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Stabilization of *jiuyao* quality for *huangjiu* brewing by fortifying functional strains based on core microbial community analysis

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ABSTRACT

Jiuyao as a generally small saccharifying and spontaneously fermenting starter is indispensable for *huangjiu* brewing. However, *jiuyao* currently in use is facing a major challenge of standardized manufacturing because of its open operating environment and rather primitive operations. This study provided a stead manufacturing procedure for *jiuyao* by controlling the processing parameters. *Saccharomycopsis fibuligera* and *Rhizopus microsporus* were revealed as the core functional species related to starch-degrading enzyme activities with single-molecule real-time (SMRT) sequencing. Two strains with high starch-degrading abilities were successfully isolated and identified as *Saccharomycopsis fibuligera* CY2111 and *Rhizopus microsporus* SM4. Meanwhile, the environmental fermentation parameters of *jiuyao* were optimized to enhance enzyme activities. The glucoamylase and α-amylase activities of fortified *jiuyao* with strain CY2111 and SM4 were 3.07 and 3.15 times that of traditional *jiuyao*, respectively. Moreover, the alcohol content increased by approximately 7.5% when fortified *jiuyao* was used for *huangjiu* brewing. The flavor profiles determined by gas chromatography–mass spectrometry indicated there was no significant difference in the key aromas between the *huangjiu* fermented with fortified *jiuyao* and that of traditional *jiuyao*. Further, fortified *jiuyao* produced more pleasant esters in *huangjiu* such as ethyl isovalerate, isoamyl acetate, and ethyl caprylate. Combined with the sensory evaluation, *huangjiu* fermented with fortified *jiuyao* presented stronger fruity aroma. These results demonstrated that careful fermentation settings combined with biofortification technology were feasible to improve *jiuyao* quality, thus promoting *huangjiu* flavor. Taken together, it provided scientific guidance to improve traditional handcrafting and scale up the production of *jiuyao* under controllable fermentation.

1. Introduction

Huangjiu, a distinctive traditional alcoholic drink in China, has a history dating back more than 5000 years (Varela et al., 2015). Traditional manual *huangjiu* is fermented with traditional *jiuyao* and wheat *qu* using glutinous rice as ingredients (Peng et al., 2022). *Huangjiu* has been widely consumed in Asia for its characteristic and desirable flavor profiles. It contains sugars (monosaccharides, polysaccharides and oligosaccharides), organic acids, amino acids, peptides, and polyphenols (Yu et al., 2019). According to the data from the National Bureau of Statistics of China, the overall sales revenue of *huangjiu* reached 12.72 billion Yuan in 2021. *Huangjiu* production is unique brewing process that requires simultaneous saccharification and alcoholic fermentation (Chen et al., 2021a).

Jiuyao, serving as the saccharifying agent of traditional manual *huangjiu*, containing core functional microorganisms and several types of enzymes related to starch metabolism, plays a crucial role in the complex aroma formation of *huangjiu* (Yang et al., 2020). *Jiuyao* is produced by natural and spontaneous fermentation only in an open summer environment, whose quality will be influenced by various

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environmental factors such as temperature and moisture (Ma, Luo, et al., 2022; Zhu et al., 2022). Moreover, microorganisms from the fermentation environment and microbial interactions simultaneously contribute to diverse microbial composition and dynamic succession, leading to intricate properties and metabolomic profiles of *jiuyao* between batches (Blasche et al., 2021; Xu et al., 2022). The process operated by traditional empirical techniques usually results in unpredictability and uncontrollability in a large-scale fermentation process, negatively affecting the quality of *huangjiu*. Currently, the *huangjiu* industry is in a critical period of transition to mechanization, modernization, and standardization (Jin et al., 2017). *Jiuyao* is also facing the challenge of transforming from manual manufacturing into intelligent manufacturing (Jiao et al., 2017). However, the development and application of *jiuyao* are limited due to obsolete tools, seasonal restrictions, high labor intensity, and low productivity as well as poor stability (Fig. 1). Therefore, it is necessary to develop a controllable manufacturing procedure for *jiuyao* to achieve standardization and efficiency.

In order to strengthen the control of the *jiuyao* fermentation process, physiochemical indexes, hydrolyzing enzymes, microbial communities, and metabolic characteristics of *jiuyao* have been uncovered extensively (Chen et al., 2020). Studies have been carried out to explore the core functional microbiota related to flavor compounds or key enzymes through multi-omics approaches (Kang et al., 2022). Some researchers have also tried to develop an artificial starter with the core microbial species of *jiuyao* for *huangjiu* brewing (Chen et al., 2021). However, there are limited studies available on controlling and improving the *jiuyao* quality. Regulating fermentation conditions accurately by monitoring the fermentation parameters (temperature, moisture, oxygen, and so on) has been thought to be a directional and applicative solution (Ban et al., 2022). Biofortification with indigenous functional strains has also been considered a feasible alternative to achieve stability and controllability (Chen et al., 2021b; Mu et al., 2023).

This study first revealed the core saccharifying microorganisms of *jiuyao* by SMRT sequencing. Then functional strains with high starch-degrading enzyme-producing abilities were screened. Subsequently, a fermentation process of fortified *jiuyao* with multiple strains was developed and optimized to stabilize the quality of *jiuyao*. Finally, the effects of fortified *jiuyao* on *huangjiu* brewing were explored.

2. Material and methods

2.1. Sample collection

Jiuyao samples were collected in different years from four *huangjiu* manufacturers (CYY, JH, SYH and TP) in Shaoxing, Zhejiang Province, China (Table S1). The whole fermentation time of *jiuyao* could be divided into four stages, including Ru gang (RG), Qian jiao (QJ), Hou jiao (HJ), and Yin gan (YG). Samples from each period were collected at 0 h (RG), 30 h (QJ), 45 h (HJ), and 147 h (YG). All samples were stored at −80 °C for sequencing analysis and 4 °C for microbial screening. Three biological replicates were used for analysis.

2.2. Microbial community analysis of jiuyao by amplicon sequencing

DNA was extracted using CTAB/SDS method. The V1–V9 region of 16S rRNA genes for bacteria was amplified using the universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GNTACCTTGTTACGACTT-3′). PCR amplification of the fungal ITS region was performed using the forward primer ITS9munngs (5′-TACACACCGCCCGTCG-3′) and the reverse primer ITS4ngsUni (5′-CCTSCSCTTANTDATATGC-3′). The amplicons were sequenced on the PacBio Sequel platform (Mosher et al., 2013).

QIIME2 2022.4 (https://docs.qiime2.org/2022.2/tutorials/) was used for amplicon sequencing bioinformatics. Raw sequences were quality filtered using the Cutadapt plugin, followed by denoising demultiplexed sequences and assigning them to Amplicon Sequence Variants (ASVs) by the DADA2 plugin (Callahan et al., 2016). Subsequently, the taxonomy of ASVs was determined with a confidence threshold of 0.9 using the Sklearn Classifier (Bokulich et al., 2018) against the Silva 138 for bacteria and UNITE database for fungi, respectively.

