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Environmental factors drive microbial succession and *huangjiu* flavor formation during raw wheat *qu* fermentation

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ABSTRACT

As the core raw material of *huangjiu* (Chinese rice wine) production and brewing, changes of physichemical factors during the fermentation process significantly affect the succession of the microbial community of raw wheat *qu* (RWQ), also playing important role in *huangjiu* quality. In this report we analyzed the community structure and driving force RWQ microorganisms with different processes (manual *qu*, MAQ; mechanical *qu*, MEQ), and evaluating the flavor of *huangjiu*. The production procedure was divided into three stages. Dominant microorganisms were *Aspergillus, Alternaria*, *Epicoccus*, *Bacillus, Pantoea, Saccharopolyspora* and *Staphylococcus*. In early stage, microbial community of MAQ was significantly correlated with temperature and water content, oxygen in middle stage, while no significant driving force in the last stage. Water content and oxygen affected MEQ community significantly in early stage, while in the final stage, fungal community was affected by both temperature and water content, and bacterial community was significantly correlated with oxygen. Glucoamylase activity reached its maximum at 50 °C, while the α-amylase activity decreased with the rise of temperature. The highest amylase activity was obtained with 19% initial water addition. Amylase activity showed positive correlation with the thickness of RWQ. The total amount of organic acids was higher in mechanical *huangjiu* at the end of pre-fermentation. Contents of n-hexanol, ethyl isovalerate, ethyl hexanoate and guaiacol in manual *huangjiu* were significantly higher than those in mechanical one, while the content of 4-vinylguaiacol was lower.

1. Introduction

Chinese *huangjiu* is the traditional rice wine with moderate alcohol content, which enjoys a high reputation in China for its bright color and sweet flavor (Fang et al., 2015). Raw wheat *qu* (RWQ) acts as one kind of starters in the fermentation process of *huangjiu*, the natural multifunctional compound that integrates aroma, flavor and color during the fermentation process (Chen & Xu, 2013). RWQ is made by natural inoculation of microorganisms after crushing, water adding, stirring, briquetting, and stacking (Fig. S1). According to the different pressing process, it is divided into manual raw wheat *qu* (MAQ) and mechanical raw wheat *qu* (MEQ). MEQ is constantly used in *huangjiu* brewing thanks to its high production efficiency. Changes of producing process has resulted in variations of microorganism and physicochemical properties in raw wheat *qu*, further affecting the characteristics of *huangjiu* (Hu et al., 2021).

Production techniques of MEQ are similar to those of MAQ, adopting the mechanical buckling process and the water adding method controlled by the metering device. Process changes has led to difference in microbiota of *qu*, thus affecting *huangjiu* flavor (Yang et al., 2017; Zhang et al., 2019). Similar to Baijiu *daqu*, RWQ microorganisms mainly come from raw materials and open production environments. Different

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production processes and environments made the microbial communities in the two RWQ owe certain differences, resulting in different enzyme activities and metabolites (Du et al., 2019; Hu et al., 2021). However, the complete dynamic changes of microorganisms in the fermentation process of RWQ is still ambiguous.

The driving forces in the fermentation environment are defined as environmental factors that are intensely related to the microbial community succession throughout fermentation, such as temperature, water content, acidity, oxygen and other physical and chemical indicators such as enzyme properties and starch content (Ryu et al., 2021). Environmental factors have a significant impact on microbial as well as sensory indicators in various fermented foods (Gao, Wu, & Zhang, 2020; Guan et al., 2020; Liu et al., 2015; Xiao et al., 2017; Zhang et al., 2020; Zhao, Su Xiao, et al., 2019). Correlation data set between RWQ microbial succession and physicochemical factors is still relatively lacking.

In order to investigate the microbial community profile of RWQ and the driving force of its succession during the fermentation process, we collected time series samples of MAQ and MEQ produced in Shaoxing in 2020, which were produced in the same area at almost the same time. All samples were placed in corresponding fermenting room for 60 days, with the physicochemical parameters tracked and recorded. Microbial construction in different parts was determined by high-throughput sequencing. Correlation between microbial communities and physicochemical indicators in different parts was analyzed, and the driving force was analyzed and verified. *Huangjiu* was brewed with different RWQs. Flavor and brewing indexes were analyzed.

2. Material and methods

2.1. Sample collection

Samples were collected in 2020 in Shaoxing, Zhejiang Province, China (30°5′N; 120°30′E). The fermentation cycle was 60 days, MAQ and MEQ samples were taken at 0 d, 2 d, 4 d, 6 d, 8 d, 10 d, 13 d, 16 d, 19 d, 25 d, 35 d and 60 d, respectively. Three repeats of each sample were randomly selected in the middle layer of the fermenting room. All the 1 cm thick surface parts were collected as surface sample, then the 1 cm thick middle part was cut off, leaving the center part of the *qu* as core sample. All samples were crushed according to the position, and 1000 g of each sample was mixed evenly and stored at −80 °C.

2.2. Microbial community analysis

Total DNA was extracted by CTAB method. 16S rDNA V3–V4 (primer 27F, 1492R) and ITS-1 region (primer ITS1, ITS4) were amplified. NGS (Thermo Fisher Ton Torrent S5, USA) sequencing platform was selected for database construction and sequencing. Quality control of raw data was carried out according to report (Xiao et al., 2017). QIIME (v 1.8.0) was used to compare bacteria and fungi in SILVA database (version 132) and UNITE database respectively. OTU clustering was performed according to 99% similarity (Garcia-Garcia et al., 2019). Blast analysis was performed on the unclassified genera. NT database was used to select the pair with the highest score, and relevant information was changed to reconstruct OTU table. The new OTU table was standardized and used in Alpha and Beta diversity analysis.

2.3. Physicochemical indexes of RWQ and huangjiu

The temperature and relative humidity were directly read by electronic temperature and humidity meter. Dry/Wet weight measurement method was applied to determine water content (Liu, Tang, et al., 2020). Enzyme activities of glucoamylase and α-amylase were determined according to report by Yu et al. (Yu et al., 2021). The oxygen content in the surface was assigned to 1 and that in the center is 0. The total sugar was measured by DNS method (Xu et al., 2018), and the alcohol content, acidity and amino acid nitrogen were measured according to methods by reports (Gong et al., 2020).

