease and cellulase activity and acidity, and lower glucoamylase and amylase enzyme activity. Metagenomic sequencing reveals that although *Daqu* and wheat *Qu* community composition have significant differences at species level, they have similar functional genes. *Daqu* were enriched in *Pediococcus pentosaceus*, *Weissella paramesenteroides*, *Rasamsonia emersonii* and *Byssochlamys spectabilis* (22.48% of the total abundance), while wheat *Qu* harbored greater abundances of *Saccharopolyspora* (54.78%, *Saccharopolyspora rectivirgula*, *Saccharopolyspora shandongensis*, *Saccharopolyspora hirsuta*, *Saccharopolyspora spinose*, and *Saccharopolyspora erythraea*). From a functional perspective, the important functions of *Daqu* and wheat *Qu* are both amino acid metabolism and carbohydrate metabolism. Meanwhile, a combined analysis among microbiota, functional genes, and dominant flavors indicated *S. shandongensis*, *S. rectivirgula*, and *S. spinose* might be the main contributor to the synthesis of flavor compounds in wheat *Qu*, while *R. emersonii*, *W. paramesenteroides*, *Leuconostoc citreum*, *Leuconostoc mesenteroides*, *Weissella cibaria* and *P. pentosaceus* may make the greatest contribution to flavor compounds synthesis in *Daqu*. This study reveals the microbial and functional dissimilarities of *Daqu* and wheat *Qu*, and helps elucidating different metabolic roles of microbes during flavor formation.

1. Introduction

Qu is the world's first artificially cultured substrate enriched with microorganisms and their metabolites. The invention of *Qu* making technology is a great achievement of the ancient Chinese working people (Zheng, Tabrizi, Nout, & Han, 2011). Wine making with *Qu* is a characteristic of Chinese wine making and a watershed between Eastern and Western wine culture. *Daqu* and wheat *Qu* are the unique raw materials for *baijiu* and *huangjiu* (Chinese rice wine) brewing in China, respectively (Xu, Wang, Fan, Mu, & Chen, 2010). *Baijiu* and *huangjiu*, the traditional indigenous fermented alcoholic drink, play indispensable roles in the Chinese dietary profile and have gained worldwide acceptance.

Qu (*Daqu* and wheat *Qu*) is a type of solid-state dry fermentation starter, the production process usually consists of 5 phases (Fig. 1): (i) Raw material. The *Daqu* is made with a blend of barely, wheat and peas, while the wheat *Qu* is made from pure wheat. (ii) Crushing and Mixing. The raw materials are crushed and blended with water. The purpose of

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this type is to break the grains to release starch and obtain a homogenous mixture. (iii) Shaping. The wetted material is transferred to a molding press and formed into a brick with different size. Generally, the size of the *Daqu* brick is 34 × 20 × 5.5 cm (Length × Width × High), and the wheat *Qu* brick is 24 × 15 × 7.5 cm (Length × Width × High). (iv) Fermentation. It can be divided into three stages: Stage I, 30 to 40 ℃ for 3–5 days, which results in the mass reproduction and growth of microorganisms. Stage II, the stage is characterized by a controlled and gradual increase of the temperature, and maintaining the relative humidity greater than 90%, which is used mainly for the accumulation of microbial metabolites. In this stage, the maximum temperature of *Daqu* is reached 50 to 65 ℃ and maintained for 7 to 8 days. The temperature of wheat *Qu* reached a maximum of 45 to 55 ℃ and was maintained for 4 to 5 days. Stage III, the aim of this stage is to remove moisture in the *Qu* core and the formation of aroma substances. For the *Daqu*, the temperature should not be lower than 45 °C for 9 to 12 days, and the relative humidity < 80%. While, for the wheat *Qu*, the temperature should not be lower than 40 °C for 10 to 12 days. (v) Maturation. After cooling down to room temperature, the maturation of *Daqu* and wheat *Qu* is 6 months and 2 months, respectively. The finished product of *Qu* is complete once this stage has finished.

Qu plays a key role in liquor fermentation and determines the flavor quality of the final product (Xu, et al., 2018; Zhang, Li, Wu, Yang, & Zou, 2019). It has been shown that wheat *Qu* and *Daqu* display similar functions in liquor fermentation, which can be summarized in three aspects: i) providing microbiota for liquor brewing. In the process of making *Qu*, the microbes in raw materials, water, air and working environment are domesticated and cultured to form stable microbiota for liquor brewing (Wang, Du, Zhang, & Xu, 2018; Xiao, et al., 2017); ii) providing brewing raw materials and flavor compounds. Wheat *Qu* is made of wheat, and the main materials of *Daqu* are wheat, or a mixture of wheat, barley and pea (Xu, Wang, Fan, Mu, & Chen, 2010). The *Qu* contains a large amount of starch, protein and others, which can provide nutrients for the growth of microorganisms. At the same time, due to the action of microorganisms during the *Qu* making process, various metabolites such as alcohols, acids and esters are produced, which are dissolved in the liquor during the fermentation process, forming the unique flavor of liquor (Xiao, et al., 2017; Yang, et al., 2021); iii) providing enzymes for liquor brewing. Various enzymes of microbial metabolism during *Qu* making, such as amylase, glucoamylase, protease and esterifying enzyme, are used in the decomposition of raw materials in liquor fermentation (Liu, Chen, Fan, Huang, & Han, 2018). Obviously, microbes and their metabolic activities in *Qu* are the most critical factors that affect the quality of liquor. Therefore, exploring the relationship between flavor compounds and microbes to determine the core functional microorganisms of fermentation is of great significance for improving the quality of liquor and controlling the fermentation process.

In this study, we aimed to provide a new method for identifying functional microorganism in traditional fermentation. Moreover, this study may also shed light on the underlying mechanisms of the differences between the two *Qu* from the aspects of microorganisms and metabolism. This study is helpful to improve the flavor and quality of traditional fermented food.

2. Material and methods

2.1. Samples collection

Daqu were collected from a traditional *baijiu*-making factory (33°74′N, 118°48′E) in Suqian, Jiangsu Province, China on September 2019. Wheat *Qu* were collected from a *huangjiu*-making factory (30°08′N, 120°49′E) in Shaoxing, Jiangsu Province, China on September 2019. In order to obtain adequate information and representation before carrying out analysis, six pieces of each type of sample were selected randomly from upper, middle and lower locations in the storage room, ground to powder in a sterile grinder and mixed as a sample. The size of Daqu is approximately 34 × 20 × 5.5 cm (length × width × high), weighing around 4.4 kg each. The size of wheat Qu is approximately 23 × 15 × 7.5 cm (length × width × high), weighing around 2.5 kg each. The *Daqu* and wheat *Qu* samples were transported to the laboratory on dry ice within 24 h of collection. Samples were stored at −20 °C before analysis of enzyme activity and flavor compounds, and were stored at −80 °C before DNA extraction. All experiments were conducted at least 3 times.

2.2. Physicochemical and enzymatic analysis of Daqu and wheat Qu

Moisture content was analysed by drying the samples at 105 °C until constant weight. Acidity was determined at a 1:2.5 (w/v) ratio in ultrapure water by titration to the endpoint of pH 8.2 with 0.1 M NaOH solution. Glucoamylase, amylase, and protease activities were determined as previously described (Liu, et al., 2020). Cellulase activity was estimated by measuring the release of reducing sugars from carboxymethylcellulose sodium (CMC-Na, 1% w/v) by crude enzyme solution in a sodium acetate buffer (50 mM, pH 4.6) at 30 °C and for 10 min, and expressed as glucose equivalents. One unit of amylase activity was defined as the amount of dry samples required for the liquefaction of 1 g starch per hour in sodium phosphate buffer (100 mM, pH 4.6) at 30 °C (U/g dry sample). One unit of glucoamylase activity was defined as the amount of dry samples required for the liberation of 1 mg glucose per hour in sodium acetate buffer (50 mM, pH 4.6) at 30 °C (U/g dry sample). One unit of protease activity was defined as the amount of dry samples required for the liberation of 1 μg of tyrosine per min in sodium acetate buffer (50 mM, pH 4.6) at 30 °C (U/g dry sample). One unit of cellulase activity was defined as the amount of dry samples required for the liberation of 1 μmol of glucose per min in sodium acetate buffer (50 mM, pH 4.6) at 30 °C (U/g dry sample).