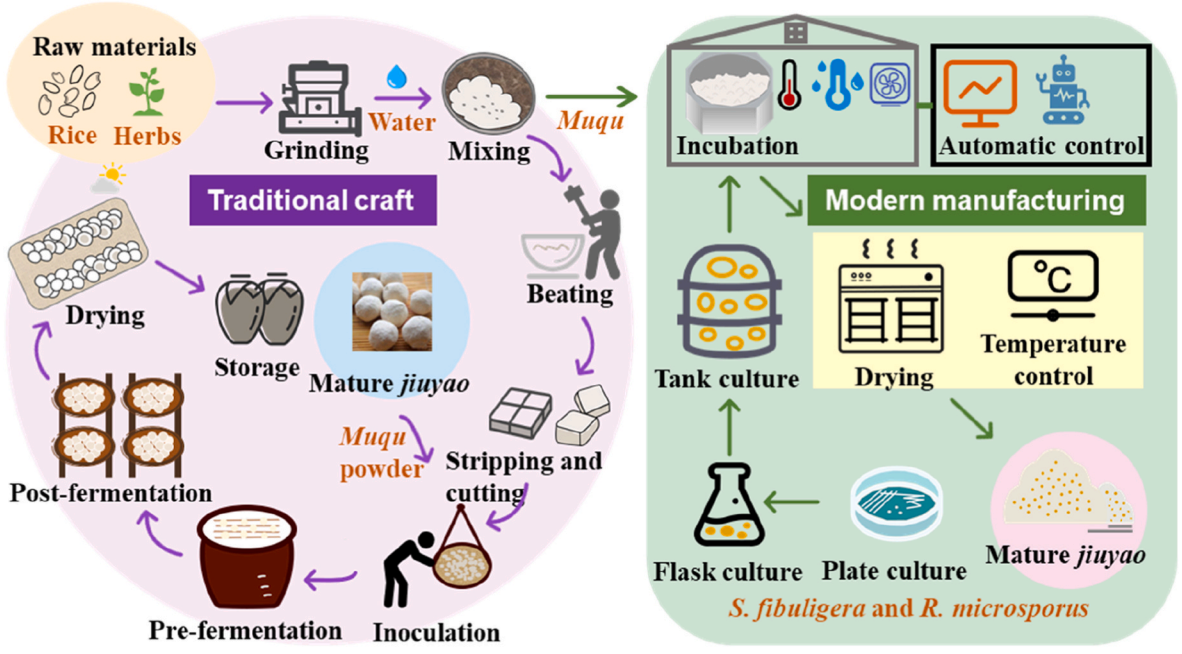

Fig. 1. Process diagram for the traditional craft of *jiuyao* and manufacturing process of fortified *jiuyao*. For the traditional craft of *jiuyao*, rice and herbs are the main ingredients, and then, after grinding, mixing with water, and shaping into a pill, it is inoculated, fermented, and dried using obsolete tools in the natural environment; for the modern manufacturing of *jiuyao*, the pretreatment of materials is the same as the traditional craft, it is inoculated with traditional *jiuyao* powder and additional core functional strains culture, then incubated in the controllable fermentation room and dried in the drying oven.

2.3. Screening and identification of the amylase-producing strain

The *jiuyao* samples were suspended in sterile distilled water for 30 s to isolate yeasts while being incubated in potato dextrose agar (PDA) for 2 h to isolate molds. Then serial dilutions were spread on yeast extract peptone dextrose (YPD) and PDA agar plates for incubation at 30 °C for 2–5 d, and colonies were isolated and purified according to their morphologies. The purified strains were preserved in 30% (v/v) glycerol at −80 °C. The starch degradation assay was performed to initially screen yeasts and molds with starch degrading ability. All strains were inoculated onto starch agar plates and cultured at 30 °C for 3 d. A transparent circle appeared by flooding the culture medium with a dilute iodine solution. The ratio of the surrounding halo diameter to colony diameter (D_h/D_c) was calculated as a measure of starch degradation (Jancic et al., 2016).

The 18 yeasts and 19 molds were cultured in liquid-state fermentation medium (LSF) and solid-state fermentation (SSF) respectively to assess their enzymes-production capacities. Yeast cultures (10^6 cells/mL) were transferred at 2% (v/v) to 50 mL of LFM medium (0.5% yeast extract, 1% peptone, 1% starch, 0.1% NaCl, 0.01% $MgSO_4$, 0.01% KH_2PO_4, 0.005% $CaCl_2$) and incubated at 30 °C for 3 d (Huang et al., 2021). The supernatant was obtained after centrifugation at 8000×*g* (12000 rpm in a 24 × 1.5/2.0 mL Rotor, Sorvall Legend Micro 17 centrifuge, ThermoFisher Scientific Co., Waltham, Massachusetts, USA) at 4 °C for 10 min for glucoamylase and α-amylase activities assays. The SFM consisting of wheat bran and water in a 1:1 ratio was inoculated with 2% (v/v) of spore suspension at a concentration of 10^6 spores/mL and incubated at 30 °C for 3 d.

The macroscopic and microscopic features were used to study the morphological characteristics of strain CY2111 and SM4. For fungal identification, ITS regions were amplified using primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and sequenced. And phylogenetic trees were also generated using MEGA11 software.

2.4. Strain culture and manufacturing of jiuyao

Each strain was grown in YPD medium (yeasts) or PDA medium (molds) at 30 °C for 2–5 d. The individual colony was then transferred to the YPD liquid medium and incubated at 30 °C for 2 d while shaking at 160 rpm, which was inoculated at a 1% (v/v) inoculation rate into the secondary seed medium (YPD) and then incubated at 30 °C for 2 d while shaking at 160 rpm. The cell pellets were adjusted to a concentration of 10^6 cells/mL for yeasts. For molds, sterile water was then added to plates, and spores were scraped off and suspended in solution to obtain suspension (adjusted to 10^6 spores/mL).

Rice was ground and sifted out over a 50 meshes sieve, mixed with 30% (w/w) wheat bran and some proper herbal powder. Solutions containing *Saccharomycopsis fibuligera*: *Rhizopus microsporus* at a ratio of 10^6:10^6 cells/mL and matured *jiuyao* were added to the mixture of the raw materials. Subsequently, some large lumps were sieved into small homogeneous pieces and piled into the frame (Φ22 cm × 10 cm) with small holes (10 mm in diameter) padded with four layers of gauze. The *jiuyao* starter was then placed in a fermentation room for 50–55 h of fermentation.

2.5. Physicochemical and enzymatic activity analysis

Glucoamylase activity and α-amylase activity were measured as previously reported (Ma, Liu, et al., 2022). One unit of glucoamylase activity was defined as the amount (in milligrams) of glucose liberated from starch by 1 g or 1 mL of sample per min at 30 °C. One unit of α-amylase activity was defined as the amount (in grams) of starch liquefied by 1 g or 1 mL of sample per hour under assay conditions. All measurements were performed in triplicates.

2.6. Application of fortified jiuyao to huangjiu fermentation

The Laboratory-scale fermentation was conducted in a 5 L beaker. Glutinous rice grains (1000 g) were soaked, drained, steamed, and cooled down with cold boiled water to room temperature (between 25 °C and 28 °C). The addition of fortified *jiuyao* was 0.5 g/100 g uncooked rice. After incubation at 28 °C for 36–48 h, 150 g of raw wheat *Qu* and 1000 mL of water were added to the fermented mash and maintained under the same condition for 96 h, then incubated at 15 °C for 240 h. Traditional *jiuyao* which collected from the factory was used as the control to conduct *huangjiu* fermentation under the same conditions. All fermentation parameters (alcohol, titratable acidity, amino acid nitrogen) were measured according to GBT13662-2018 huangjiu. Reducing sugar was determined using the 3,5-dinitrosalicylic acid (DNS) method (Zhang et al., 2021). Organic acids and amino acids were determined by HPLC as described before (Liu, Sun, et al., 2021; Wang et al., 2014). Volatile flavor compounds were quantified by HS-SPME/GC-MS according to the previously described method (Liu et al., 2019; Zhao, Liu, Han, et al., 2022). All experiments were performed in triplicate.