2.4. Analysis of chemical compounds in huangjiu by GC-MS and HPLC

The fermentation method of *huangjiu* is shown in **Supplementary text**. Detection of physicochemical indicators of *huangjiu* (alcohol, total sugar, acidity, and amino acid nitrogen) were referred to report (Gong et al., 2020).

Determination of organic acids was referred to report (Gong et al., 2020) with slight modifications. Add 0.2 mL zinc sulfate (30 g/L) and 0.2 mL potassium ferrocyanide (10.6 g/L) solution to 1 mL sample, shake well and dilute to 5 mL, settle for 1 h and then centrifugal at 8000 r/min for 10 min, the supernatant was passed through a 0.22 μm water membrane and determined by high performance liquid chromatography (Waters e2695 high performance liquid chromatography, waters technology, USA). The mobile phase was 0.025 mol/L sodium dihydrogen phosphate solution (pH adjusted to 2.70 with phosphoric acid). The equal ratio elution method was adopted, with 0.8 mL/min flow rate and 210 nm detection wavelength.

Measurement of higher alcohols was referred to report with slight modifications (Zhou et al., 2020). Take 3.5 mL *huangjiu* sample, add 3.5 mL pure water, 1 mL acetonitrile, 600 μL dichloromethane, 50 μL 4-methyl-2-pentanol solution (internal standard, 16800 mg/L), vortex for 1 min, mix and centrifuge at 5000 r/min for 5 min. Collect the bottom organic phase. After passing through a 0.22 μm filter membrane, the standard solution was processed in the same way for GC-MS determination. Volatile flavor substances were extracted as described above (Chen et al., 2019), and determined using HS-SPME combined with GC-MS (Thermo Fisher trace gas chromatography-mass spectrometry, Thermo company, USA) with a 50/30 μm DVB/CAR/PDMS extraction head.

2.5. Verification of driving force

With reference to former reports (Liu et al., 2021) and the factory's MEQ manufacturing process, each piece of wheat *qu* used 2.50 kg of crushed wheat and the water content was 21%–22%. Different driving force control methods are shown as follows: In the dampening stage before fermentation, different proportions of water were added to the wheat raw materials (18%, 19%, 20%, 21%, 22% and 23% of the weight of wheat) then pressed the block curve (length 28.0 cm, width 18.0 cm) through the pressing mold. Control the pressure to make the block curve with thickness of 6.5 cm, 7.0 cm, 7.5 cm, 8.0 cm. Wheat *qu* were fermented for 2 days under temperature and humidity control conditions before being cultured under different temperature conditions (natural temperature, 25 °C, 37 °C, 50 °C, 55 °C, 60 °C) until the end of fermentation, and the entire fermentation cycle was 8 days. The same group of wheat *qu* was completely crushed and mixed, and then 1000 g was sampled. Three samples from each group were taken in parallel and stored under −20 °C before testing.

2.6. Statistical analysis

Canoco 5 was used to analyze the contribution of physicochemical parameters on microbial succession, and Monte Carlo replacement test was carried out to calculate the interpretation rate of environmental factors on the formation of microbial community structure in RWQ. Mantel test was carried out to analyze the correlation between the community structure and the corresponding environmental factors through vegan software package of R (version 3.6.3). Spearman correlation coefficient was applied to characterize the correlation. Microbial community structure data was Bray Curtis dissimilarity coefficient matrix, and that of environmental factors was Euclidean distance matrix. Analysis of molecular variance (AMOVA) combined with Stamp software was used to compare the significant differences in community structure, then the microbial genera with significant differences in

different RWQ were obtained. One way analysis of variance (ANOVA) was used to test the significance of community structure, enzyme activity and water content in the results of driving force validation, using Graphpad prism 7 for visualization. The Bray Curtis distance was selected for cluster analysis by the Past software (version 4.10). All environmental variables were standardized and analyzed after dimensionless removal.

3. Results

3.1. Microbial community structure

3.1.1. Alpha diversity of MAQ and MEQ

Through next-generation sequencing, 8,472,249 high-quality sequences (50,194 OTUs) and 8,640,892 high-quality sequences (22,693 OTUs) were obtained from the 16S rDNA and ITS1 regions, respectively. During the MAQ fermentation process, significant difference was found in the microbial community diversity and bacterial richness between the surface and the core (Shannon, $p < 0.01$) (Fig. 1). Furthermore, the bacterial community diversity between the surface and core of MEQ showed significant differences ($p < 0.05$).

3.1.2. Bacterial community structure of MAQ and MEQ

Bacillus, *Pantoea, Saccharopolyspora, Staphylococcus* and *Weissella* were the main genera of MAQ (Fig. 2). In the MAQ surface, the relative abundance of *Saccharopolyspora* and *Bacillus* reached 54.8% and 48.7% on 2 d and 4 d, respectively, becoming the dominant bacteria in MAQ. The relative abundance of *Weissella* rapidly increased from 1.9% to 58.2% (0-6d), and rapidly reduced to less than 1% (13–16 d) and 32.7% in the end. The dominant bacterial community in MAQ core was similar to surface, while the average relative abundance of *Saccharopolyspora* was higher than that in surface (53%), with the lower relative abundances of *Weissella*, *Bacillus* and *Pantoea* (19.0%, 9.6% and 17.9% respectively), which was possibly related to lower oxygen levels. *Bacillus, Pantoea, Saccharopolyspora* and *Staphylococcus* were the dominant bacteria in MEQ, with an average relative abundance of 81.0% at the end of fermentation. *Curtobacterium*, *Erwinia*, *Kosakonia*, *Lactobacillus*, *Lactococcus*, *Massilia, Pseudomonas* and *Sphingomonas* also dominated the community in core, with a total relative abundance of 8.2% (60 d). The proportion of bacteria assigned to others (<1%) in MEQ was significantly higher than that in MAQ (0–8 d), indicating that the bacterial community in the fermentation process of MEQ was more complex and diverse.