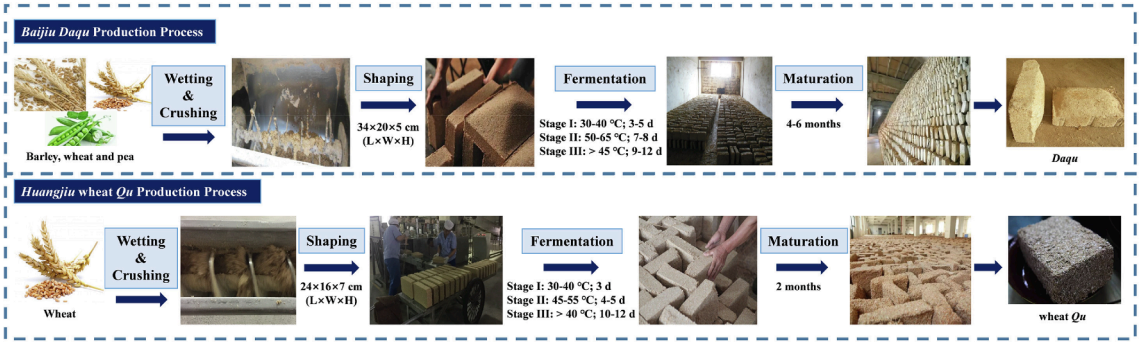

Fig.1. Manufacturing process of Daqu and wheat Qu.

2.3. Genomic DNA extraction and whole-metagenome sequencing

Genomic DNA was extracted from *Daqu* and wheat *Qu* samples as our previously described (Liu, Chen, et al., 2019). DNA concentrations were assessed using a UV–Vis Spectrophotometer Q5000 (Quawell, San Jose, USA). DNA was stored at −80 °C until further processing. 1 μg of genomic DNA was used as input material for library preparation. DNA libraries were generated using NEBNext® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA) following manufacturer's recommendations. Library quality was evaluated on Agilent 2100 Bioanalyzer (Agilent) system and quantified using real-time PCR. DNA libraries were paired-end sequenced using Illumina HiSeq4000 sequencing platform (Novogene, Tianjin, China).

2.4. Bioinformatic analysis

Sequence data processing was conducted as described in ref (Liu, Chen, et al., 2019). Sequence reads were first screened to remove low-quality reads (quality value<38 ≥ 40 bp, and ≥ 10 % N containing reads) by Readfq (V8, https://github.com/cjfields/readfq). Then reads aligned to the wheat, rice, pea, barley and human genome (Bowtie 2.2.4, parameters: –end-to-end, –sensitive, -I 200, -X 400) were removed from the raw data set (Karlsson, et al., 2013). The remaining high-quality reads of each DNA sample was assembled by the SOAP denovo (Version 2.04, parameters: -d 1, -M 3, -R, -u, -F, -K 55) (Brum, et al., 2015; Feng, et al., 2015; Qin, et al., 2014). Genes were predicted using MetaGeneMark (Version 2.10) software with default parameters (Nielsen, et al., 2014; Qin, et al., 2014; Villar, et al., 2015). Genes were annotated using DIAMOND software (Version 0.9.9) with the non-redundant database (NR) (Buchfink, Xie, & Huson, 2015), Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.kegg.jp/kegg/), Evolutionary genealogy of genes, Non-supervised Orthologous Groups (eggNOG, http://eggnogdb.embl.de/#/app/home), Carbohydrate Active enzymes Database (CAZy, http://www.cazy.org/) (Cantarel, et al., 2009; Huerta-Cepas, et al., 2016; Kanehisa, Furumichi, Tanabe, Sato, & Morishima, 2017).

A total of 26.26 Gbp raw reads were generated. After strict quality control and filtering of host DNA reads (wheat, rice, pea, barley and human genome), these clean reads of *Daqu* and wheat *Qu* were assembled into a total of 21,400 and 90,600 scaftigs with corresponding average length of 1403 bp and 1516 bp, and the N50 were 1769 bp and 2146 bp, respectively. 151,360 and 40,480 ORFs were found in *Daqu* and wheat *Qu* with 68.42% and 42.20% GC percent, respectively. For gene annotation, all unigenes of *Daqu* microbiome were aligned to several databases, 110,897 in NR, 51,849 in KEGG, 3,969 in CAZy and 80,144 in eggNOG. In wheat *Qu*, 32,525 of the unigenes were annotated to NR, 13,115 were annotated to KEGG, 1,273 were annotated to CAZy, and 28,363 were annotated to eggNOG. Sequencing, assembly statistics and gene prediction are shown in Table 1.

Metabolic pathways and enzymes involved in dominant flavor compounds of *Qu* were constructed by using KEGG with modifications (Liu, Chen, et al., 2019). Based on the results of taxonomic assignment and function annotation, these enzymes were connected with *Qu* microbiota by gene ID. When a gene ID from a microorganism was simultaneously annotated as an enzyme-coding gene, then the connection of enzyme and microbiota was built.

2.5. Flavor metabolites analyses

Daqu or wheat *Qu* (10 g) were mixed with sterile water (20 mL) in a 50 mL sterilized centrifugal tube by rotational shaking at 150 rpm for 2 h at room temperature, then filtered through a double layer of Whatman filter paper (Whatman, USA). Part of the filtrate were deproteinised with 10 % trichloroacetic acid (TCA, w/v) and centrifuged at 8000 g for 15 min. The supernatant was used for organic acids and amino acids analysis.

Table 1
Statistics of sequencing and bioinformatics analysis.

Parameter	*Daqu*	wheat *Qu*
Type of sequencing	**Illumine Hiseq 4000**	
Sequencing		
Raw data (Mbp)	12,052.74	14,209.64
Clean data (Mbp)	10,971.94	8,809.38
Clean Q20 (%)	97.55	96.94
Clean Q30 (%)	93.09	92.12
Clean GC (%)	45.22	56.76
Coverage (%)	99.49	99.76
Assembly		
Scatigs number	90,600	21,400
Scatigs average length (bp)	1,516.15	1,403.00
N50 length (bp)	2,146	1,769
N90 length (bp)	604	618
Gene Prediction		
Number of ORFs	151,360	40,480
Number of unigenes	158,876	102,014
GC (%)	42.20%	68.42%
Annotation		
Annotated on NR (% of ORFs)	110,897 (73.27%)	32,525 (80.35%)
Annotated on KEGG (% of ORFs)	51,849 (34.26%)	13,115 (32.40%)
Annotated on CAZy (% of ORFs)	3969 (2.62%)	1273 (3.14%)
Annotated on eggNOG (% of ORFs)	80,144 (52.95%)	28,363 (70.07%)

2.5.1. Analysis of organic acids by HPLC

Eight organic acids contents (α-ketoglutaric acid, pyruvic acid, oxalic acid, acetic acid, malic acid, tartaric acid, citric acid, lactic acid) of the samples were analysed by RP-HPLC (Waters e2695, Milford, MA) with an Athena C18-WP column (250 × 4.6 mm, 5 μm). The column temperature was maintained at 30℃. The detection wavelength was 210 nm. The mobile phase was phosphate buffer (0.025 M NaH_2PO_4, pH 3.1), and the flow rate was 0.7 mL/min.

2.5.2. Analysis of free amino acids by HPLC

Seventeen free amino acids contents (Aspartic acid (Asp), Glutamic acid (Glu), Serine (Ser), Histidine (His), Glycine (Gly), Threonine (Thr), Arginine (Arg), Alanine (Ala), Tyrosine (Tyr), Cysteine (Cys), Valine (Val), Methionine (Met), Phenylalanine (Phe), Isoleucine (Ile), Leucine (Leu), Lysine (Lys), Proline (Pro)) of the samples were analysed according to the method reported with modifications (Wang, et al., 2014). Briefly, the supernatant was analyzed using an Agilent series 1100 instrument (Agilent Technologies, Palo Alto, CA, USA) equipped with an ODS HYPERSIL column (250 × 4.6 mm, 5 μm) and a UV detector. The composition of the mobile phase A was crystallized sodium acetate: triethylamine:water = 6.5 g:200 μL:1000 mL, mobile phase B was crystallized sodium acetate:acetonitrile:methanol:water = 6.5 g:400 mL:400 mL:200 mL, and pH was adjusted to 7.20 ± 0.05. The column temperature was maintained at 40℃. The detection wavelengths were 338 nm and 262 nm, and the flow rate was 1.0 mL/min.