2.7. Sensory evaluation

The sensory panel test was conducted in tasting panel at the Sensory Analysis Laboratory with uniform source of lightning, absence of noise and distracting stimuli following GB/T 13662-2018. A total of 12 sensory attributes of aroma (alcoholic, fruity, honey, cereal, herb, smoky, and *Qu* aroma) and taste (sweet, sour, bitter, astringent, and umami) were chosen to characterize the sensory properties of *huangjiu* (Zhao, Liu, Yang, et al., 2022). Ten trained panelists (five females and five males, aged between 20 and 35 years) from the School of Food Science and Engineering, Jiangnan University were selected and asked to score on a five-point scale according to intensity, ranging from 0 (none) to 5 (strong). All of *huangjiu* samples were marked randomly. Two types of *huangjiu* fermented with different *jiuyao* were evaluated by the panel. All the procedures followed the guidelines of the Declaration of Helsinki. All participants volunteered to take part in the study and received financial compensation for participation.

2.8. Statistical analysis

All data were tested by one-way analysis of variance (ANOVA) followed by Duncan's test using IBM SPSS Statistics (v.21.0, Armonk, NY, USA). Although a $p < 0.05$ was generally used, the authors have also chosen to use 0.01 for some of the data to indicate the greater significance of the differences. Origin 2022 64Bit (OriginLab Corporation, Northampton, MA, USA) was used for image processing. Molecular Ecological Network Analysis (MENA) based on Random Matrix Theory (RMT) was used to construct the microorganism network for identifying the key species (Deng et al., 2012). The Spearman correlation was calculated in R software (v.4.1.2, Vienna, Austria) to reveal the relationship between the core functional species and enzyme activities which was carried out by "psych" and "pheatmap" packages (Pang et al., 2020), as well as the interactions among microorganisms, and a highly correlated coefficient ($|\rho|>0.7$, $p < 0.05$) was filtrated and visualized using Gephi v.0.9.2 software (https://gephi.org). The SIMCA 14.1 software (Umetrics, Umea, Sweden) was used for OPLS-DA analysis and variable importance in projection (VIP) calculation.

3. Results

3.1. Identification of core functional microbiota related to starch-degrading activities

The microbial diversity of *jiuyao* collected from various production workshops in Shaoxing city was profiled (Supplementary Table 1). A

fungal core community consisting of *Rhizopus* species (mainly *Rhizopus arrhizus* and *Rhizopus microsporus*), *Saccharomycopsis fibuligera* and *Saccharomyces cerevisiae* was found, which together accounted for >95% of the total abundance of over half of all samples (Fig. 2A). Only a few aroma-producing yeasts as *Candida* species, *Pichia* species, and *Wickerhamomyces* species were different in the fungal communities of *jiuyao*. The bacterial communities were mainly composed of lactic acid bacteria (usually *Pediococcus pentosaceus* and *Weissella* sp.), despite differing in the relative abundance of each specie (Fig. 2B). Microbial network analysis is a popular approach to detect important species or keystones with communities (Feng et al., 2022; Tang et al., 2022). Similar results were observed in the microbial network analysis based on spearman correlation (Fig. 2C). The Zi-Pi plot (Fig. 2D) showed that the majority of fungal and bacterial ASVs located in the peripherals area (Zi < 2.5, Pi <

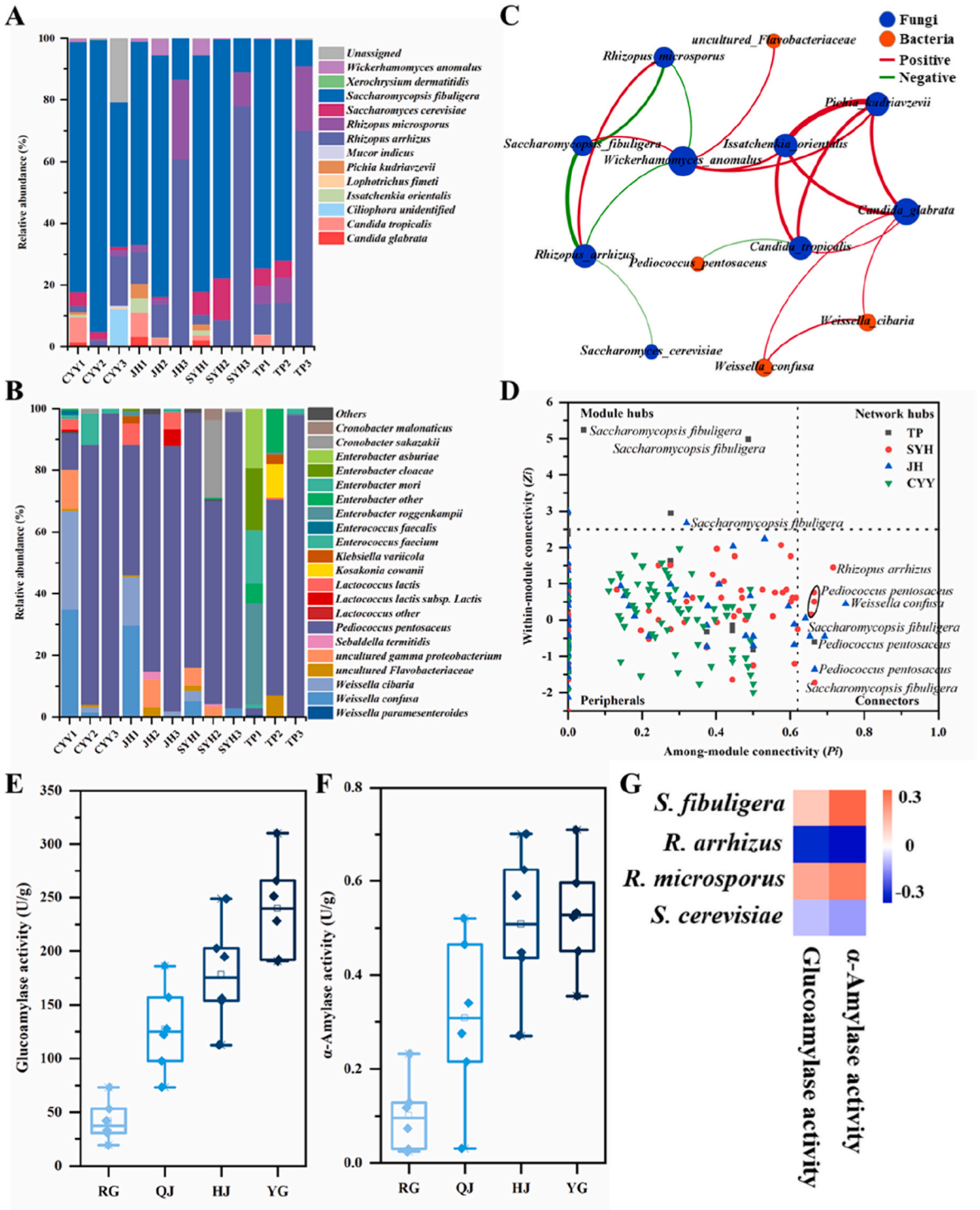

Fig. 2. The analysis of keystone microbes in *jiuyao*. (A) Fungal and (B) bacterial communities at the species level of different *jiuyao*; (C) Co-occurring community network of *jiuyao* based on spearman correlation analysis, the red and green lines represent the positive correlation ($\rho > 0.7$ and $p < 0.05$) and the negative correlation ($|\rho| > 0.7$, $\rho < 0$, and $p < 0.05$), respectively; (D) The distribution of ASVs based on their module-based topological roles; Changes of glucoamylase activity (E) and α-amylase activity (F) during *jiuyao* fermentation; (G) The correlation by the Spearman's correlation coefficient between core functional community and enzyme activities.