3.1.3. Fungal microbial community structure of MAQ and MEQ

The fungal genera of MAQ were mainly composed of *Alternaria, Aspergillus, Cryptococcus, Emericella, Epicoccum, Fusarium, Rhizomucor, Thermomyces* and *Xeromyces*. *Thermomyces* dominated fungal community from 2 d, with the highest abundance reaching 88.7%, but then *Aspergillus* turned dominated species from 4 d. *Aspergillus* predominated MAQ surface, while the core was mainly consisted of *Thermomyces*, which may be related to the difference in fermentation speed (Tan et al., 2019). Fungal composition was more complex in surface than core, which may be reasoned the surface could be affected by environmental microorganisms such as in air in later fermentation stage, while core maintained a relatively stable community structure under the isolation of physical accumulation. The fungal community of MEQ surface succeeded to a community structure dominated by *Aspergillus*, increasing significantly from 3.1% to 78.9%, which was similar to MAQ. Unlike surface, *Rhizomucor* was the main microorganism in 4–19 d of MEQ core,

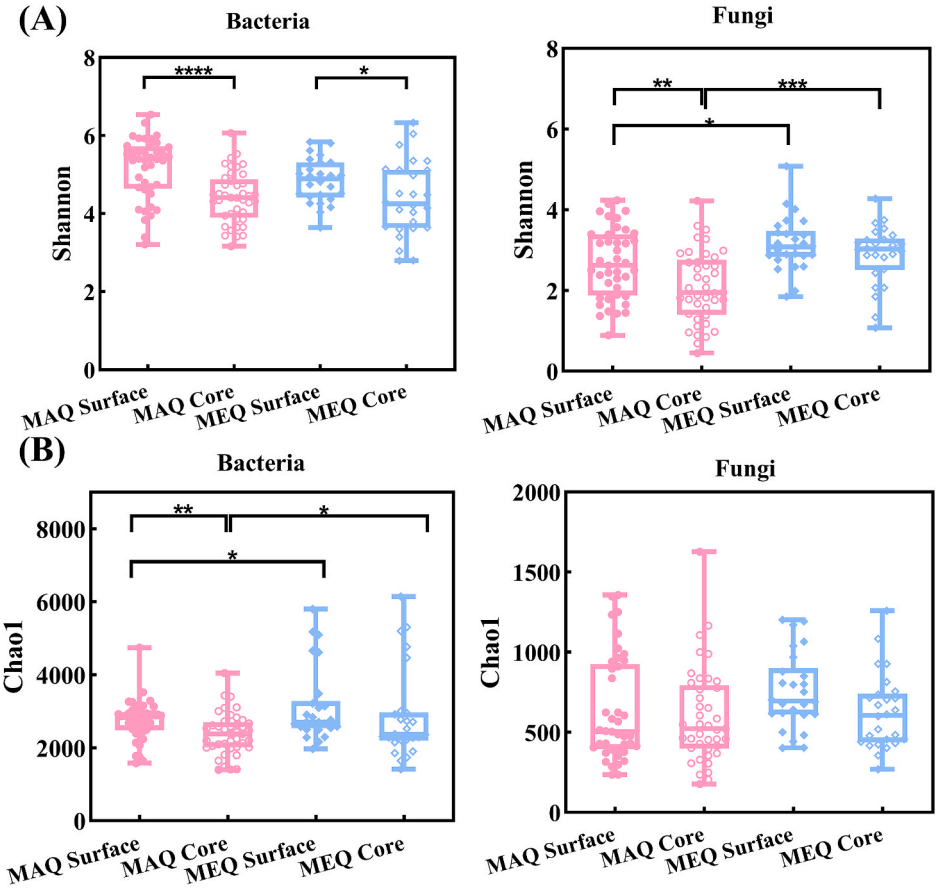

Fig. 1. Alpha diversity in RWQ with different producing procedures. Shannon (A) and Chao1 (B) indices of the surface and core of RWQ. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