2.5.3. Analysis of volatile compounds by HS-SPME/GC–MS

The volatile compounds were determined by using headspace solid-phase microextraction (HS-SPME) and analyzed using GC–MS instrument (Trace 1300, ISQ LT, Thermo Scientific, San Jose, CA, USA) according to the method reported with modifications (Mo, Fan, & Xu, 2009). The supernatant (5 mL) were placed in a 20 mL SPME glass vial together with 2.5 g of sodium chloride and 10 μL of 2-octanol (41.2 mg/L in absolute ethanol) as an internal standard. The vial was incubated for 50 min at 50 °C. After extraction, the fiber was introduced into the injection port of the GC–MS system (at 250 ℃ for 7 min) and the analytes extracted from the fiber were thermally desorbed. The volatile compounds of *Qu* was extracted using a 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber (Supelco, Bellefonte, PA, USA). Separation was carried out on a TG-Wax (30 m × 0.25 μm × 0.25 mm) with 1.0 mL/min helium as the carrier gas. The program temperature was 40 °C for 3 min, raised at 6 °C/min to 230 °C

and held for 7 min. Mass specta were acquired in the electron ionization (EI) mode at 70 eV and with a mass range of *m/z* 33–350. The ion source and transfer line temperatures were set at 230 °C and 250 °C, respectively. Mass spectra and retention indices (RI) of compounds detected by GC–MS analysis were compared with published data and those in the MS library of National Institute for Standards and Technology (NIST, Search Version 2.0). The quantificantion was carried out using the internal standard method.

2.6. Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). All data were subjected to analysis of variance using GraphPad Prism software (Vision 8.02). Statistical significance was calculated using one-way analysis of variance, followed by Tukey tests. A P value of < 0.05 was considered statistically significant.

3. Results and discussion

3.1. Physicochemical parameters and enzymatic activities of Daqu and wheat Qu

The physicochemical parameters and enzymatic activities are very important indicator for judging the quality of *Qu* (Fan, et al., 2020; Zhang, Li, Wu, Yang, & Zou, 2019). Table 2 shows the physicochemical parameters and enzymatic activities of *Daqu* and wheat *Qu*. Moisture content and acidity of *Daqu* and wheat *Qu* were different, but they all met the quality requirement of *Qu* (Fan, et al., 2020; Xu, Wang, Fan, Mu, & Chen, 2010). Compared with wheat *Qu*, *Daqu* has higher protease and cellulase activity, lower glucoamylase and amylase enzyme activity ($P < 0.05$). Previous studies showed that the lower moisture content and acidity can reduce or inhibit the growth and metabolism of microorganisms, which is helpful to the safe storage of *Qu* (Fan, et al., 2020; Liu, Zhang, et al., 2019). The formation of acidity mainly results from the organic acid metabolism of microorganisms and degradation of fat, starch, and protein (Fan, et al., 2018), therefore it can qualitatively reflect the metabolites in *Qu*. Higher glucoamylase, amylase, and cellulase activities can promote the conversion of starch, cellulose and other polysaccharides to glucose in raw materials. Protease can decompose the protein to peptides and amino acids, which is beneficial for the growth of microorganism and the formation of flavors. The difference in microbial composition, raw materials and manufacturing technique between *Daqu* and wheat *Qu* might be the reason for the different enzyme activities (Liu, Chen, Fan, Huang, & Han, 2018; Zhang, Zhao, & Du, 2014).

Daqu is made of barely, pea, and wheat through solid-state fermentation, and wheat *Qu* is fermented with wheat as the raw material. Barley contains more husks, high cellulose content, about 58%-65% starch content, 11.1–12.6% protein content (Pahlavan, Sharma, Pereira, & Williams, 2016). Wheat is rich in nutrients, with a starch content of about 53% to 70% and a protein content of about 10.1 to 16.4% (Pahlavan, Sharma, Pereira, & Williams, 2016). The protein content of peas is about 21% to 28%, the starch content is about 28–56%, and it is sticky and easy to stick together into lumps (Gallardo, Thompson, & Burstin, 2008). Polysaccharides like starch and cellulose the most abundant, natural reserve polysaccharide and primary source of stored energy in cereal grains, which can be degraded into glucose. Glucose is one of the most important sources of energy and nutrients for microbial growth and reproduction. It can be involved in the glycolysis pathway of the central carbon metabolism, and is closely related to the synthesis of various metabolites such as pyruvate. Protein in raw materials is the nitrogen source of microorganisms. It will be decomposed into amino acids in the fermentation process, which is one of the main nutrients of microorganisms, and is used to form the precursors of various enzymes and flavor compounds (Wang, Li, et al., 2020). Studies have shown that the moderate amount of nitrogen can increase the growth and reproduction of *Aspergillus* and *yeast* and then increase the amount of enzyme produced (Lu, Yang, Shen, Qian, & Li, 2019). Compared with wheat *Qu*, *Daqu* components is more abundance in protein, which makes microorganism get more nitrogen sources. The difference in raw material may have an important impact on the flavor, the quantity and variety of microbiota in *Qu*.

In addition to providing various nutrients, the different raw materials also have individual characteristic flavors. A previous study showed that 35 flavor compounds were detected from barley, mainly diethyl succinate, ethyl palmitate, isoamyl alcohol, heptanoic acid, octanol, pentanoic acid, etc (Zhu, Wu, Li, & Xu, 2015). The main flavor compounds in wheat are β-phenethyl alcohol, phenol, ethyl hexanoate, and hexanol, while the main flavor compounds in pea are hexanol, hexanal, phenylethylene, 2-butanone, dimethyl sulfide, 3-carene, ethyl acetate and 2,3-dimethyl-5-methylpyrazine, etc. These bound flavor compounds in raw materials will decompose into free states during the fermentation of *Daqu* and *baijiu*, and become part of the flavor of *baijiu* (Azarnia, Boye, Warkentin, & Malcolmson, 2011; Zhu, Wu, Li, & Xu, 2015). Additionally, analysis of the sources of microorganisms in Daqu found that the bacterial community mainly came from raw materials, while the fungal community mostly came from the production environment (Du, Wang, Zhang, & Xu, 2019). Therefore, different *Qu* raw materials carry different microbial communities to participate in the fermentation of *Qu*, thus affecting the community composition of *Qu*. Accordingly, the differences in raw material, physicochemical properties and enzyme activities indirectly reflect the significant differences in microbial composition, quantity and metabolism between *Daqu* and wheat *Qu*.

Table 2
Physicochemical parameters and enzymatic activities of *Daqu* and wheat *Qu*

Parameter	*Daqu*	wheat *Qu*
Moisture (%)	12.62 ± 0.17 [a]	12.58 ± 0.03 [a]
Acidity (mmol/10 g dry sample)	0.62 ± 0.02 [a]	0.47 ± 0.01 [a]
Amylase activity (U/g dry sample)	2.07 ± 0.11[b]	2.93 ± 0.02 [a]
Glucoamylase activity (U/g dry sample)	831.36 ± 14.53[b]	1429.85 ± 35.74 [a]
Protease activity (U/g dry sample)	85.77 ± 4.63 [a]	48.11 ± 2.80[b]
Cellulase activity (U/g dry sample)	23.52 ± 2.17[a]	11.71 ± 1.65[b]

*Different superscripts (a, b) in a column refer to significant differences ($P < 0.05$).

3.2. Microbiota in wheat Qu and Daqu revealed by whole-metagenome sequencing

Firmicutes, Ascomycota, and Mucoromycota massively dominated the microbial community in *Daqu* at the phylum level, and their total relative abundance reached 84.34% (Fig. 2**A**). Further analysis at the species level showed there were 341 fungal species and 906 bacterial species identified in *Daqu*, of which 33 species had relative abundance greater than 0.1% (Fig. 2**A and Table S1**). The dominant microbes were defined as the microbial species with more than 1% of relative abundance. In *Daqu* microbiota, the dominant microbes were *P. pentosaceus* (6.01%), *R. emersonii* (5.75%), *W. paramesenteroides* (5.57%), *B. spectabilis* (5.15%), *Lichtheimia ramosa* (4.87%), *L. citreum* (3.72%), *W. cibaria* (2.54%), *Lactobacillus paralimentarius* (1.76%), *Weissella confuse* (1.75%), *L. mesenteroides* (1.37%), *Lactobacillus plantarum* (1.31%), *Pichia kudriavzevii* (1.20%), *Staphylococcus gallinarum* (1.12%) and *Lactobacillus brevis* (1.02%). Molds (*R. emersonii, B. spectabilis* and *L. ramosa*) were regarded as the main source for enzymes production, and the secreted enzymes can promote the degradation of wine raw materials, which is conducive to the continuous progress of fermentation(de Morais, et al., 2018; Garcia, et al., 2015; Tanney & Seifert, 2013). *P. kudriavzevii* is the core strain of liquor fermentation, which can contribute to the functionality (alcohols, acids and esters) and improve the sensory and some functional properties of the cereal-based substrate during fermentation (Wang, Wu, Nie, Wu, & Xu, 2019). Intersetingly, the dominant bacteria in *Daqu* are all Firmicutes. The high temperature,

