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Environmental factors drive microbial community succession in biofortified wheat *Qu* and its improvement on the quality of Chinese *huangjiu*

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Wheat *Qu* plays the role of saccharification fermentation, providing microorganisms and flavor in the fermentation of *huangjiu*, and the use of functional microorganisms to fortify wheat *Qu* is becoming increasingly popular. Yet, the mechanisms promoting microbial successions of wheat *Qu* remain unclear. In this study, we first correlated microbial community succession with physicochemical factors (moisture, temperature, acidity, glucoamylase and amylase) in inoculated raw wheat *Qu* (IRWQ) with *Saccharopolyspora rosea*. The Mantel test was performed to investigate the significance and found that temperature ($r = 0.759$, $P = 0.001$), moisture ($r = 0.732$, $P = 0.006$), and acidity ($r = 0.712$, $P = 0.017$) correlated significantly with the bacterial community in phase 1 (0–40 h). Meanwhile, temperature correlated significantly with the fungal community in phases 1 and 2 (40–120 h). To confirm the effect of temperature on microbial communities, the artificial reduction of bio-heat (37°C) in IRWQ also reduced the relative abundance of heat-resistant microorganisms including *Bacillus* and *Saccharopolyspora*. A higher abundance of *Saccharopolyspora* (87%) in IRWQ was observed following biofortified inoculation of *S. rosea*, in which glucoamylase activity increased by 40% compared to non-inoculated raw wheat *Qu* (NIRWQ) (1086 U/g vs 776 U/g). Finally, the IRWQ was employed to mechanized *huangjiu* fermentation and it was found to reduce the bitter amino acid and higher alcohol content by 27% and 8%, respectively, improving the drinking comfort and quality of *huangjiu*.

[**Key words:** *Saccharopolyspora*; *Huangjiu* wheat *Qu*; Bio-heat; Microbial community succession; Drinking comfort]

Huangjiu, one of the three oldest brewed wines (*huangjiu*, wine, beer) in the world, is an alcoholic beverage mainly fermented by glutinous rice, water, wheat *Qu* and brewer's yeast (1). In recent years, mechanized *huangjiu* with stable product quality and high production efficiency has slowly replaced traditional handmade *huangjiu* (2). However, the high concentration of higher alcohol (3) and heavy bitterness (2) in mechanized *huangjiu* affects its drinking comfort and hinders the development of the *huangjiu* industry. Some strategies, such as supplementing assimilable nitrogen in *huangjiu* fermentation (4), and overexpressing *ALD*6, a key gene for higher alcohol production by *Saccharomyces cerevisiae* (5), were used to reduce higher alcohols in *huangjiu*. Up to now, few researchers have used wheat *Qu* to regulate the higher alcohol and bitterness of mechanized *huangjiu*.

Inoculated raw wheat *Qu* (IRWQ) is a new type of starter produced by inoculating specific functional microorganisms inoculated into raw wheat, and used in *huangjiu* fermentation IRWQ is a kind of wheat *Qu* with specific functions fermented by inoculating functional microorganisms into raw wheat (6). In *huangjiu* brewing, inoculated raw wheat *Qu* has gradually become a substitute for *huangjiu* wheat *Qu* due to its high enzymatic activity, short fermentation time, no seasonal restrictions, and high production efficiency. Compared with traditional raw wheat *Qu*, the glucoamylase, amylase, and protease activities of a raw wheat *Qu* inoculated with *Aspergillus oryzae* SU-16 were increased by 17%, 36% and 67%, respectively, indicating that inoculation with functional microorganisms can significantly improve the enzyme activity of wheat *Qu* (6). More studies on the application of functional microbial bioaugmentation are mainly focused on *Daqu* production, grain fermentation, and artificial pit mud culture to increase the flavor and specific functional characteristics of *Daqu* (7,8). However, similar studies about bioaugmented wheat *Qu* applied in *huangjiu* are scarce owing to the understanding deficiency of key microorganisms.