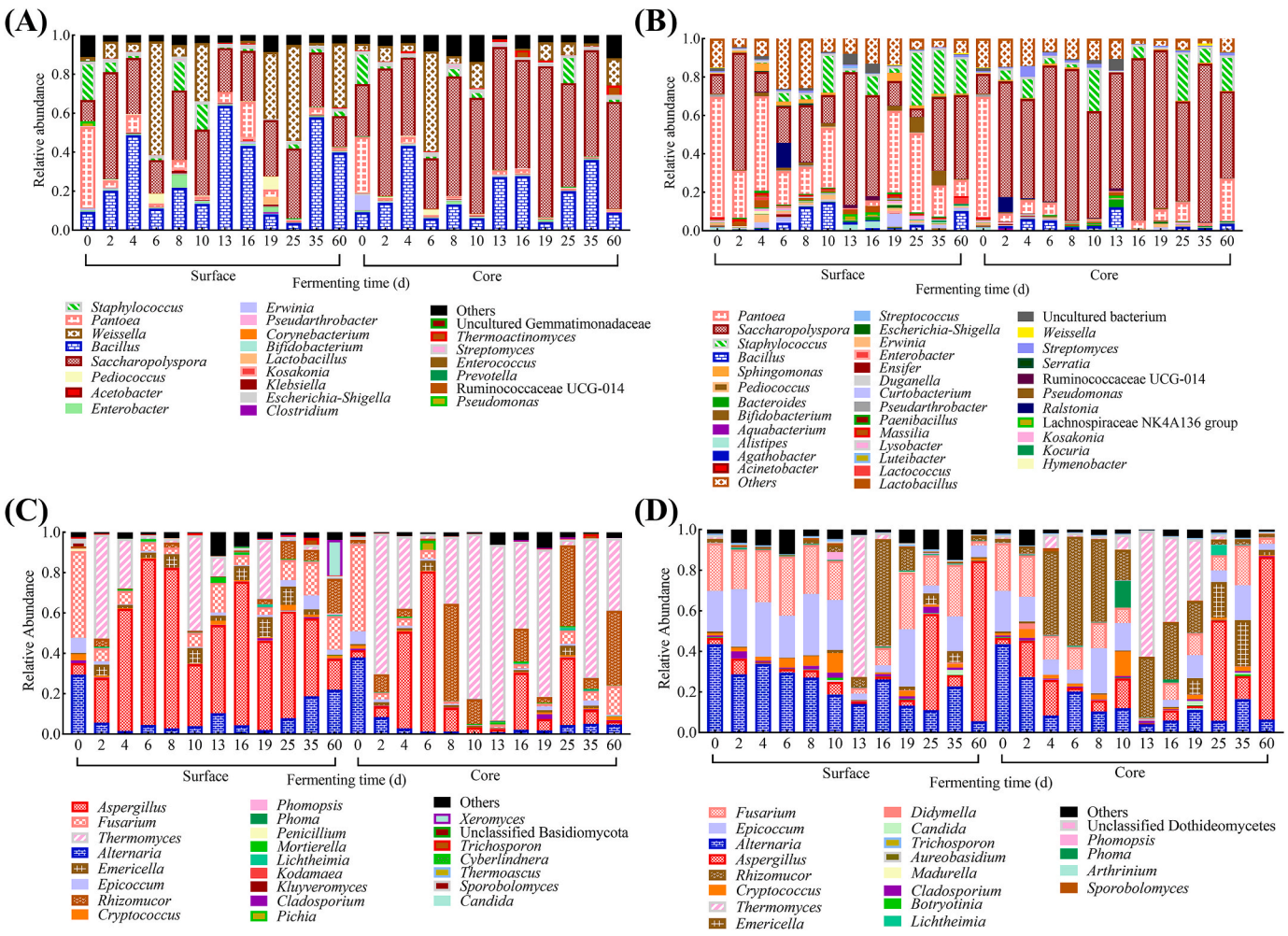

Fig. 2. Microbial community structure of MAQ and MEQ. Bacterial structure of MAQ (A) and MEQ (B). Fungal structure of MAQ (C) and MEQ (D).

reaching a maximum value of 53.4% at 6 d, and then decreased steadily. *Thermomyces* experienced a succession process of rapid rise and rapid decline in the middle stage of fermentation, similar to *Rhizomucor*. Subsequently, filamentous fungi such as *Aspergillus*, *Emericella* and *Epicoccus* invaded from the surface, rendering the fungal community structure of surface and core gradually consistent.

3.1.4. Cluster analysis

Cluster analysis showed that fermentation process of raw wheat *qu* was divided into 3 stages (Fig. S2, Table S1). There was almost no difference in the fermentation stage of bacterial and fungal communities, which can all be divided into three stages, similar to the traditional fermentation process, slow in the initial stage, stable in the middle, and slow in the final stage.

3.2. Physicochemical factors of MAQ and MEQ

Over the past decades, the factors affecting the microbial community in the fermentation process of *qu* have been well documented, which are usually attributed to temperature, moisture, pH, acidity, oxygen and so on (Zhu et al., 2022). Previous studies have provided the influence of temperature, acidity and other factors on the microbial community of *qu*, mainly based on time factors (microbiota succession), while space factors (microbiota in different positions) were ignored due to the difficulty of measurement. In addition, the influencing factors and driving factors of community formation in *huangjiu* RWQ are still not fully understood. The data of microbial community structure in fermentation process obtained by amplicon or metagenome research methods are not enough, and the corresponding physical and chemical factors need to be matched to form a data set, otherwise it will form an imperfect meta-analysis.

3.2.1. Temperature, moisture and water content

The fermentation temperature of MAQ and MEQ reached their peaks at 6 d and 4 d, respectively, then dropped to room temperature and stabilized gradually due to the ventilation step in the process (Fig. S3). The relative humidity and water content of the two RWQ decreased continuously. Particularly, the relative humidity of MEQ increased to a certain extent at 35 d. There was a certain mismatch between the moisture content of the RWQ surface and the core. In MAQ, the surface moisture was significantly higher than that of core in 6 d, and tended to be the same for the rest of fermentation. The surface moisture content of MEQ was always lower than that of core and turned consistent in the late fermentation period.

3.2.2. Glucoamylase and α-amylase activity

The glucoamylase activities of MAQ and MEQ firstly increased, then decreased and tended to be stable. There was a crossover between the surface and core glucoamylase activities of the two RWQs, which was consistent with the distribution of the community clustering stages. As for α-amylase activity, MAQ and MEQ generally showed a trend of firstly decreasing and then increasing, and dropped to the lowest values at 2 d and 4 d, respectively. The α-amylase activity in different parts of RWQ fluctuated very frequently, which may be related to the continuous

influence of environmental factors on α-amylase-producing microorganisms during the fermentation process.

3.3. Analysis of driving force

3.3.1. The spearman correlation between environmental factors and microbial community

Mantel test was applied to assess Spearman's correlations between environmental factors and microbial community succession. Obviously, MAQ microorganisms were significantly affected by temperature and water content in fermentation stage I ($p < 0.05$), and the subsequent time was only affected by oxygen content (Table 1, Table S2). In MEQ, bacterial communities were significantly affected by water content and oxygen in stage I, while fungal communities were only affected by oxygen content. In stage III, bacterial communities were constrained by oxygen levels, while fungal communities were influenced by temperature and water content. Notably, the MEQ microbial community was not significantly associated with environmental factors at stage II.

3.3.2. RDA analysis of driving forces

RDA results analysis showed that temperature and water content were the main driving forces affecting the succession of RWQ microbial communities (Fig. 3). In MAQ, environmental factors were highly correlated with samples in stage I. In particular, temperature showed a negative correlation with the community succession direction after 13 d. Glucoamylase activity and α-amylase activity in MAQ surface showed significantly positive correlation with *Erwinia, Pseudomonas, Penicillium, Pantoea, Staphylococcus, Fusarium, Alternaria, Emericella, Clostridium* and so on. α-amylase activity in core was positively correlated with *Pseudomonas, Streptococcus, Thermomyces* and *Pichia*, while the glucoamylase activity was positively correlated with *Pantoea, Erwinia, Epicoccus, Fusarium* and *Alternaria*. Fermentation progress in MEQ surface was negatively correlated with water content and temperature, especially after 6 d ($p < 0.05$). For most genera, water content acted as main driver together with temperature, which showed synergistic effect with each other. It is worth noting that the glucoamylase activity was almost absolutely negatively correlated with the α-amylase activity, indicating that in the MEQ core rise in the glucoamylase activity often meant reduction in the α-amylase activity.