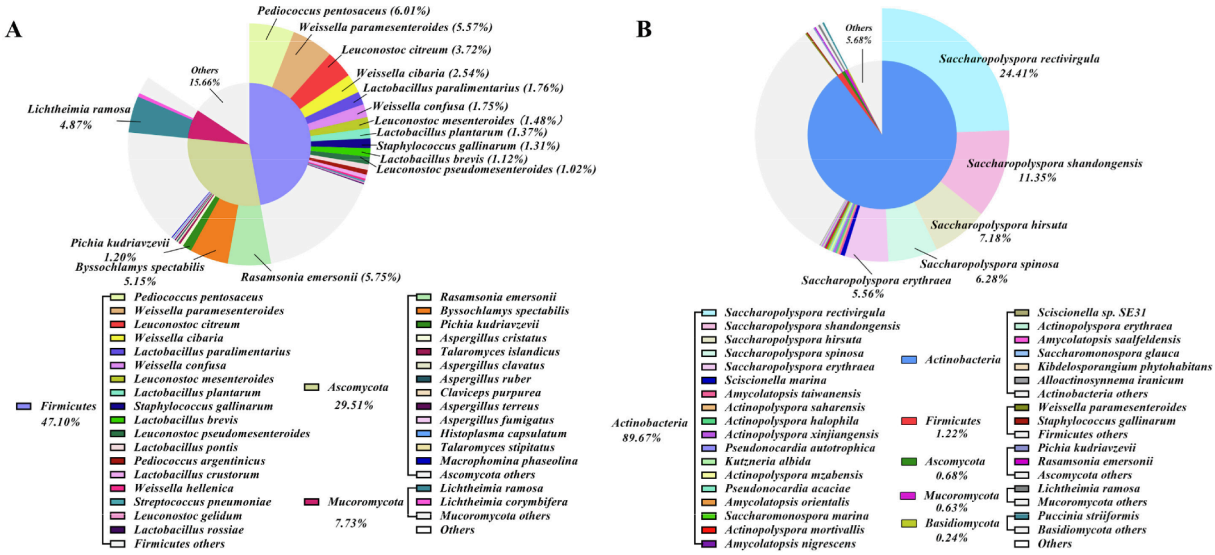

Fig.2. Microbial communities of Daqu at the phylum (inner circles), and species levels. (outer circles) (A); Microbial communities of wheat Qu at the phylum (inner circles),. and species levels (outer circles) (B). Species with a relative abundance larger than 0.1%. are displayed.

high humidity and acidic fermentation environment of *Daqu* will cause the extinction of a large number of microorganisms (Xiao, et al., 2017), while Firmicutes have been found to be abundantly distributed in environments with extreme temperature, pH environments and in seawater (Li, et al., 2020; Selvarajan, et al., 2019). Bacteria can not only secrete enzymes, but also a potential source of flavor metabolites (Wang, Liu, Shen, & Lian, 2018). The lactic acid produced by lactic acid bacteria (LAB) in the fermentation process can make full-bodied taste and improve the harmony of aroma profile, and the ethyl lactate formed by the esterification of lactic acid and ethanol is one of the characteristic flavor substances in the wine (Chen, et al., 2021; Jiang, Lu, & Liu, 2020; Moreno-Arribas & Polo, 2005; Xiao, et al., 2014). The metabolites of *Weissella*, such as lactic acid and acetic acid, are the precursors of important flavor substances in wine (Centeno, Tomillo, Fernandez-Garcia, Gaya, & Nunez, 2002). *S. gallinarum* can secrete acidic protease, saccharification enzyme and lipase, etc (Jia, et al., 2020; Zheng, et al., 2015).

In contrast, wheat *Qu* possessed a relatively simple microbiota structure, and the total relative abundance of Actinobacteria and Firmicutes reached 90.89% (Fig. 2**B**). A total of 243 fungal species and 1011 bacterial species were identified in wheat *Qu*, of which 30 species had relative abundance greater than 0.1% (Fig. 2**B and Table S2**). Among them, all the dominant microbes belonged to *Saccharopolyspora* (54.78%), including *S. rectivirgula* (24.41%), *S. shandongensis* (11.35%), *S. hirsuta* (7.18%), *S. spinose* (6.28%), and *S. erythraea* (5.56%). Clearly, the dominant microbes of *Daqu* and wheat *Qu* were significantly different, especially *Saccharopolyspora* strongly dominated the wheat *Qu* microbiota. *Saccharopolyspora* belong to Actinobacteria, which can produce enzymes, vitamins, cellulose degradation promoting factors, and are a class of safe biological resource bacteria (Sayed, Abdel-Wahab, Hassan, & Abdelmohsen, 2020). Previous studies showed that *Saccharopolyspora* was also the dominant bacteria in the fermentation process of *huangjiu* (Liu, Chen, et al., 2019). This result indicated that *Saccharopolyspora* in *huangjiu* fermentation may originate from wheat *Qu*. Earlier studies have demonstrated that *Saccharopolyspora* is the dominant microbial genus in Moutai starter, and it is also essential for generating flavoring substances in Moutai liquor production (Gan, et al., 2019). In addition, *Saccharopolyspora* sp. are known to produce thermostable a-amylase enzyme, glucoamylase, protease and cellulase (Chakraborty, et al., 2011; Meena, Rajan, Vinithkumar, & Kirubagaran, 2013; Xu, Liao, Yao, Ye, & Ye, 2016). This finding implies that *Saccharopolyspora* in wheat *Qu* has great application value. However, the mechanism of *Saccharopolyspora* in *huangjiu* fermentation is still unclear. Function and application of *Saccharopolyspora* in food fermentation can be further studied in the future.

3.3. *Functional gene category by blasting to eggNOG, CAZy, KEGG databases*

To explore the functional feature and differences between *Daqu* and wheat *Qu* microbiota, the whole-metagenomic data were annotated using the KEGG, CAZy and eggNOG database, and analysed at the functional and metabolic pathway levels (Fig. 3). According to the KEGG database annotation at level 1, all the genes were classified to 6 categories, of which Metabolism (ME) was the most abundant category in both wheat *Qu* and *Daqu* microbiota, occupied 18.54% and 12.73% of all annotated genes, respectively.

Among the 45 level 2 pathways, the most abundant pathway in *Daqu* microbiota were carbohydrate metabolism (3.63%), global and overview maps (2.68%), amino acid metabolism (2.46%), energy metabolism (2.40%) and translation (2.13%) (Fig. 3**A**). Global and overview maps (5.30%), amino acid metabolism (4.98%), carbohydrate metabolism (4.61%), energy metabolism (3.22%) and metabolism of cofactors and vitamins (2.82%) were primary functions of wheat *Qu* microbiota in KEGG annotations. The KEGG-based analysis showed that *Daqu* and wheat *Qu* microbiota is enriched with genes involved in carbohydrate metabolism, amino acid metabolism, and energy metabolism. This result suggests that the *Daqu* and wheat *Qu* microbiota have great potential for raw materials degradation (rice, wheat and barley) and flavor compounds metabolism, which is consistent with the function of *Qu* (Xu, Wang, Fan, Mu, & Chen, 2010). Remarkably, metabolism of cofactors and vitamins (e.g. pantothenate and CoA biosynthesis, nicotinate and nicotinamide metabolism, and vitamin B6 metabolism) were also primary functions of wheat *Qu* microbiota.

According to CAZy database, these genes were annotated into six categories including Glycoside Hydrolases (GH), Glycosyl Transferases (GT), Carbohydrate-Binding Modules (CBM), Carbohydrate Esterases (CE), Auxiliary Activities (AA) and Polysaccharide Lyases (PL) (Fig. 3**B**). These enzymes can assemble or degrade carbohydrates (such as starch for energy storage, cellulose for structure maintenance, sugar complexes) that are widespread in nature to form a variety of carbohydrates. Among them, GH and GT had the highest relative abundance both in *Daqu* and wheat *Qu*, and PL had the least. GHs are a group of important enzymes that hydrolyze glycosidic bonds, which contain enzymes