A study based on the dynamics of bacterial succession during the fermentation of *huangjiu* found *Saccharopolyspora* to be the most abundant bacterial genus in the fermentation process (18%–21%) (9). Illumine-based metagenomic sequencing was used to investigate the bacterial community composition of wheat *Qu*, and

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results indicated that actinobacteria (40.7%), which consisted mainly of *Saccharopolyspora*, was the main microorganisms at the phylum level (10). A metagenomic functional annotation was used to explore the relationship between *Saccharopolyspora* and *huangjiu* flavor, and results revealed that *Saccharopolyspora* could not only annotate amylase and cellulase, but also annotate the most enzymes involved in the synthesis of flavor substances such as alcohols, esters, amino acids and fatty acids (11). These results also indicated that *Saccharopolyspora* had a biological regulation effect on the decomposition and utilization of raw materials and the synthesis of flavor substances during *huangjiu* fermentation. Studies also have found that *Saccharopolyspora* in *Maotai Daqu* mainly participates in the catabolism of macromolecular substances through the production of enzymes, and then forms flavor substances (12). *Saccharopolyspora rosea* is an amylase, glucoamylase and protease-producing strain obtained by our laboratory from wheat *Qu*, which can be used to hydrolyze starch and protein and provide more nutrients for the growth of yeast during the *huangjiu* fermentation. Furthermore, *S. rosea* has the potential to reduce biogenic amines, and the degradation rate of total biogenic amines in the medium can reach up to 74.6% (13). The whole genome sequencing also annotated the enzymes including monoamine oxidase (EC 1.4.3.4) and primary-amine oxidase (EC 1.4.3.21) related to biogenic amine degradation (14).

Environmental factors such as temperature, moisture, and acidity affect the microbial succession and quality of wheat *Qu* or *Daqu*. Temperature impacts the development of microorganisms, microbial community composition, enzyme activity, metabolite profile, and fragrance characteristics of *Daqu* during the fermentation process (15). Bio-heat is a phenomenon in which the carbon dioxide produced by the metabolism of microorganisms in the fermentation process causes the temperature of the system to increase (16). Furthermore, bio-heat, acidity, humidity, ethanol, and temperature are the main factors driving microbial evolution in *Baijiu Daqu* (17–19). However, there are few studies on the factors that drive microbial community succession in *huangjiu* wheat *Qu*, which need to be investigated further.

The main objectives of this paper are: (i) to study the succession patterns of microbial communities in raw wheat *Qu* inoculated with *S. rosea*, (ii) to establish the relationship between physicochemical parameters and microbial community succession, and (iii) to demonstrate and validate the key drivers of microbial community succession in IRWQ, (iv) to investigate the improvement of drinking comfort of mechanized *huangjiu* by the application of IRWQ.

MATERIALS AND METHODS

Inoculated raw wheat *Qu* making process and sample collection The process of making IRWQ is shown in Fig. 1, and the detailed production process is described in the previous research (20). The process of making non-inoculated raw wheat *Qu* (NIRWQ) was similar to IRWQ except that the activated 5% *S. rosea* A22 (NCBI repository, accession number OP218373) seed culture (10^6 spores/mL) (per milliliter of seed liquid/gram of wheat) was replaced with water. *S. rosea* A22 was cultured on Actinomycetes medium (KNO_3 1.0 g/L, KH_2PO_4 0.5 g/L, $MgSO_4$ 0.5 g/L, $FeSO_4$ 0.01 g/L, NaCl 0.5 g/L, starch 20.0 g/L, agar 15.0 g/L). The two types of wheat *Qu* (IRWQ and NIRWQ) were then transferred to a wheat *Qu* fermentation room for 120 h fermentation. During the first 40 h, samples were taken every at 0, 8, 16, 24, 32, and 40 h with 3 replicates. Between 40 and 120 h, samples were taken at 48, 72, 96, and 120 h. Meanwhile, similar samples in triplicate were performed in NIRWQ as a control group.

Analysis of physicochemical parameters, enzyme activity in IRWQ and NIRWQ The temperature of wheat *Qu* during fermentation was monitored