0.62). Ecologically, peripherals represent specialists, whereas module hubs and connectors represent generalists (Xu et al., 2019). The results indicated that many topological features varied across samples from different factories. However, *S. fibuligera* and *P. pentosaceus* species could be considered as keystone taxa among different factories, which played the role of connecting other species.

The functional enzyme activities of *jiuyao* at different stages were measured. The glucoamylase activity of *jiuyao* increased continuously from 41.95 U/g to 239.65 U/g with the extension of fermentation time (Fig. 2E), which was attributed to the growth, reproduction, and metabolism of the main saccharifying microbiota. And the α-amylase activity of *jiuyao* showed the consistent trend in the early fermentation stages, then maintained relatively stable (about 0.50 U/g) in the later fermentation (Fig. 2F). The correlation between core functional community and enzyme activities showed that *S. fibuligera* and *R. microsporus* had a positive correlation with glucoamylase activity and α-amylase activity based on spearman correlation analysis (Fig. 2G). This result was also found in traditional fermentation starters, such as Guizhou *Xiaoqu* (Wang et al., 2020), Chuanfa *Xiaoqu*, Yichang *Xiaoqu* (Wu et al., 2017), Shaoxing *jiuyao* (Chen et al., 2020), and Xinhua *Xiaoqu* (Xiao et al., 2022). *Saccharomycopsis* and *Rhizopus* have been considered as the main powerful producers of enzymes during the saccharifying fermentation. Currently, performing biofortification inoculation with functional strains in spontaneous starter fermentation has been an effective approach to improve the quality of starter and fermented alcoholic products (He et al., 2020; Mu et al., 2022). Thus, the quality of *jiuyao* and *huangjiu* was controlled in this way.

3.2. Screening of strains for the starch degrading ability

29 yeast and 19 mold strains with the ability to hydrolyze starch were isolated from *jiuyao* samples (Tables S2 and S3) in total. In this study, 18 yeast strains ($D_h/D_c > 2.0$) were further tested for their starch-degrading enzyme-producing abilities. The assay used to evaluate enzyme activities in LSF of yeasts showed that strain CY2111 had higher α-amylase and glucoamylase activities, with values of 0.059 U/mL and 3.85 U/mL, respectively (Fig. 3A). Meanwhile, strain CY2111 exhibited higher enzyme activities in SSF using wheat bran as substrates (Table S4), which revealed activities for glucoamylase activity (1130.84 ± 55.18 U/g) and α-amylase activity (1.51 ± 0.06 U/g). For further tests

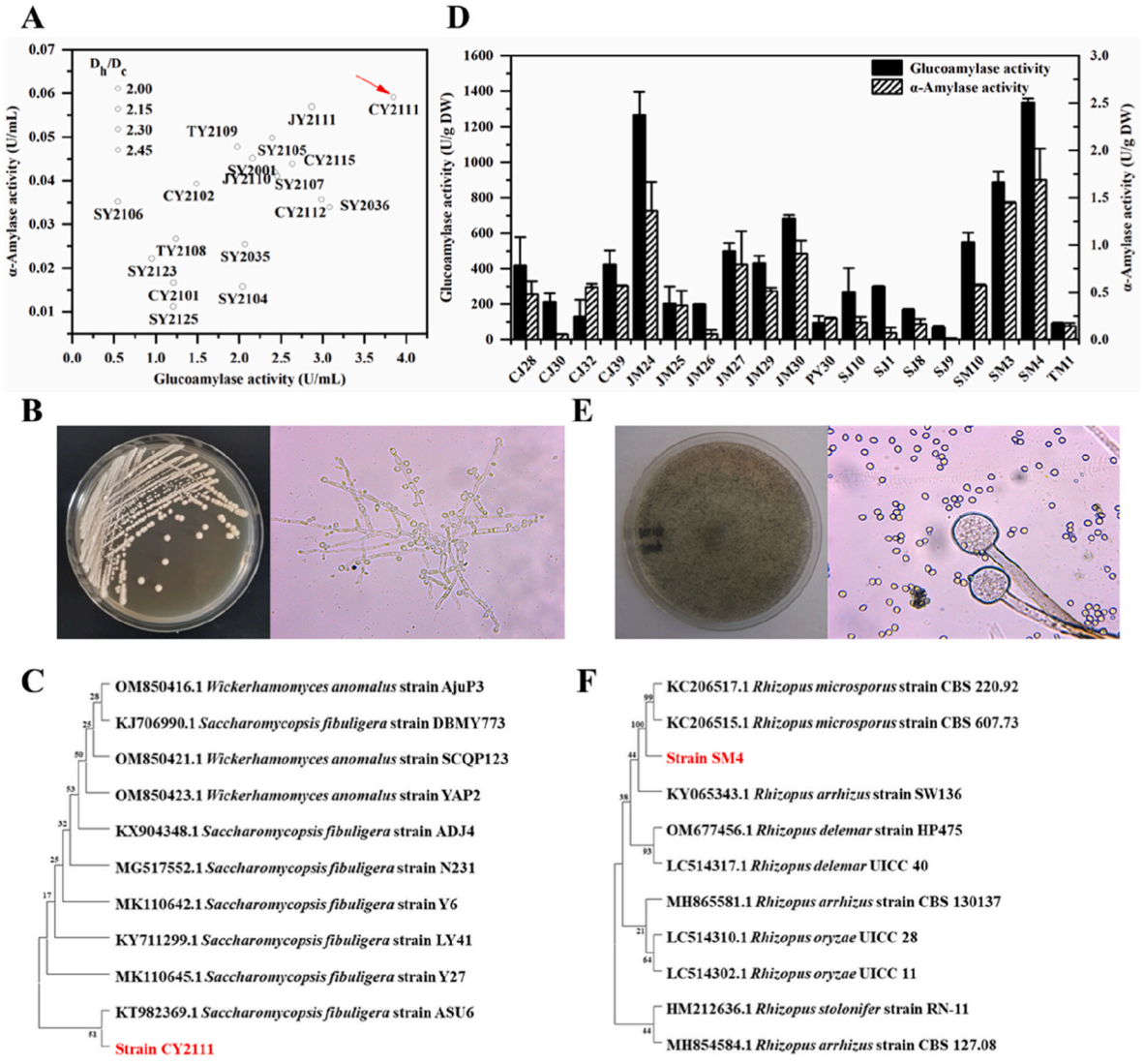

Fig. 3. The screening of the core microbes with starch-degrading enzyme-producing abilities from *jiuyao*. (A) The α-amylase and glucoamylase activities of 18 yeast strains inoculated for 3 d in the LSF; (B) The morphological characteristics of CY2111 on YPD solid medium and microscopy morphology of budding cells, pseudohyphae, and hyphae by the light microscope under 400 × magnification; (C) The neighbor-joining phylogenetic tree of strain CY2111 and related taxa; (D) The α-amylase and glucoamylase activities of 19 mold strains inoculated for 3 d in the SSF; (E) The colonial morphology of strain SM4 on PDA solid medium and microscopy morphology of conidiophores and spores by the light microscope (400 ×); (F) The neighbor-joining phylogenetic tree of strain SM4 and related taxa.