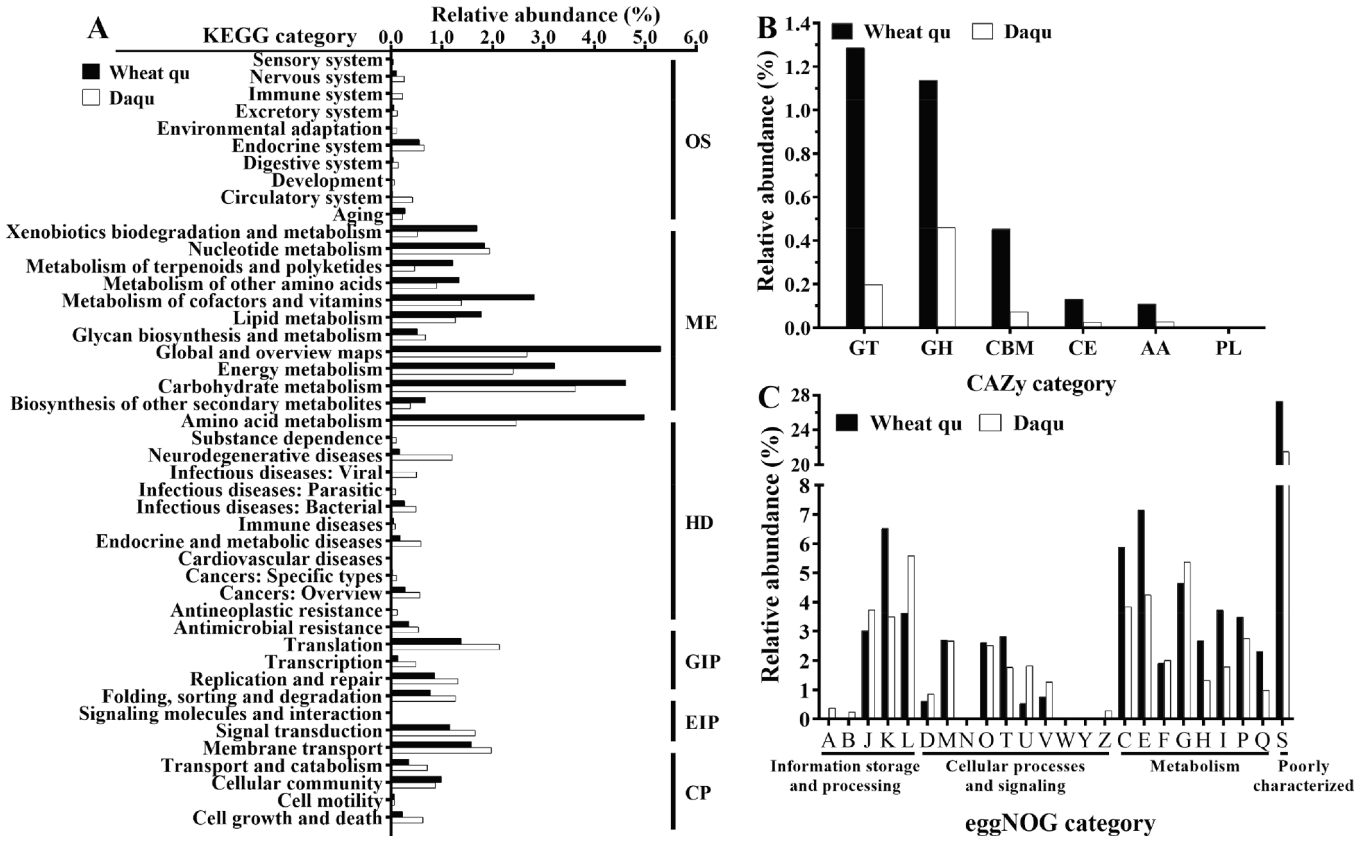

Fig. 3. Functional of Diversity of Daqu and wheat Qu. (A) KEGG. ME: Metabolism; GIP: Genetic Information Processing; EIP: Environmental Information Processing; CP:Cellular Processes; OS: Organismal Systems; HD: Human Diseases; (B) CAZy. GH:Glycoside Hydrolases; GT: Glycosyl Transferases; CBM: Carbohydrate BindingModules; AA: Auxiliary Activities; CE:Carbohydrate Esterases; PL: PolysaccharideLyases. (C) eggNOG. A: RNA processing and modification; B: Chromatin structureand dynamics; J: Translation, ribosomal structure and biogenesis; K: Transcription; L:Replication, recombination and repair; D: Cell cycle control, cell division, chromosome partitioning; M: Cell wall/membrane/envelope biogenesis; N: Cell motility; O: Posttranslational modification, protein turnover, chaperones; T: Signal transductionmechanisms; U: Intracellular trafficking, secretion, and vesicular transport; V: Defensemechanisms; Y: Nuclear structure; Z: Cytoskeleton; C: Energy production and conversion; E: Amino acid transport and metabolism; F: Nucleotide transport and metabolism; G: Carbohydrate transport and metabolism; H: Coenzyme transport and metabolism; I: Lipid transport and metabolism; P: Inorganic ion transport and metabolism; Q: Secondary metabolites biosynthesis, transport and catabolism; S:Function.

associated with starch liquefaction, saccharification and cellulose degradation. The solid-state fermentation of *Qu* is to transform the utilization of cereal raw materials into fermentable sugars for microbial utilization. Therefore, the number of genes annotated to the GHs account for a large proportion. GT is related to the biosynthesis of oligosaccharides and polysaccharides (Kim, et al., 2013), and the higher relative abundance indicates that polysaccharide-producing microorganisms might exist in *Daqu* and wheat *Qu*. Polysaccharides have antioxidant, immunomodulatory, tumor suppressive and intestinal microbial regulation effects (Shen, Mao, Chen, Meng, & Ji, 2015). Previous studies have reported that the content of polysaccharides in *huangjiu* reaches 683 mg/L (Peng, Liu, Ji, Chen, & Mao, 2019). CBM are distinct structural folds of a stretch of amino acids within carbohydrate-active enzymes having carbohydrate binding activity (Boraston, Bolam, Gilbert, & Davies, 2004; Pollet, Delcour, & Courtin, 2010). Many carbohydrate-hydrolyzing enzymes, such as cellulases and hemicellulases, are modular proteins with at least two distinct modules: the catalytic module and the CBM (Arantes & Saddler, 2010). Studies have shown that CBM helps in binding of a cazyme to its carbohydrate substrate (cellulose and starch etc.), thereby facilitating the enzyme's activity. Thus, the higher relative abundance of CBM can promote the degradation of cellulose and starch in the raw materials of *Qu*. CE are a large group of carbohydrate-active enzymes that catalyse the removal of ester substituents from the glycan chains of polysaccharides (Adesioye, Makhalanyane, Biely, & Cowan, 2016), including feruloyl esterase, acetylxylan esterases and pectinesterase. PL mainly degrade glycosaminoglycans and pectin. CE, GH cooperate with PL in plant polysaccharide degradation to overcome the complexity of the plant cell walls (Delmas, et al., 2012; Xie, et al., 2016). *Qu* is a spontaneous solid-state cereal fermentation. All of them were beneficial for the degradation of cereal raw materials.

All these genes were clustered into 24 eggNOG categories, the top four categories in wheat *Qu* were S (Function unknown), E (Amino acid transport and metabolism), K (Transcription) and C (Energy production and conversion) (Fig. 3**C**). S (Function unknown), L (Replication, recombination and repair), G (Carbohydrate transport and metabolism) and E (Amino acid transport and metabolism) were the main categories of *Daqu*. About 27.30% in wheat *Qu* and 21.54% in *Daqu* of these genes belong to category S, meaning that their functions are unknown and novel, which can continue to be analyzed and studied in the future. The production of *Qu* was an open fermentation process with lots of unculturable and unstudied microorganisms (14.95% in *Daqu* and 33.10% in wheat *Qu*), and the novel genes may be from them. Categories K, C and L are closely related to the reproduction and survival of microorganisms. Categories G and E are closely related to carbohydrate and amino acid metabolism. This result is consistent with the KEGG annotation result, which confirms that carbohydrate and amino acid metabolism play an important role in *Qu* fermentation.