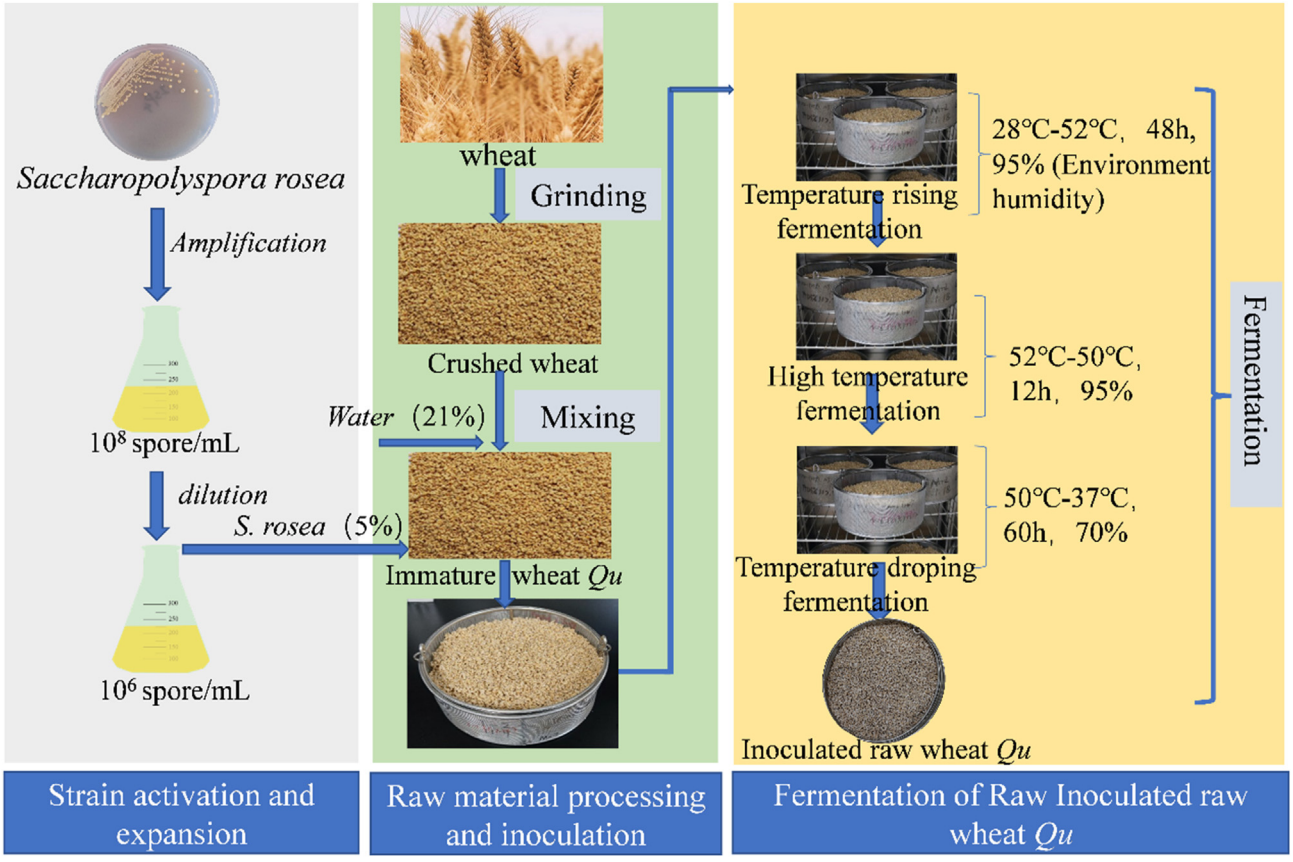

FIG. 1. The principle of the process for inoculated raw wheat *Qu*.

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by the electronic thermometer, and the ambient temperature and ambient humidity are measured by a handheld temperature-hygrometer. The moisture of wheat *Qu* is calculated by measuring its mass loss before and after drying at 101°C–105°C until constant weight. The determination of acidity, amylase activity, glucoamylase activity, and is performed by the previously described method (6,19).

Quantification of biomass in wheat *Qu* by qPCR The DNA of wheat *Qu* samples was extracted by the previously described method (11). Quantification of biomass was performed in triplicate on a qTOWER^{3}G Real-Time PCR System (Analytik Jena, Jena, Germany). Primers P1/P2 (P1: 5′-CCTACGGGA GGC AGCAG-3′; P2: 5′-ATTACCGCGGCTGCTGG-3′) (21) were used to quantify total bacterial biomass, and 10-fold serial dilutions of the linearized clone A-qRT-2 (GenBank: KM492828) containing the partial 16S rRNA gene fragments with a linear range of 8.03×10^3 to 8.03×10^{10} (copies/μL) were used as a standard with correlation coefficients R^2 of 0.9966 (efficiency = 107%). Primers Y1/Y2 (Y1: 5′-GCGGTAATTCCAGCT CCAATA G-3′; Y2: 5′-GCCACAAGGACTCAAGGT TAG-3′) (22) were used to quantify total fungal biomass, and a 10-fold serial dilution of the linearized clone Y-qRT-1 (GenBank: KM492830) containing the partial 18S rRNA gene fragments with a linear range of 8.26×10^2 to 8.26×10^9 (copies/μL) were used as a standard with correlation coefficients R^2 of 0.9995 (efficiency = 105%). Each PCR reaction system contained 10 μL TB Green *Premix Ex* Taq (Takara, Dalian, China), 0.4 μL of forward/reverse primer (10 mM), and 2 μL of 10-fold serial dilution of bacterium clone A-qRT-2, fungus clone Y-qRT-1. The qPCR reaction procedure was as follows: pre-denaturation at 98°C for 2 min, 40 cycles of 98°C for 10 s, 60°C for 15 s, 68°C for 30 s.