of the abilities to produce enzymes of molds, their enzyme activities in SSF were evaluated. The strain SM4 showed the highest α-amylase activity (1.69 ± 0.33 U/g) and glucoamylase activity (1338.30 ± 22.44 U/g) among initially screened molds (Fig. 3D). The morphology of the strain CY2111 on YPD plates and the strain SM4 on PDA agar plates incubated at 28 °C after 3 d was studied. Dimorphic characteristics of the strain CY2111 were observed as a round or oval cellular morphology, and they could form mycelia by budding reproduction (Fig. 3B). The colony of the strain SM4 was loose, flocculent, with black spores on the top of well-developed mycelium (Fig. 3E). Microscopic characteristics of the strain SM4 was observed that the conidiophore was erect without branches and the sporangia were spherical with the approximately spherical spores. The strain SM4 was preliminarily classified as *Rhizopus* based on morphological characteristics. Then the CY2111 and SM4 ITS nucleotide sequences showed 99.67% and 99.40% homology with *S. fibuligera* (Fig. 3C) and *R. microsporus* (Fig. 3F) respectively in the GenBank database using BLAST. Together with morphological analysis, CY2111 was identified as *S. fibuligera* and SM4 as *R. microsporus*. *S. fibuligera* can secret various enzymes, such as amylase, β-glucosidase and acid protease (Thanh et al., 2016). Different *S. fibuligera* strains were separated from different fermentation starters for industrial application (Xie et al., 2021), such as *Daqu* (Li et al., 2018), *Xiaoqu*, *Nuruk* (Carroll et al., 2017), and *Dombea*. Recent studies have shown that *S. fibuligera* has been recognized as not only a powerful producer of amylase and protease but also great contributors to excellent flavor compounds (mainly esters and aromas) of various alcoholic beverages such as *baijiu* (Xie et al., 2021), rice wine (Son et al., 2018), or *huangjiu* (Yang et al., 2020). Furthermore, *Rhizopus* as the main agent in the saccharification process with strong ability to produce amylase, lactic acid, and other aromatic esters, also enriches the flavor and taste of *huangjiu* (Huang et al., 2019; Sakandar et al., 2020). Therefore, *S. fibuligera* CY2111 and *R. microsporus* SM4 were selected as the two functional strains to control the quality of *jiuyao*, thereby contributing to *huangjiu* fermentation and flavor formation.

3.3. Selection of the shape and substrate for fortified jiuyao preparation

To ensure the traditional production process of *jiuyao* which was more conducive to preservation, *jiuyao* was made as a sphere, namely JYW on a laboratory scale under controllable conditions. The results showed that although the glucoamylase and α-amylase activities of JYW groups increased by 32.51% and 56.78%, respectively, no significant difference ($p > 0.05$) was observed compared with the control group (Fig. 4A and B). However, the preparation of JYW is usually time-consuming and difficult to be applied to modern manufacturing. Additionally, the growth of strains tends to be inconsistent and inhomogeneous as a result of *jiuyao* varying in volume and compactness (Wang et al., 2018). Hence, *jiuyao* was then made into a diffused shape (SQ). Obviously, the enzyme activities of the SQ group significantly increased compared with the control group. SQ could be manufactured and fermented in a round machine based on intelligent technology (Wang et al., 2021). In general, SQ was better than JYW to be carried out in modern production. *Jiuyao* is traditionally produced using rice as the main ingredient (Wang et al., 2020). But the enzyme activities of the selected single strain using wheat bran as substrates were significantly higher than that of rice (Fig. 4C and D), which was consistent with previous studies (Carroll et al., 2017). The formation of volatile and nonvolatile metabolites was reported to be affected by the different sources of starchy materials (Hong & Kim, 2020; Lee et al., 2017, 2018). Based on the above results, both wheat bran and rice were chosen for the

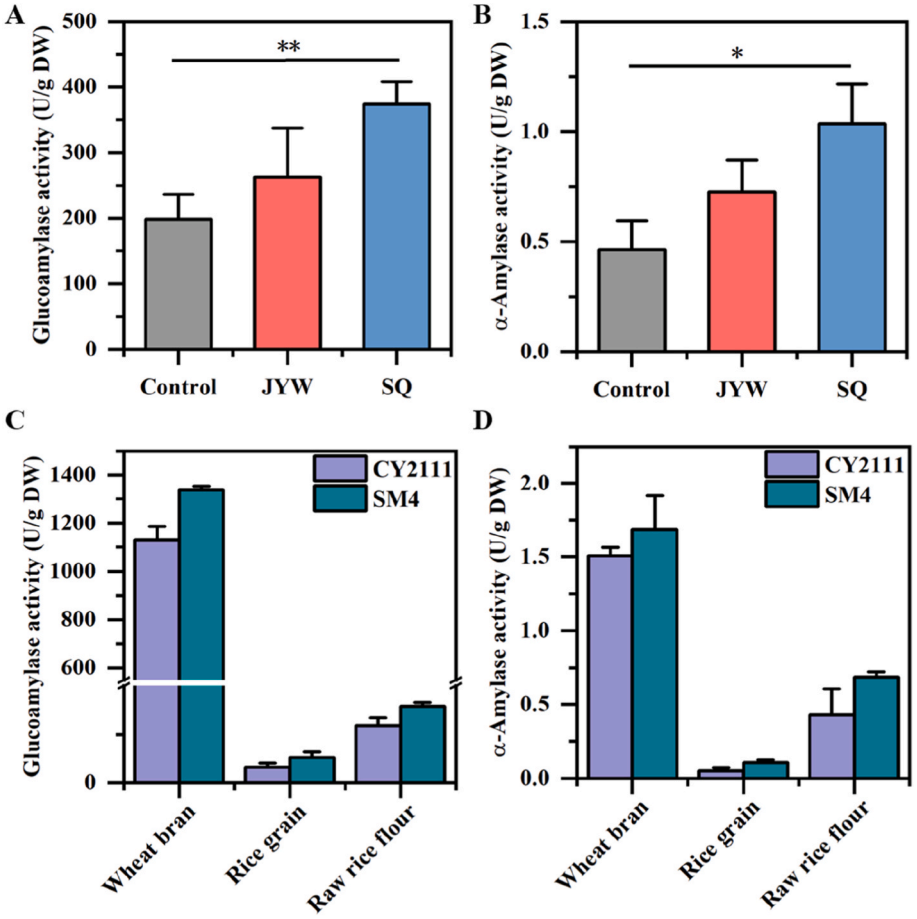

Fig. 4. The selection of shape and substrate for fortified *jiuyao* preparation. The glucoamylase (A) and α-amylase (B) activities of different shapes of the *jiuyao*, the control group means traditional *jiuyao* which is collected from the factory in the natural fermentation environment. The JYW represents *jiuyao* which has the same sphere as traditional *jiuyao* under controllable fermentation conditions on a laboratory scale. And SQ means a diffused shape *jiuyao* under the same conditions as JYW; The glucoamylase (C) and α-amylase (D) activities of selected strains in different substrates. * $0.01 < p < 0.05$, ** $p < 0.01$.

preparation of the *jiuyao* starter to improve the enzyme activities and enrich metabolomic profiles.