3.4. Verification of driving force by single factor design

3.4.1. Effect of temperature on community structure and enzyme activity of wheat qu

The community structure and enzyme activity of RWQ fermented under different temperature including natural temperature (Tn) were compared. The diversity of microbial communities was higher at low temperatures, possibly because lower temperature promoted the growth of a large number of microorganisms, meanwhile leading to the increasing of harmful and even pathogenic bacteria (Fig. 4). As the temperature rose, thermophilic communities grew significantly in RWQ, such as *Bacillus, Saccharopolyspora, Aspergillus, Alternaria, Rhizomucor*, *Kosakonia* and *Staphylococcus*. Culture temperature below 50 °C had little effect on glucoamylase and α-amylase activity, and both of them decreased significantly at 55 °C, when glucoamylase activity reached lower than 500 U/g, which was unable to continue the fermentation process. The results showed that the optimum temperatures of glucoamylase and α-amylase activity in RWQ were about 50 °C and 37 °C respectively.

Table 1
Mantel test analysis of MAQ and MEQ using Spearman correlation. Physicochemical parameters determined by Spearman correlation ($p < 0.05$) test were marked with "√".

Group	Stage	Type	Temperature	Water content	Oxygen
MAQ	Stage I (0-6d)	Bacterium	✓	✓	
		Fungus	✓	✓	
	Stage II (6-19d)	Bacterium			✓
		Fungus			✓
	Stage III (19-60d)	Bacterium			✓
		Fungus			✓
MEQ	Stage I (0-10d)	Bacterium		✓	✓
		Fungus			✓
	Stage II (10-19d)	Bacterium			
		Fungus			
	Stage III (19-60d)	Bacterium			✓
		Fungus	✓	✓	

3.4.2. Effect of initial water addition on community structure and enzyme activity of raw wheat qu

Effects of initial water addition in RWQ on community structure and enzyme activity were compared. Water addition higher than 20% led to a high-humidity and low-oxygen environment inside RWQ, thus affecting the composition of the microbial community. As the water addition rose, the proportion of *Saccharopolyspora* showed a decreasing trend, while *Pantoea* increased gradually. The relative abundance of *Aspergillus* reached the maximum at 20% water addition, and *Alternaria, Cryptococcus* and *Epicoccus* increased significantly after that. The glucoamylase activity reached the minimum at 21%, while the α-amylase activity reached the maximum at 20%.

3.4.3. Effect of thickness on community structure and enzyme activity of raw wheat qu

The thickness of RWQ was relevant to oxygen content inside. As the thickness increased, the space between the grains became larger and the oxygen content increased. Bacterial richness was higher when the thickness was less than 7.5 cm. As the thickness reached 8.0 cm, the proportion of *Saccharopolyspora* was higher than that of other samples, acting as the dominant genera with *Bacillus* and *Pantoea*. No significant difference was found in the fungal composition, and *Aspergillus* and *Alternaria* dominated in all samples. With the increase of thickness, the glucoamylase activity gradually increased, and the α-amylase activity was also approximately positively correlated with the thickness, indicating that the enzymatic activity of RWQ was positively correlated with aerobic bacteria.

3.5. Winemaking and evaluation of MAQ and MEQ

Two kinds of RWQ were used in *huangjiu* brewing, and the differences in physicochemical indicators during fermentation were compared. During the 5-day period of pre-fermentation, no significant difference showed in the rate of alcohol production. The total sugar content of MEQ brewing mash in 24 h was significantly higher than that of MAQ ($p < 0.05$), indicating a higher saccharification efficiency in the early stage of fermentation. During 15 days of post-fermentation, due to the decrease of temperature, production rate of ethanol, organic acids and amino acid nitrogen decreased, while the consumption of biochemical reactions such as tricarboxylic acid cycle and esterification gradually increased, resulting in reduction of acidity and amino acid nitrogen content over time. At the end of fermentation, no significant difference existed between two groups.

Fusel was one of the main metabolic by-products produced by *Saccharomyces cerevisiae* during the fermentation, and also one of the main substances in *huangjiu* that caused deep intoxication (Sun et al., 2020). Fusel in MAQ and MEQ (including n-propanol, isobutanol, isoamyl alcohol and phenethyl alcohol) showed no significant difference ($p > 0.05$). Organic acid is considered to be an important flavor substance to ensure the uniquely sweet and sour taste of *huangjiu*, but also leading to an increase in acidity, inhibiting the ability of yeast to produce alcohol. At the end of pre-fermentation, the total organic acid content in MEQ brewing mash reached more than 356.90 mg/L higher than that in MAQ, while almost the same content was reached in both