Illumina HiSeq sequencing and data processing The DNA of 5 g of wheat *Qu* samples was extracted by using the CTAB/SDS method, and DNA quality was examined using 1% agarose electrophoresis. Each PCR reaction system contained 15 μL of Phusion High-Fidelity PCR Master Mix (New England Biolabs, Ipswich, MA, USA), 0.2 μM of forward and reverse primers, and 10 ng template DNA. The qPCR reaction procedure was as follows: pre-denaturation at 98°C for 2 min, 30 cycles of 98°C for 10 s, 50°C for 30 s, and 72°C for 30 s. The final PCR products were detected by 2% agarose gel electrophoresis.

Sequencing libraries were generated using the TruSeq DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, CA, USA) following the manufacturer's instructions. The DNA library quality was evaluated on Agilent Bioanalyzer 2100 system (Agilent Technologies, Inc., Santa Clara, CA, USA). Finally, the DNA library was paired-end sequenced on an Illumina NovaSeq platform.

The raw sequence data were performed to remove low quality reads including length <150 bp, average Phred scores <20, mononucleotide repeats >8 bp and ambiguous bases (23) according to the QIIME (V1.9.1) quality-controlled process (24). Meanwhile, the effective tags were finally obtained by comparing with the SILVA ribosomal RNA gene database (https://www.arb-silva.de/) (25). The trimmed sequences were clustered into operational taxonomic units (OTUs) with 97% sequence identity using UCLUST under the confidence threshold of 90% (26).

Application of IRWQ to *huangjiu* fermentation The *huangjiu* fermentation process was modified referring to the previous method by replacing all the wheat *Qu* with IRWQ (13.6%, IRWQ weight: uncooked rice weight) except for the control group (wheat *Qu* including 1.8% cook wheat *Qu* and 11.8% raw wheat *Qu*, wheat *Qu* weight: uncooked rice weight) (20,27). The brief process of *huangjiu* brewing is as follows: The yeast starter of *huangjiu* was prepared by transferring *S. cerevisiae* HJ activated by YPD liquid medium to the previously prepared *huangjiu* saccharification solution, and the sample was then cultured at 28°C for 36 h at 150 rpm (28). *Huangjiu* fermentation was performed by mixing 1125 g steamed rice, 937 mL water, 104 g *Saccharopolyspora*-inoculated raw wheat Qu and 86 mL *S. cerevisiae* (20). The primary fermentation of *huangjiu* was performed at 28 ± 1°C for 120 h, and then the post-fermentation temperature was changed to 15 ± 1°C for 360 h (4). Reducing sugar content was determined by using the dinitro salicylic acid (DNS) method. Total acid and amino nitrogen were determined by using titration according to the *huangjiu* national standards GB/T 13662-2018. The content of ethanol and higher alcohol was determined as previously described (27). The volatile flavor compounds were determined by GC-MS using a Thermo Trace 1300 gas chromatograph coupled to a Thermo SO7000 mass detector with DB-wax chromatographic column (30 m × 0.25 mm, 0.25 um, Thermo Fisher Scientific, Waltham, MA, USA) (11). An Agilent 1100 high-performance liquid chromatography (HPLC) system equipped with a C18 column (250 × 4.6 mm, 5 μm) and UV detector was used to determine the content of free amino acids (20).

Statistical analysis Alpha diversity (24) and beta diversity (29) were conducted in QIIME (v1.9.1). Paired t-tests and Wilcoxon tests were performed within the R package (v3.2.4) to investigate differences in alpha and beta diversity. Microbial functions of wheat *Qu* were predicted based on PICRUSt (huttenhower.sph.harvard.edu/galaxy). The Mantel test was performed within the R package (v3.2.4) to investigate the significance between the environmental variables and the microbial community. Principal component analysis (PCA) was performed and analyzed using SIMCA 13.0. One-way analysis of variance (ANOVA) followed by Duncan's test was carried out to evaluate significant differences ($P < 0.05$) using SPSS 19.0 software. All heatmaps were plotted in the R (version 4.1.1) via the heatmap package.