3.4. Organic acids biosynthetic potential of wheat Qu and Daqu microbiota

Organic acids are mainly derived from the metabolism of microorganisms in Koji (*Qu*) and have the functions of eliminating bitterness, reducing astringency, buffering taste and stabilizing aroma in wine (Wang, et al., 2014). As shown in Fig. 4**A**, the total organic acid content in *Daqu* (28.48 mg/g) were significantly higher than in wheat *Qu* (7.08 mg/g) ($P < 0.05$). Lactic acid, acetic acid and tartaric acid were the main organic acids in both *Daqu* and wheat *Qu*. Their total amounts are 22.04 mg/g and 5.64 mg/g, respectively, accounting for more than 77.41% and 79.70% of the total contents of the 8 organic acids detected. Based on the metagenomics annotation data in KEGG, there are 6 possible pathways of acetic acid synthesis (Fig. 4**B and 4C**) in *Daqu* and wheat *Qu*. Interestingly, *Daqu* and wheat *Qu* shares the same acetic acid synthesis pathways, but there was significant difference in the enzymes and microbial species. In addition, the functional microbes were defined as the microbial species involved in the main flavors development in *Qu*. In *Daqu*, based on the abundance of enzymes, there are two main metabolic pathways of potential acetic acid production using acetaldehyde and acetyl-P (Fig. 4**B and 4C**). *L. ramosa, P. kudriavzevii, R. emersonii, B. spectabilis* and *S. gallinarum* might transform acetaldehyde to acetic acid by aldehyde dehydrogenase (EC1.2.1.3), while *W. paramesenteroides, L. citreum* and *L. paralimentarius* might produce acetic acid with acetyl-P as the substrate under the action of acetate kinase (EC 2.7.2.1) and acyl-phosphatase (EC 3.6.1.7). In wheat *Qu*, pruvate, acetyl adenylate, acetyl-CoA, acetaldehyde, lactic acid and acetyl-P were the immediate precursor of acetic acid biosynthesis, and it can be synthesized through 8 enzymatic pathways (EC 1.2.1.-, 2.7.2.1, 3.6.1.7, 1.2.5.1, 2.8.3.1, 2.8.3.18, 3.1.2.1, and 6.2.1.1). Many microorganisms might participate in the formation of acetic acid, including *S. shandongensis*, *S. rectivirgula*, *S. erythraea*, *S. hirsuta*, *Saccharopolyspora antimicrobica, Actinopolyspora halophile, and S. gallinarum*. Clearly, *Saccharopolyspora* might be the main producer. In *Daqu*, *W. paramesenteroides, L. citreum, L. mesenteroides*, *W. confuse*, and *W. cibaria* might transform pyruvate to lactic acid by lactate dehydrogenase (EC1.1.1.27, 1.1.1.28) and malolactic enzyme (EC4.1.1.101), while *L. citreum* and *W. paramesenteroides* might produce lactic acid with malate as the precursor by the activity of lactate dehydrogenase (EC1.1.5.12). In wheat *Qu*, hydroxyacylglutathione hydrolase (EC 3.1.2.6) was the primary enzyme catalyzing the conversion of (R)-S-lactoyglutathione into lactic acid, and *S. erythraea* might be the main producer. *Sciscionella* sp.*SE31* could reduce oxaloglycolate to tartaric acid by tartrate dehydrogenase (EC1.1.1.93) in wheat *Qu*. Although tartaric acid was a dominant organic acid in *Qu*, relative abundance of enzyme genes in the biosynthetic pathway of tartaric acid was quite low in wheat *Qu*, and there are no related enzyme genes annotated in *Daqu* at the species level. Tartaric acid in *Qu* might mainly originate from the degradation of raw materials or other species that have not been annotated. This analysis revealed that there was a significant difference between the organic acid biosynthesis pathway and the main functional microbial of the two *Qu*. In addition, bacteria were the main producer of organic acids in *Daqu* and wheat *Qu*.

3.5. Free amino acids biosynthetic potential of Daqu and wheat Qu microbiota

Free amino acids are one of the nitrogen sources for the growth and metabolism of microorganisms, and they are important precursors of

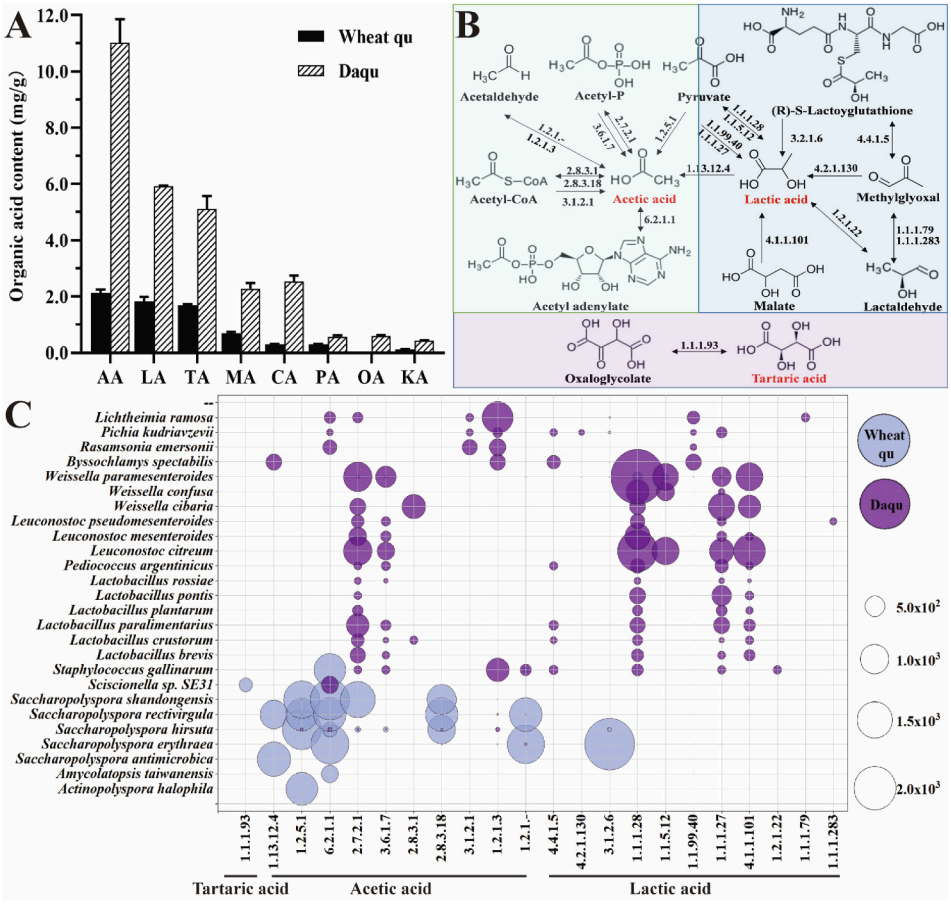

Fig. 4. Organic acid production of Daqu and wheat Qu by microorganisms and its.relative enzymes. (A) Content of organic acids; Acetic acid (AA); Lactic acid (LA); Tartaric acid (TA); Malic acid (MA); Citric acid (CA); Pyruvic acid (PA); Oxalatc (OA);α-Ketoglutaric acid (KA); (B) Pathway of organic acids production; (C) Therelationship between microorganisms and enzymes involved in different metabolicpathways.

higher alcohols, esters and other flavor compounds (Gambetta, Cozzolino, Bastian, & Jeffery, 2017). Amino acids of the *Qu* might originate from proteins or peptides in raw materials, or synthesized by microorganisms (Wang, et al., 2021). The free amino acids content of the *Qu* are shown in Fig. 5**A**. The main amino acids in wheat *Qu* were Pro, Glu, Arg, Asp, Lys, Ala, His and Phe, the contents of 8 main amino acids were 763.23 mg/kg, accounting for 80.98% of total amino acid content. Glu, Ala, Pro, Phe, Asp, Val, Lys, Ser and Leu were the main amino acids of *Daqu*, which accounted for 75.93% of the total amino acids at a content of 4113.01 mg/kg. Obviously, the content of amino acids in *Daqu* is significantly higher than that in wheat *Qu* ($P < 0.05$). There may be two possible reasons for this. First, the raw materials are different. Wheat *Qu* uses wheat as the main raw material, and *Daqu* uses wheat, barley and peas as the main raw materials, which makes the protein content of *Daqu* higher than that of wheat *Qu*. Second, the production temperature of wheat *Qu* and *Daqu* is different. The production temperature of *Daqu* (58 ~ 65℃) was significantly higher than that of wheat *Qu* (≤55℃), and high temperature culture will promote protein decomposition (Deng, et al., 2020).