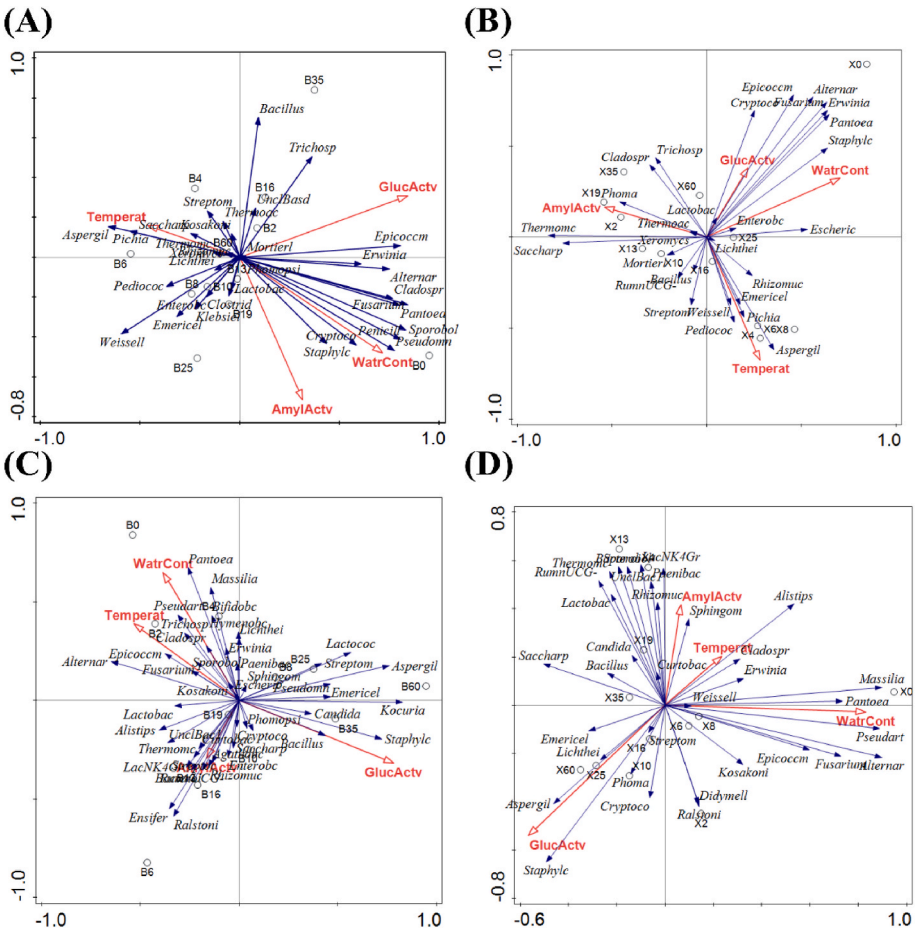

Fig. 3. Redundant analysis (RDA) of main microorganism and physicochemical factors in the surface of MAQ (A) and MEQ (C) and core of MAQ (B) and MEQ (D).

groups at the end of post-fermentation.

Alcohols, acids and esters were the skeleton composition of *huangjiu* aroma components (Wang et al., 2014). The content of n-hexanol in MAQ *huangjiu* was significantly higher than that in MEQ ($p < 0.05$) (Table S3). Esters are the most abundant flavor compounds in *huangjiu*. The content and ratio of ethyl acetate and ethyl lactate is an important parameter for the coordination of flavor (Gao, Liu, et al., 2020). Contents of ethyl isovalerate and ethyl hexanoate in MAQ *huangjiu* were higher than those in MEQ ($p < 0.05$). No significant difference was found in aldehyde contents between two groups, mainly containing benzaldehyde and phenylacetaldehyde. Higher concentrations of isovaleric acid were also detected in *huangjiu*, while there was no significant difference in acid contents between groups. The 4-vinylguaiacol content of MEQ *huangjiu* was higher than that of MAQ ($p < 0.05$), which may be related to the higher proportions of genera including *Fusarium, Aspergillus, Cladosporium,* and *Kosakona*, usually associated with ferulic acid esterase, turning ferulic acid in wheat into phenol such as 4-vinylguaiacol (Maraval et al., 2008; Rosazza et al., 1995; Yin et al., 2018; Zhang et al., 2015).

4. Discussion

RWQ is essential starter for the bilateral fermentation process of Chineses *huangjiu*, which can form various flavor compounds through microbial metabolic pathways such as amino acid metabolism, lactose metabolism and ketone degradation with starch, fat and protein (Liu, Hu, et al., 2020). Being produced in natural and open environment, RWQ quality is affected by many factors, especially physicochemical factors. By comparing the RWQ community structure succession characteristics and driving forces of different producing procedure and analyzing the sensory and physicochemical characteristics of *huangjiu* can provide a reference for the improvement of MEQ process, which is of great significance for promoting the modernization of RWQ production, thus improving the quality of *huangjiu*.

Bacillus, Saccharopolyspora, Alternaria, Streptomyces, Aspergillus, Rhizomucor and *Thermomyces* were the common dominant microorganisms shared by two kinds of RWQ, which were also detected in Korean wheat Nuruk and sake koji (Bal et al., 2014, 2016; Bokulich et al., 2014). Microbial differences between the core and the surface might be attributed to the variance in water content and temperature (Ji et al., 2018). Overall, thermophilic genera such as *Saccharopolyspora*, *Thermomyces* and *Rhizomucor* accounted for a higher proportion of in cores with high temperature and humidity. Proportions of *Aspergillus, Pantoea* and *Epicoccum* in the surface was higher than those in the interior, which could survive under low humidity, thus gradually becoming dominant genera (Yuan et al., 2020).

Differential genera in RWQ with different process procedures were further analyzed. It had the most abundant differential microorganisms at the beginning (0 d) (Fig. S4), mainly due to differences in the production environment (such as production equipment and worker skin surfaces) (Du et al., 2019). As fermentation proceeded, it was estimated physicochemical factors gradually drove the microbiota to converge among different process. For instance, proportion of *Fusarium* in MAQ decreased to significantly lower than that in MEQ, which may be related to the sudden decrease of water content in surface (Wilson et al., 1975), also leading to a significant increase in *Aspergillus*. Differences in the distribution of microbiota were also reflected in changes in enzyme activity. The relatively higher temperature and lower water content