3.4. Optimization of processing parameters in the fortified jiuyao fermentation

To determine the optimum parameters of *jiuyao* fermentation, the changes in enzyme activities under different fermentation conditions were probed. *Jiuyao* had the highest glucoamylase activity and α-amylase activity (368.59 ± 19.42 U/g and 0.61 ± 0.11 U/g, respectively) when it was cultivated in the combination of raw rice flour and wheat bran (Fig. 5A). Both temperature and humidity had been proven to be important factors affecting microbial growth and survival (Li et al., 2017; Xiao et al., 2017). The *jiuyao* fermentation is a variable process, so the temperature and humidity curve of *jiuyao* under the natural fermentation was tracked first (Fig. 5B). Notably, Pre-fermentation (QJ) and post-fermentation (HJ) were the two critical periods in the fermentation process. Temperature and humidity showed similar change trends in QJ and HJ, rising first and then falling. The temperature increased rapidly during 0–17 h, reaching a peak at 17 h (33.78 °C), and the humidity peaked at the same time (93.57%). Then the straw cover was gradually opened to lower the *jiuyao* temperature. The temperature reached another peak (37.3 °C) when *jiuyao* was in a closed room during the HJ period. Therefore, the temperature and humidity control system were divided into two stages to simulate the *jiuyao* fermentation better. Simultaneously, a fermentation environment with constant temperature and humidity was also regulated throughout the process to avoid the natural rise of temperature caused by the generation of massive biological heat (Kang et al., 2022). The humidity levels were set at 95% and 80% for the pre-fermentation and post-fermentation, respectively. The results presented that the glucoamylase and α-amylase activity of *jiuyao* were both higher at 28 °C than that at 30 °C

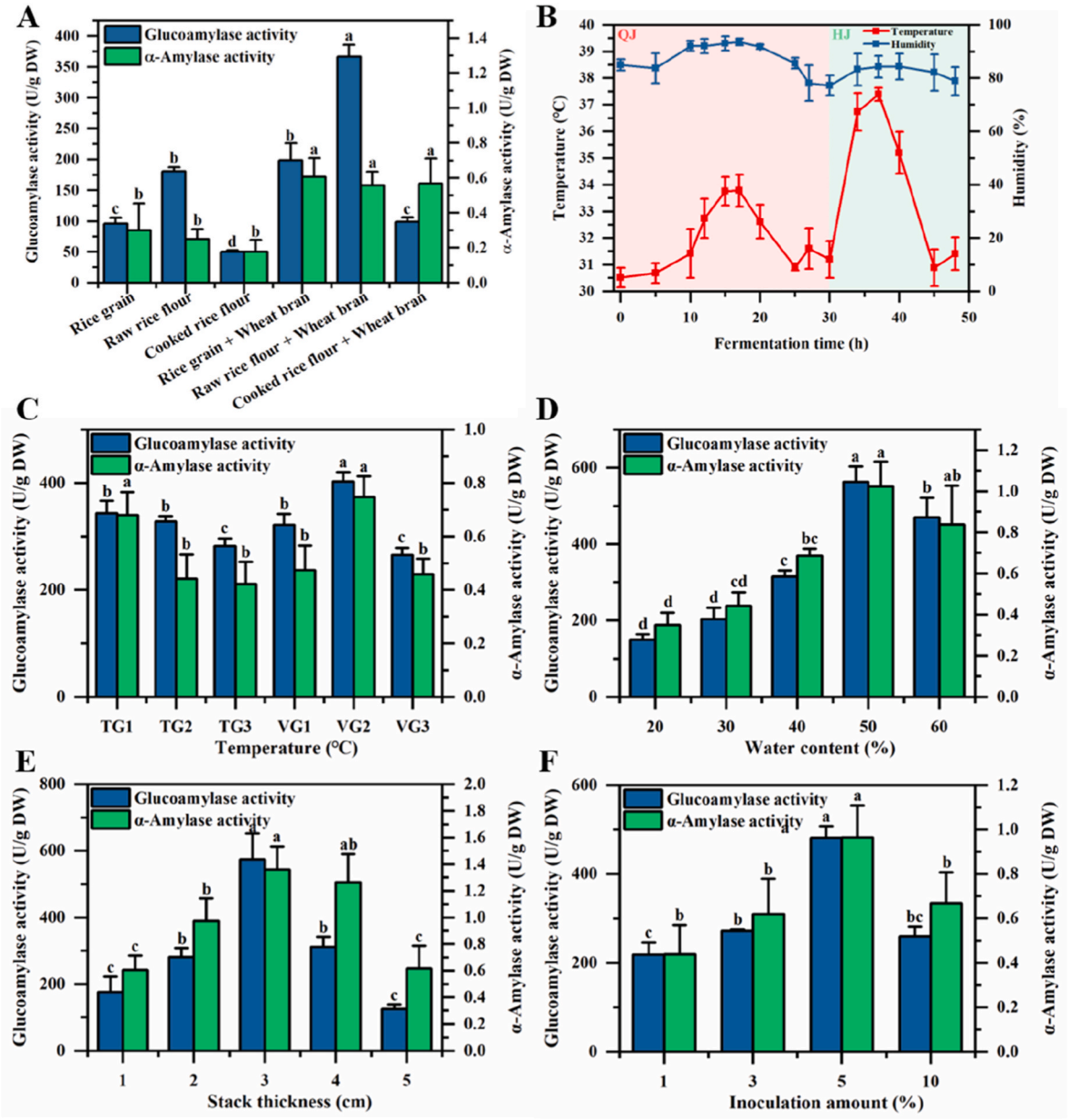

Fig. 5. The optimization of fermentation conditions of fortified *jiuyao*. (A) The enzyme activities of *jiuyao* made with different substrates; (B) Changes in temperature and humidity during *jiuyao* fermentation. Effects of temperature (C), water content (D), stack thickness (E), and inoculation amount (F) on the saccharifying activities of *jiuyao*. In A, TG1, TG2, and TG3 means thermostatic temperature 28 °C, 30 °C, and 32 °C, respectively; VG1, VG2 and VG3 means 28 °C in pre-fermentation, while 30 °C, 32 °C, and 34 °C in post-fermentation, respectively. The humidity was set to 95% in pre-fermentation and 80% in post-fermentation.

and 32 °C. Based on this result, 28 °C was chosen as the optimum temperature in QJ and the temperature in HJ was changed according to the actual measurements. The higher enzyme activities were achieved when the HJ temperature was up to 32 °C (Fig. 5C). Therefore, a variable temperature and humidity system (28 °C, 95% in QJ and 32 °C, 80% in HJ) was implemented to control the *jiuyao* fermentation.

Water content is an essential and critical factor for microbial spore germination, hyphal extension, and metabolism under SSF (Jin et al., 2019). The enzyme activities of *jiuyao* were investigated by varying the water content (20%–60%). Both the glucoamylase and α-amylase activity exhibited a similar trend of rising first and then falling as the water content increased. They reached a maximum value of 562.43 ± 40.53 U/g and 1.02 ± 0.12 U/g with 50% water content (Fig. 5D). Therefore, the optimum water content for *jiuyao* fermentation was determined to be 50%.

Stack thickness can affect the oxygen content, heat dissipation, and moisture retention by altering the ventilation (Yamashita, 2021), thus affecting the growth and enzyme production of microorganisms. The highest glucoamylase activity of 574.13 ± 78.63 U/g was observed at 3 cm, and the α-amylase activity also peaked at 3 cm with a value of 1.36 ± 0.17 U/g (Fig. 5E). The optimum stack thickness was thus determined to be 3 cm.