According to KEGG annotations, the biosynthesis of 11 main free amino acids (His, Ser, Ala, Leu, Phe, Val, Lys, Glu, Asp, Arg, and Pro) in *Daqu* and wheat *Qu* involves 32 and 27 enzymes, respectively (Fig. 5**B and 5C**). Histidinol dehydrogenase (EC 1.1.1.23) might catalyze the oxidation of histidinol into His. Serine hydroxymethyl transferase (EC 2.1.2.1) and phosphoserine phosphatase (EC 3.1.3.3) were the key enzymes in the synthesis of Ser, catalyzing the conversion of glycine or phosphoserine to Ser (Jouhten, et al., 2016). In addition, hydroxypyruvate and glycine can also be converted to Ser by serine-glyoxylate transaminase (EC 2.6.1.45). Aminotransferases, also known as transaminases, catalyze the transfer of amino groups from amino donors to amino acceptor compounds (Koech, et al., 2019). Phenylpyruvate can be converted to Phe under the catalysis of multiple transaminases (EC 2.6.1.5; 2.6.1.9; 2.6.1.57; 2.6.1.58; 2.6.1.21). Prephenate can also be converted to Phe in two steps, catalyzed by prephenate dehydrogenase (EC 4.2.1.51; 4.2.1.91) and transaminase. Interestingly, branched-chain amino acid aminotransferase (EC 2.6.1.42) not only catalyzes the conversion of 4-methyl-2-oxopentanoate to Leu, but also 2-oxoisovalerate to Val. By reductive amination, pyruvate can be converted into Ala by alanine transaminase (EC 2.6.1.2) and alanine dehydrogenase (EC 1.4.1.1). Asparaginase (EC 3.5.1.1), aspartate aminotransferase (EC 2.6.1.1) and asparagine synthase (EC 6.3.5.4) can catalyze the synthesis of Asp. Notably, previous research has shown that asparagine synthase is an important enzyme in the synthesis of Asp (Sun, et al., 2016). Diaminopimelate decarboxylase (EC 4.1.1.20) and saccharopine dehydrogenase (EC 1.5.1.7) may be involved in the biosynthesis of Lys. 2-Oxoglutarate was known as a substrate for inorganic nitrogen assimilation for the conversion to Glu via glutamate dehydrogenase (EC 1.4.1.2; 1.4.1.3; 1.4.1.4) and glutamate synthase (EC 1.4.1.13; 1.4.1.14) (Takahashi & Kohno, 2016). Arg can be synthesized form citrulline via either arginine deiminase (EC 3.5.3.6) or nitric-oxide synthase (EC1.14.14.47). Additionally, Arg may also be synthesized via the aspartate-argininosuccinate shunt by argininosuccinate lyase (EC 4.3.2.1), which joins the TCA cycle with the urea cycle (Fei, Lee, McCarry, & Bowdish, 2016). In the Pro biosynthesis pathway, ornithine cyclodeaminase (EC 4.3.1.12) catalyses the conversion of ornithine to proline. Moreover, Pro also can be potentially synthesized from pyrroline 5-carboxylate using pyrroline 5-carboxylate reductase (EC 1.5.1.2). 11 main functional microorganisms were involved in the biosynthesis of amino acids in *Daqu*, including 3 fungi (*R. emersonii*, *B. spectabilis*, *L. ramosa)* and 8 bacteria (*P. pentosaceus*, *W. paramesenteroides*, *L. citreum*, *S. gallinarum*, *W. cibaria*, *L. paralimentarius*, *L. mesenteroides and L. brevis)*. There were 9 main functional microorganisms involved in the synthesis of amino acids in wheat *Qu*, all of which were bacteria, including *S. rectivirgula*, *S. shandongensis*, *S. spinose*, *S. erythraea*, *S. hirsuta*, *Saccharopolyspora flava*, *S. antimicrobica*, *Micromonospora echinaurantiaca and Actinobacteria bacterium IMCC26207*. It is well known that *Daqu* and wheat *Qu* play similar roles in fermented liquors, but this study found that the microbes involved in amino acid synthesis

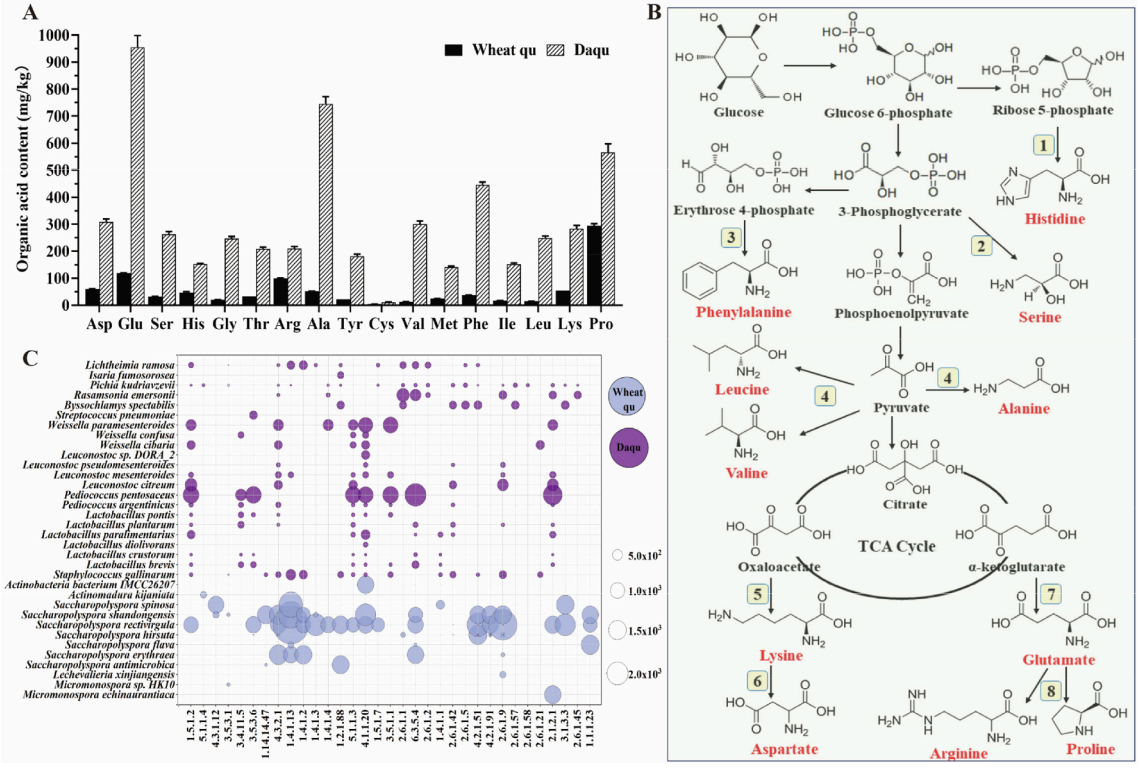

Fig. 5. Amino acids production of Daqu and wheat Qu by microorganisms and its relative enzymes. (A) Content of amino acids; (B) Pathway of amino acids production, (C) The relationship between microorganisms and enzymes involved in different metabolic pathways.

were significantly different. Notably, *Saccharopolyspora* were the main participants in wheat *Qu*, while the species in *Daqu* were more abundant. This indicated that genes from different microbes perform similar functions. *Daqu* microorganisms have more amino acid synthesis pathways than wheat *Qu*, which may be due to the fact that *Daqu* functional microorganisms have more species, especially some fungi.

3.6. Volatile compounds biosynthetic potential of Daqu and wheat Qu microbiota

Flavor compounds are very important indicators of *Qu* quality (Pang, et al., 2020). In addition to affecting the flavor compounds formation during *baijiu* and *huangjiu* fermentation, the flavor compounds of *Qu* can directly bringing into them to exert an important influence on the complex aroma of the final production (Chen & Xu, 2013; Yang, et al., 2021). The volatile compounds detected in *Daqu* and wheat *Qu* are shown in Fig. 6**A**. The *Qu* contains three classes of major volatiles including alcohols, esters and aldehydes. The most abundant volatile compounds in *Daqu* were phenethyl alcohol, 2-octene-1-ol, hexanol, ethanol, isoamyl alcohol, ethyl caprylate, phenylacetaldehyde, benzaldehyde and 4-vinylguaiacol, with a total content of 970.42 μg/kg, accounting for 76.60% of total volatile compounds detected. Phenethyl alcohol, 1-octen-3-ol, 2,3-butanediol, 2-octene-1-ol, hexanol, ethanol, heptanol, isoamyl alcohol, ethyl hexanoate, ethyl acetate, hexanal and tetramethylpyrazine (TMP) were the most abundant volatile compounds of wheat *Qu*, with a total content of 1021.81 μg/kg, at 74.73% of the total volatile compounds detected.

Alcohols, especially higher alcohols are important flavor substances in *Qu* (Mo, Fan, & Xu, 2009). For example, isoamyl alcohol is responsible for fruity-floral flavor, phenethyl alcohol for roses and sometimes honey flavor and 1-octen-3-ol for mushrooms-like flavor (Tang, Liang, Song, Lin, & Luo, 2019; WangYuan, et al., 2020). Higher alcohols are produced from amino acid catabolic metabolism (Ehrlich pathway) as well as sugar anabolic metabolism (Harris pathway) (Wang, He, Pan, Duan, & Wang, 2018). In the Erich pathway, amino acids are catalyzed by transaminase and decarboxylase to sequentially generate α-keto acids and aldehydes, and the produce higher alcohols under the catalysis of alcohol dehydrogenase. If α- ketoacid is derived from carbohydrate metabolism, it is called the Harris pathway (Wang, et al., 2021). In this study, phenylacetaldehyde is the immediate precursor in the biosynthesis of phenylethylalcohol though aryl-alcohol dehydrogenase (EC 1.1.1.90) (Fig. 6**B and 6C**). Butanediol dehydrogenase (EC 1.1.1.76, 1.1.1.14) and butanol dehydrogenase (EC 1.1.1.-) were the primary enzyme catalyzing the conversion of 2-acetoin into 2,3-butanediol. Acetaldehyde is mainly metabolized to ethanol by alcohol dehydrogenase (EC 1.1.1.1, 1.1.1.2, 1.1.2.8). 9 functional microorganisms were involved in the biosynthesis of higher alcohols in *Daqu*, including *W. paramesenteroides, L. citreum, P. pentosaceus, L. mesenteroides, R. emersonii, Lactobacillus pontis, L. brevis, W. cibaria, Leuconostoc pseudomesenteroides*. There were 8 functional microorganisms involved in the synthesis of higher alcohols in wheat *Qu*, including 4 *Saccharopolyspora* (*S. spinose, S. rectivirgula, S. hirsuta and S. shandongensis*) and *Kutzneria albida, Amycolatopsis jejuensis, Streptomyces xylophagus, Microvirgula aerodenitrificans*.