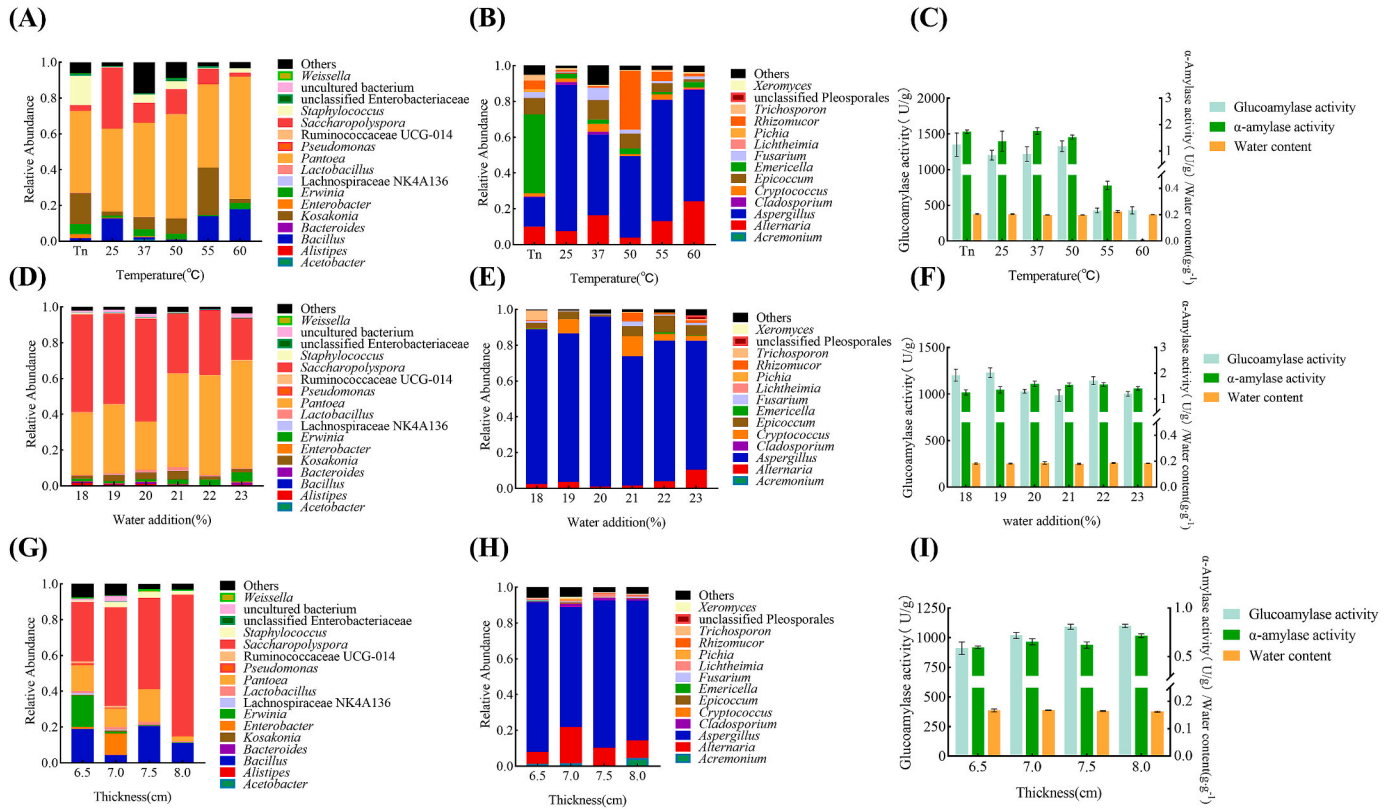

Fig. 4. Verification of driving force by single factor design. Influence of temperature on bacterial (A), fungal structure (B) and enzyme activity (C) of *qu*; Tn: room temperature. Influence of water addition on bacterial (D), fungal structure (E) and enzyme activity (F) of *qu*. Influence of thickness on bacterial (G), fungal structure (H) and enzyme activity (I) of *qu*.

resulted in a greater proportion of *Thermoactinomyces*, *Enterococcus* and *Aspergillus* in MEQ, which could produce more glycosylases for *huangjiu* brewing. It was worth mentioning that the higher microbial diversity in MAQ in the end may explain the more complex flavors in manual *huangjiu* (Yang et al., 2017).

The analysis of driving force showed that the effects of temperature, water content and oxygen on the RWQ community structure varied in different fermenting stage. In MAQ fermentation stage I, microbiota quantities increased rapidly (Shannon, Simpson), resulting in a rapid rise in temperature and decrease in water content due to bioheat generation, promoting growth of thermophilic microorganisms such as *Bacillus, Weissella*, and *Thermomyces* (Fig. 1, Fig. S3). Meanwhile, water content and temperature were main driving factors for community succession during this stage ($p < 0.05$). From 8d to the end, water content and temperature gradually decreased and tended to rarely fluctuating, when microbiota was only effected by the oxygen concentration ($p < 0.05$) (Table S2). Microbial structure was distinctly correlated to enzyme activity. According to RDA results, glucoamylase activity was positively correlated with genera including *Epicoccus, Erwinia, Streptococcus, Pantoea, Fusarium, Staphylococcus* and *Aspergillus*. *Aspergillus* was proved to produce extracellular glucoamylase, releasing D-glucose from the non-reducing end of starch (Gao et al., 2019). Moreover, α-amylase activity was positively correlated with *Rhizomucor, Saccharopolyspora, Thermomyces, Phoma, Ralstonia, Bacillus* and so on. It has been reported that some species of *Bacillus* and *Saccharopolyspora* could produce α-amylase to hydrolyze the starch in the raw materials thus continuously providing glucose for microorganism growth (Chakraborty et al., 2011; Chen et al., 2014).

RWQ is the primary liquefying and saccharifying agent in *huangjiu* brewing. Single factor analysis was used to further study the effects of physicochemical factors on microbiota and enzyme in RWQ. Activities of glucoamylase and amylase reached its maximum at 50 °C and 37 °C respectively, similar to previous studies (Fig. 4) (Urbanova et al., 1993). When the temperature exceeded 55 °C, activity of glucoamylase decreased apparently, while that of amylase almost disappeared, indicating enzyme activities were affected by both enzyme-producing microorganism distribution and temperature. Initial water addition had less obvious impact on RWQ microbiota. With the increase of addition, the humidity increased and the oxygen content decreased, growth of *Saccharopolyspora* was inhibited while the abundance of *Pantoea* increased. Glucoamylase activity reached its peak at 19% of water addition, while amylase activity increased with water addition rising, which may be related to the increase of *Pantoea* (Heydarian et al., 1996). Interestingly, thicknesses of *qu* had a greater impact on bacterial community, as the diversity decreased with the thickness increasing. Oxygen content in RWQ rose with the increase of thickness, meanwhile, proportion of *Saccharopolyspora*, glucoamylase and α-amylase activity all increased. It was estimated *Saccharopolyspora* was the major genus producing glucoamylase in RWQ.