Inoculation amount was well known to be closely related to the quality of *jiuyao*. The glucoamylase and α-amylase activities increased first and then decreased with the change of inoculation amount from 1%–10%. They both reached the maximum when the inoculation amount was 5%. After that, the enzyme activities started to decrease gradually with the inoculation amount increasing, which was attributed to the growth of microorganisms being accelerated, resulting in earlier entry into the aging period, thus adverse to the synthesis and secretion of metabolites such as enzymes. Finally, the optimum processing parameters of jiuyao fermentation were summarized as inoculation amounts of 5%, 28 °C with 95% of humidity in QJ, 32 °C with 80% of humidity in HJ, water contents of 50%, and stack thickness of 3 cm. The glucoamylase and α-amylase activities of fortified *jiuyao* increased by 207.82% and 215.22% than traditional *jiuyao* under the optimum conditions, respectively.

3.5. Effects of fortified jiuyao on physicochemical indexes and flavor compounds of huangjiu

To understand how fortified *jiuyao* (Fjiuyao) influenced *huangjiu* fermentation, the physicochemical parameters and flavor compounds during *huangjiu* fermentation were profiled. All of the indexes complied with the Chinese national standard (GB/T 13662-2018). The alcohol content of the Fjiuyao group significantly increased by 7.5%, and the reducing sugar content was significantly lower than the control group ($P < 0.05$) (Fig. 6A). This indicated that Fjiuyao with higher enzyme activities could accelerate the hydrolysis of starch to create a high osmotic pressure environment suitable for the growth of yeasts with a high potential for ethanol production. Eventually, the nutrients were consumed and utilized more completely. There was no significant difference in the titratable acidity between *huangjiu* fermented with Fjiuyao and that of traditional *jiuyao* (Fig. 6A).

As one of the flavor precursors of *huangjiu*, amino acids could be converted into alcohols, esters, and aldehydes via the microbial metabolic pathway (He et al., 2014). *S. fibuligera* and *R. microsporus* had been mainly identified as optimal protease producers (De Barros Ranke et al., 2020), which explained why the amino acid contents of the Fjiuyao group were higher than the control group (Fig. 6B).

In addition, organic acids were important indicators of *huangjiu* quality, affecting color, flavor, and biological stability (Zhao, Liu, Han, et al., 2022). The total organic acid contents of the Fjiuyao group (13.58 ± 0.32 g/L) significantly increased by 28.51% against the control group (10.57 ± 0.86 g/L) (Fig. 6C), especially in succinic acid (121.25%), critic acid (70.36%), and oxalic acid (65.17%). These acids might originate from the microbial metabolites of *S. fibuligera* and filamentous fungi (Lee et al., 2018). The functional strains also provided an acidic environment during earlier saccharification by producing various organic acids, which selected superior yeasts and lactic acid bacteria with high endurance (Liu, Ma, et al., 2021). This was also the reason why the Fjiuyao group had higher alcohol content.

Towards disentangling the contribution of fortified *jiuyao* to volatile flavor compounds of *huangjiu* fermentation, a total of 46 volatile compounds were detected, including eight alcohols, fifteen esters, six acids, nine aldehydes, five phenols, one ketone, one pyrazine and one sulfur-containing compound (Fig. 6D). No significant difference was discovered in the total concentration of the major classes of flavor substances between the Fjiuyao group and control group (Table S5). However, the concentration of specific flavors greatly differed. A total of 22 differential aromatic substances were presented according to VIP>1, including five alcohols, six esters, two acids, six aldehydes, two phenols, and one sulfur-containing compound (Fig. 6E).

As shown in Table 1, individual OAVs of differential aromatic substances were detected. The volatile compounds with OAVs≥1 can be regarded as the main contributors to the aroma composition of *huangjiu*. The results showed that 11 volatile compounds with OAVs≥1 were identified. The concentration of isovaleraldehyde, phenethyl alcohol, and ethyl acetate in the Fjiuyao with OAVs>5 were similar to the control group (Table 1). In line with previous studies (Peng et al., 2022), representative higher alcohol phenethyl alcohol and ester ethyl acetate were recognized as core aromas of traditional manual *huangjiu,* which present floral and honey-like aromas (Lin et al., 2018) and pineapple-like flavor (Chen et al., 2019), respectively. Yeast can produce phenylethyl alcohol from the conversion of L-phenylalanine via the amino acid catabolic pathway or de novo synthesize it (Zhao et al., 2023). Previous studies showed that there were positive correlations between *Saccharomycopsis* and phenyl ethanol as well as ethyl butyrate, *Rhizopus* and some ethyl esters in *jiuyao* (Chen et al., 2020), exhibiting the potential contribution of two species to *huangjiu* flavor. At the same time, the contents of several esters such as ethyl isovalerate, isoamyl acetate, and ethyl caprylate in the Fjiuyao group were significantly higher than the control group, enriching the fruity aroma of *huangjiu*. Further, the similar phenomenon was also found in *makgeolli* fermented with *koji* inoculated with *Saccharomycopsis fibuligera* and *Aspergillus oryzae* (Son et al., 2018). *S. fibuligera* contributed to the formation of amino acids, fusel alcohols, acetate esters, and ethyl esters. Therefore, the fortified *jiuyao* was a potential substitute for the traditional *jiuyao* based on its positive effect on the fragrance of *huangjiu*.

3.6. Sensory evaluation of huangjiu samples

Sensory evaluation of *huangjiu* was investigated (Fig. 7). The results showed that the overall aroma and taste profiles of the *huangjiu* samples brewed with traditional *jiuyao* and fortified *jiuyao* is relatively similar. The honey attributes score did not differ significantly among *huangjiu* samples, which might be attributed to the content of phenethyl alcohol in control group close to that of fortified *jiuyao* (Table 1). *Huangjiu* fermented with fortified *jiuyao* had higher scores for fruity aroma. This might be because of the higher contents of fruity esters, such as ethyl acetate, ethyl isovalerate, isoamyl acetate, and ethyl caprylate. Furthermore, the alcoholic and sour sensory score for fortified *jiuyao* was higher than the traditional *jiuyao*, while the sweet sensory score was lower, which was consistent with the results of Fig. 6A and B. Therefore, the sensory evaluation results together with volatile compound analysis indicated the fortified *jiuyao* had the potential to produce high-quality *huangjiu* with a stable flavor profile for industrial application.

4. Conclusions

Saccharomycopsis fibuligera and *Rhizopus microsporus* were the core saccharifying microbiota at species-level taxonomic resolution.