Aldehydes are important flavor compounds in *Qu*. In *Daqu*, aryl-alcohol dehydrogenase (EC 1.1.1.90) was the primary enzyme catalyzing the conversion of benzyl alcohol into benzaldehyde, and *P. pentosaceus* might be the main producer. In wheat *Qu*, *A. jejuensis* might transform benzyl alcohol to benzaldehyde by aryl-alcohol dehydrogenase (EC1.1.1.90), while *S. spinose, Actinomadura oligospora, S. rectivirgula* and *S. shandongensis* might produce benzaldehyde with phenylglyoxylic acid as the precursor by the activity of benzoylformate decarboxylase (EC4.1.1.7) (Fig. 6**B and 6C**). In *Daqu*, phenylacetaldehyde has a variety of production pathways, which can be produced under the catalysis of decarboxylase (EC 1.1.1.90; 1.2.1.5; 4.1.1.-) using phenylethylalcohol, phenylacetate and phenylpyruvate as precursors, *P. pentosaceus* and *R. emersonii* might be the main producer. *R. emersonii* and *B. spectabilis* could oxidizes phenethylamine to phenylacetaldehyde by oxidase (EC 1.4.3.4; 1.4.3.21). *S. spinose*, *A. jejuensis* and *K. albida* were the related functional microorganism in wheat *Qu*. Hexanal can be synthesized form hexenol via alcohol dehydrogenase (EC 1.1.1.1).

Esters mostly present fruity, sweet and floral aromas, which can bring pleasant aromas to *Qu* and wine. Esters may be generated through the esterification reaction of alcohol and acid, or by condensation of acetyl-CoA with alcohols catalyzed by alcohol acetyltransferase (Campayo, de la Hoz, Garcia-Martinez, Salinas, & Alonso, 2020). Alcohol dehydrogenase (EC1.1.1.1) and lipase (EC 3.1.1.3) may be involved in the synthesis of esters (Fig. 6**B and 6C**). *K. albida, S. rectivirgula, S. shandongensis* might be the main functional microorganism in wheat *Qu*, while the related functional microorganism in *Daqu* are *R. emersonii, L. citreum, W. cibaria, W. paramesenteroides, L. mesenteroides*. 4-

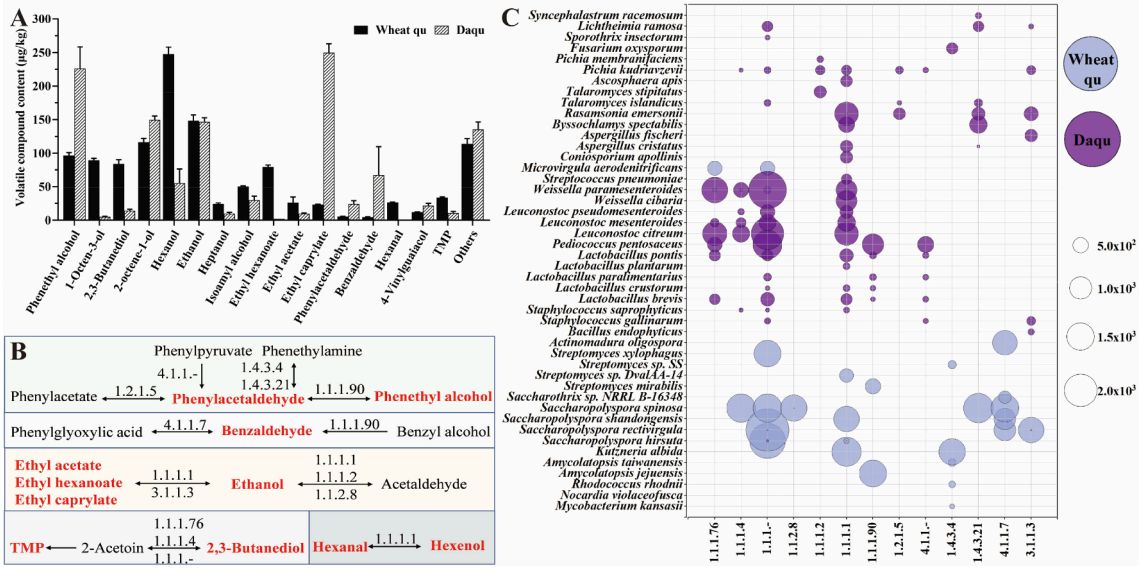

Fig. 6. Volatile compounds production of Daqu and wheat Qu by microorganisms and. its relative enzymes. (A) Content of volatile compounds; (B) Pathway of volatile compounds production; (C) The relationship between microorganisms and enzymes involved in different metabolic pathways.

Vinylguaiacol is an important aroma compound in *Qu* and wine, showing spicy, smoke and clove flavor. 4-Vinylguaiacol can be produced by enzymatic or thermal decarboxylation from ferulic acid (Mo & Xu, 2010). In this study, phenacrylate decarboxylase (EC 4.1.1.102) related genes and microorganisms were not annotated. Therefore, it may be generated through non-enzymatic reactions. TMP has the functions of dilating blood vessels, improving microcirculation and inhibiting platelet accumulation (Wu, et al., 2020). It is mainly produced through non-enzymatic reactions with acetoin as the precursor in *Qu* making and wine fermentation.

In summary, *R. emersonii*, *W. paramesenteroides*, *L. citreum*, *L. mesenteroides*, *W. cibaria*, *P. pentosaceus*, *B. spectabilis*, *L. ramosa*, *S. gallinarum*, *L. paralimentarius*, *L. brevis*, *L. pontis*, *L. pseudomesenteroides*, *W. confuse*, and *P. kudriavzevii* were all the functional microbes may involve in the main flavors development in *Daqu*. In wheat *Qu* microbiota includes *S. shandongensis*, *S. rectivirgula*, *S. spinose*, *S. erythraea*, *S. hirsuta*, *K. albida*, *A. jejuensis*, *S. antimicrobica*, *A. halophile*, *S. gallinarum*, *S. flava*, *S. sp.SE31*, *M. echinaurantiaca*, *A. bacterium* IMCC26207, *S. xylophagus*, *M. aerodenitrificans*, and *A. oligospora*. However, a combined analysis among microbiota, functional genes, and dominant flavour compounds showed that some maicroorganisms may be involved in the synthesis of only a few flavor compounds ($\leq$3). And they can be replaced by other microorganisms. Therefore, the combined analysis among microbiota, functional genes, and dominant flavour compounds indicated *R. emersonii*, *W. paramesenteroides*, *L. citreum*, *L. mesenteroides*, *W. cibaria* and *P. pentosaceus*, may be the main functional microbes to flavor compounds synthesis in *Daqu*, while *S. shandongensis*, *S. rectivirgula*, and *S. spinose* might be the main functional microbes to the synthesis of flavor compounds in wheat *Qu*.

4. Conclusion

Identifying the accurate relationship between flavor and microorganisms is essential to realize the transformation from natural fermentation to controlled fermentation, which is the premise for stable making high-quality foods. *Qu* is a reproducible and typical natural fermentation product, which can be used as a model to explore the relationships between flavor and microorganisms. In this study, whole-metagenome shotgun sequencing was combined with metabolite analysis to reconstruct the synthesis pathway of flavor substances in *Qu*, and find that the dominant microbes in *Qu* may not be the main functional microbes. Therefore, this study provides a new perspective to help clarify the different metabolic roles of microbes in flavor formation during fermentation. However, bioinformatic data are only predictive and require more experimental verification. In addition, *Saccharopolyspora* can be used as a new resource to further explore its role and application in fermented food.

CRediT authorship contribution statement

Jing Zhang: Conceptualization, Methodology, Formal analysis, Investigation, Visualization, Writing – original draft. **Shuangping Liu:** Supervision, Writing – review & editing. **Hailong Sun:** Data curation, Writing – review & editing. **Zhengfei Jiang:** Data curation, Writing – review & editing. **Yuezheng Xu:** Data curation, Writing – review & editing. **Jieqi Mao:** Supervision, Writing – review & editing. **Bin Qian:** Data curation, Writing – review & editing. **Lan Wang:** Data curation, Writing – review & editing. **Jian Mao:** Funding acquisition, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2021.110707.

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