Multi-omics analytical methods, including metabolomics and microbiomics have been widely used in food analysis (Li et al., 2021). In this study, HPLC and GC-MS were applied to compare brewing characteristics of *huangjiu* with different RWQs. The initial acidity, organic acid and total sugar content were higher in MEQ fermentation mash, which was speculated to come from MEQ with higher proportions of *Pantoea* and *Alternaria* (Fig. 5) (Vijaya Kumar & Rao, 1976). Except succinic acid, contents of various organic acids in MEQ fermentation mash at the end of pre fermentation were higher than that in MAQ, which may be related to the higher abundance of acid-producing bacteria such as *Pseudomonas* and *Candida* in wheat *qu* (Cheng et al., 2009; Lockwood & Stodola, 1946). Organic acids not only guaranteed the unique taste in *huangjiu* but also inhibited the growth of spoilage bacteria (Zhao et al., 2019a, 2022). In our study, higher content of organic acid led to the increase of acidity in the mash, thus inhibiting the ethanol production capacity of

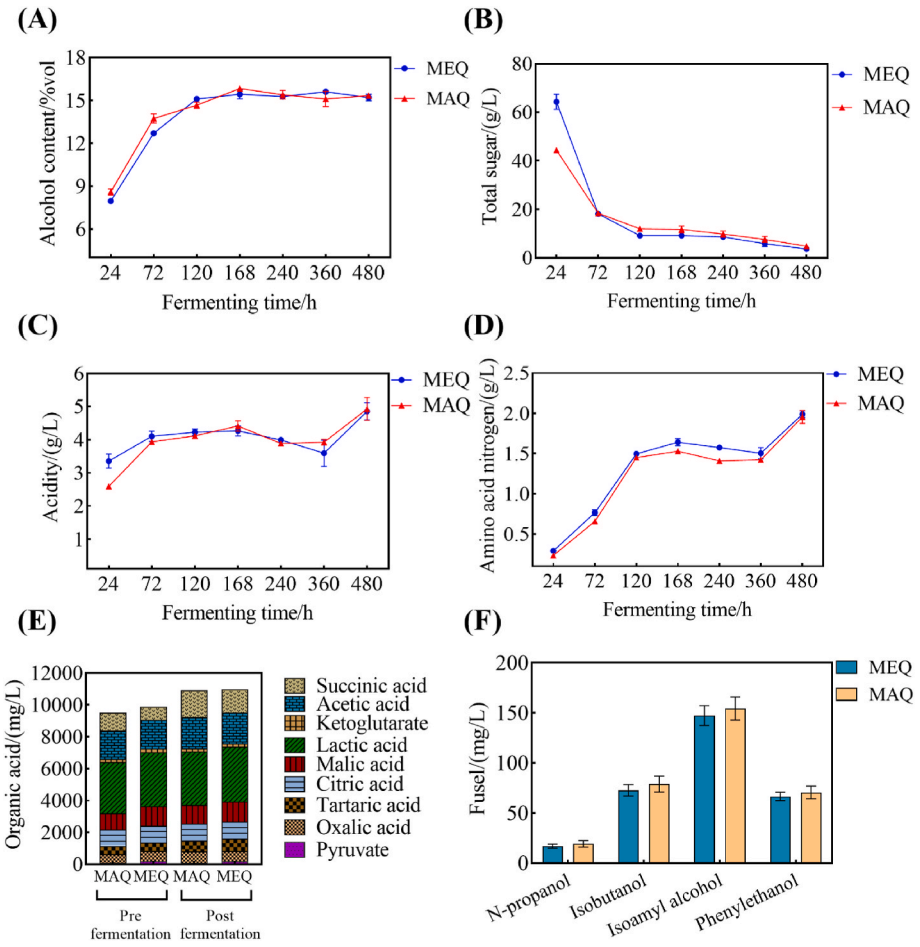

Fig. 5. Physicochemical factors and flavor of *huangjiu* made by MAQ and MEQ. Alcohol (A), total sugar (B), acidity (C), amino acid nitrogen (D), Organic acid (E) and fusel (F) content of *huangjiu* during fermentation.

yeast, resulting in the low alcohol content of MEQ mash during pre-fermentation (Chowdhury et al., 1997). Meanwhile, growth of harmful bacteria was inhibited due to the increase of acidity, thus ensuring the quality of *huangjiu* (Chen et al., 2022a, 2022b; Inatsu et al., 2017). Contents of all organic acids rose compared to those in the beginning except for ketoglutaric acid. No significant difference was detected in each index in the end.

Differences in aroma composition of *huangjiu* were further analyzed. Contents of hexanol, ethyl isovalerate, ethyl caproate and guaiacol in manual *huangjiu* were higher than that in mechanical one ($p < 0.05$), consistent with organic acid contents at the end of fermentation, with more fruity and mellow odors pronounced, while the content of 4-vinyl guaiacol was significantly lower than that in mechanical *huangjiu* (Fig. 5, Table S3). Relationship between RWQ microorganisms and flavor components was further studied by Spearman correlation analysis. Apparently, hexanol, ethyl caproate and guaiacol contents in *huangjiu* showed positive correlation ($r > 0.8$, $p < 0.05$) with genera such as *Acinetobacter, Botryotinia, Bacteroides and Paenibacillus*, with no significant difference in the percentage of these microorganisms except for *Botryotinia*, suggesting that *huangjiu* aroma might be more effectively influenced by the flavor components and enzymes of RWQ (Mo et al., 2010) (Fig. S5). Moreover, higher abundance of *Aspergillus* were detected in MEQ, which was able to promote the production of 4-vinyl guaiacol (Yang et al., 2021). *Fusarium* metabolizes ferulic acid to a transient intermediate 4-vinyl guaiacol, which is further metabolized to vanillin, vanillic acid, and protocatechuic acid. This may explain the low percentage of *Fusarium* in MEQ and the high content of 4-vinyl guaiacol in the corresponding *huangjiu* (Nazareth & Mavinkurve, 1986).

In summary, microbiota in different part in RWQ varied due to differences in water and oxygen distribution. The initial microbiota and the evolution pattern in RWQ with different process showed variations too. Temperature, water and oxygen content drove the final formation of microbiota in RWQ. Differences in microbiota and enzyme activity also led to differences in *huangjiu* flavor. Process improvement of MEQ was also investigated in this study, which might help to improve the flavor characteristics of *huangjiu*.

CRediT author statement

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Declaration of competing interest

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

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