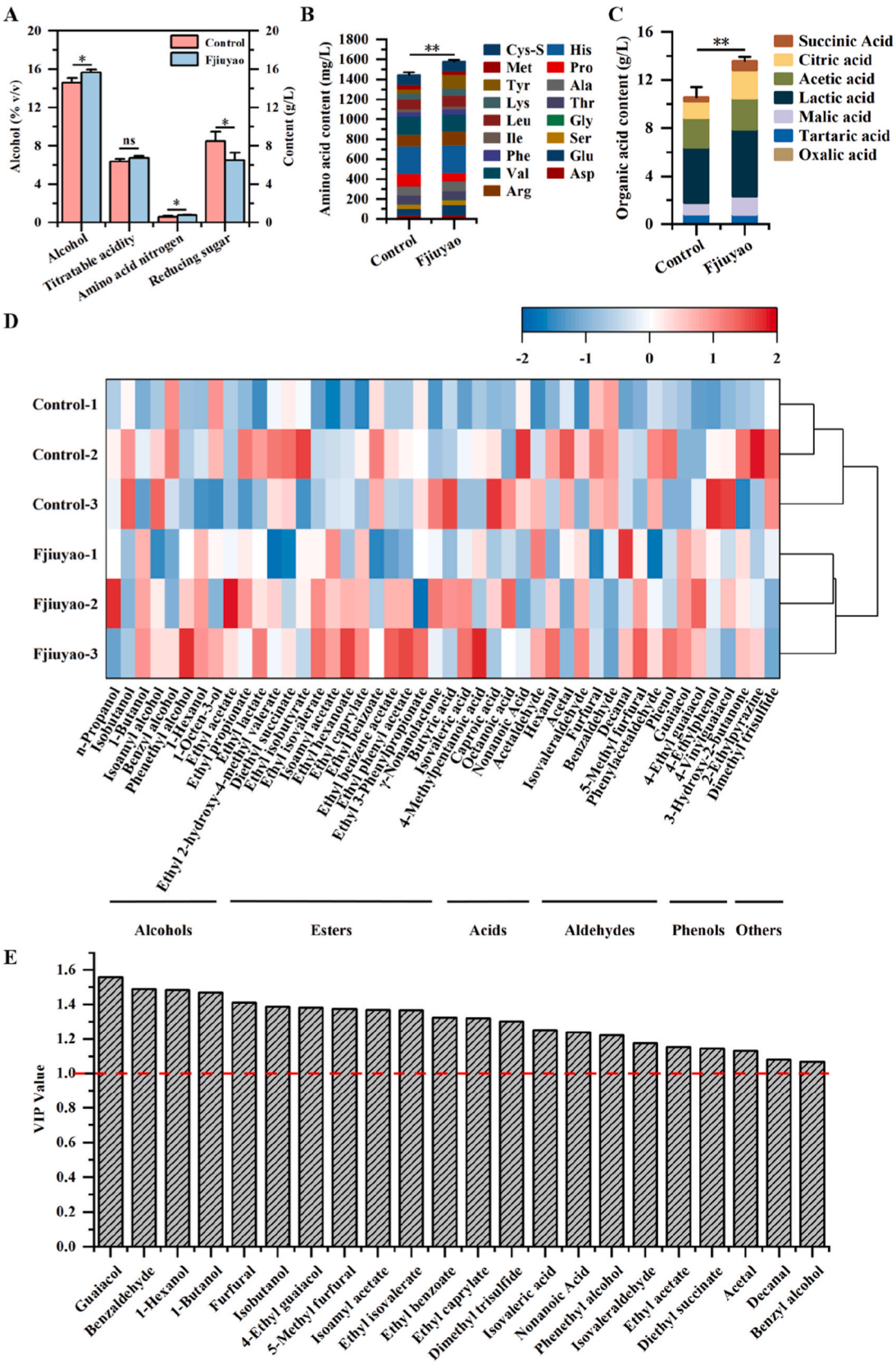

Fig. 6. The influences of fortified *jiuyao* on *huangjiu* brewing. The physicochemical parameters (A), amino acid content (B), organic acid content (C), and volatile compounds (D) of *huangjiu* fermented with different *jiuyao*. (E) The analysis of the differential aroma components based on VIP value calculated by OPLS-DA, and aromatic substances with VIP>1 are defined as differential metabolites.

Table 1
OAVs of differential volatile compounds calculated in *huangjiu* fermented with different *jiuyao*.

No	Compounds	*P*-value	OAV (Control)	OAV (Fjiuyao)	Threshold (μg/L) (Chen et al., 2013, 2019)
1	Guaiacol	0.000	< 1	< 1	9.5
2	Benzaldehyde	0.004	< 1	< 1	990
3	1-Hexanol	0.004	< 1	< 1	8000
4	1-Butanol	0.006	< 1	< 1	150000
5	Furfural	0.016	< 1	< 1	14100
6	Isobutanol	0.020	2.166	1.604	40000
7	4-Ethyl guaiacol	0.021	< 1	1.022	33
8	5-Methyl furfural	0.023	< 1	< 1	20000
9	Isoamyl acetate	0.023	2.905	3.399	30
10	Ethyl isovalerate	0.025	1.923	2.464	3
11	Ethyl benzoate	0.038	< 1	< 1	575
12	Ethyl caprylate	0.036	3.424	3.943	5
13	Dimethyl trisulfide	0.037	1.667	1.500	0.18
14	Isovaleric acid	0.059	< 1	< 1	3000
15	Nonanoic Acid	0.064	–	–	–
16	Phenethyl alcohol	0.069	15.618	20.672	8500
17	Isovaleraldehyde	0.089	47.043	59.116	120
18	Ethyl acetate	0.096	7.718	9.195	7500
19	Diethyl succinate	0.107	< 1	< 1	200000
20	Acetal	0.107	1.393	1.007	1000
21	Decanal	0.132	1.298	1.351	10
22	Benzyl alcohol	0.140	< 1	< 1	900

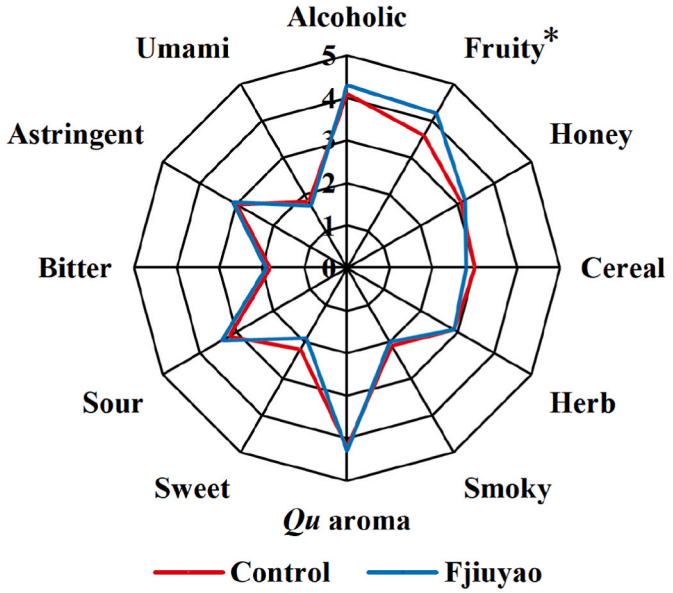

Fig. 7. The aroma and taste profiles of *huangjiu* fermented with traditional *jiuyao* (control) and fortified *jiuyao* (Fjiuyao). * $0.01 < p < 0.05$, ** $p < 0.01$.

Saccharomycopsis fibuligera CY2111 and *Rhizopus microsporus* SM4 with the high ability to produce starch-degrading enzymes were isolated from *jiuyao*. Fortified *jiuyao* with two strains was prepared. The addition of two strains enhanced the glucoamylase and α-amylase activities of *jiuyao*. The controllable fermentation conditions were determined to improve the *jiuyao* quality. Additionally, fortified *jiuyao* showed promising potential for *huangjiu* brewing because of its strong saccharification and fermentation capacity. The flavor evaluation of *huangjiu* showed that fortified *jiuyao* had positive effects on enriching the aromatic flavor during fermentation. In summary, this study provided clues for biofortification with specific functional strains to improve the quality of *jiuyao* and *huangjiu*. It would provide a foundation to develop a systematic procedure for *jiuyao* manufacturing on an industrial scale for stability and efficiency.

CRediT authorship contribution statement

Ying Zhu: Conceptualization, Data curation, Formal analysis, Methodology, Software, Visualization, Writing - original draft. **Shangping Liu**: Investigation, Supervision, Writing - review & editing. **Donglin Ma:** Conceptualization, Methodology, Writing - review & editing. **Chen Yang:** Conceptualization, Methodology, Writing - review & editing. **Yuezheng Xu:** Supervision, Writing - review & editing. **Jian Mao:** Funding acquisition, Project administration, Resources.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2023.102370.

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