RESULTS

Effects of inoculation of *S. rosea* on the physical and chemical parameters and biomass of raw wheat *Qu* Two stages of fermentation, phase 1 (0–40 h) and phase 2 (40–120 h), were assessed based on the central temperature of wheat *Qu* (Fig. 2A). The central temperature of both IRWQ and NIRWQ increased in phase 1, then decreased and fluctuated at 45°C in phase 2. The central temperature of IRWQ increased from 28°C at 0 h to the highest at 52°C at 36 h, which is 7°C higher than those of NIRWQ. This suggested that IRWQ inoculation with *S. rosea* produced a higher bioheat than NIRWQ. In contrast, the moisture content of both IRWQ and NIRWQ decreased rapidly in phase 1, and continue to decrease slowly and eventually to equilibrium at 16% (Fig. 2B). No significant differences in the core temperature and moisture content between IRWQ and NIRWQ at the end of fermentation.

In this study, the change in glucoamylase activity was similar for both IRWQ and NIRWQ, with a slow increase in phase 1 and a sharp increase in phase 2. The final glucoamylase activity for IRWQ (1086 ± 78 U/g) was 40.0% higher than those of NIRWQ (776 ± 85 U/g), which were 4.40 and 3.88 folds higher than the initial glucoamylase activity of IRWQ and NIRWQ (Fig. 2C). The amylase activity of both IRWQ and NIRWQ increased slowly during fermentation, and the final amylase activity for IRWQ and NIRWQ were 1.22 ± 0.03 U/g and 1.08 ± 0.01 U/g, respectively (Fig. 2D). There was a significant increase in amylase activity compared to the initial amylase activity for both IRWQ and NIRWQ (56% and 27.5%, respectively). It can be seen that the acidity fluctuates in phase 1, with 0.155 ± 0.002 mmol/g and 0.154 ± 0.002 mmol/g for IRWQ and NIRWQ respectively at 0 h. In phase 2, both IRWQ and NIRWQ showed a rapidly decreasing trend in acidity with 0.128 ± 0.001 mmol/g and 0.125 ± 0.001 mmol/g at 120 (Fig. 2E), respectively. These results indicated that the inoculation of *S. rosea* had little effect on the acidity of the wheat *Qu*.

The biomass of total bacteria was rapidly increased from 7.94 ± 0.03 log copies g^{-1} (0 h) to 9.73 ± 0.03 log copies g^{-1} (40 h), and then maintained a range of 9.73–10.16 log copies g^{-1} until the end of fermentation in IRWQ. The biomass of total bacteria increased from 5.09 ± 0.12 log copies g^{-1} (0 h) to 8.59 ± 0.05 log copies g^{-1} (120 h) in NIRWQ, which was significantly lower than the bacterial biomass of IRWQ (6.1% lower at 120 h) (Fig. 2F and Table S1). The dynamics of fungal biomass in NIRWQ increased consistently from 4.97 ± 0.04 log copies g^{-1} at 0 h to 8.61 ± 0.09 log copies g^{-1} at 120 h, significantly higher than the bacterial biomass in IRWQ (Fig. 2G and Table S1). The biomass of fungi in IRWQ only increased during the first 24 h (from 4.48 ± 0.02 to 8.35 ± 0.02 log copies g^{-1}), and then declined slowly to 7.4 ± 0.03 log copies g^{-1} (at the end of phase 1) and 7.51 ± 0.03 log copies g^{-1} at 120 h, respectively. The main reason for the difference in bacterial biomass in the two types of wheat *Qu* was the inoculation of *S. rosea*, and the fungal difference came from the microorganisms present in the raw wheat and the bioheat generated during fermentation.

Effects of inoculation of *S. rosea* on the microbe community structure of raw wheat *Qu* *Saccharopolyspora*, *Bacillus*, *Lactobacillus*, and *Staphylococcus* were the top 10 genera shared by IRWQ and NIRWQ, with a change in the composition of the remaining top 10 microorganisms. The relative abundance of *Saccharopolyspora* in IRWQ increased more than 3 folds within 24 h (26% at 0 h and 78.6% at 24 h, respectively), which was 87% higher than those in NIRWQ at 120 h (55%) (Fig. 3A and B). After 24 h of fermentation, *Saccharopolyspora* had become the dominant bacterium in IRWQ, however it was only after 72 h of fermentation that

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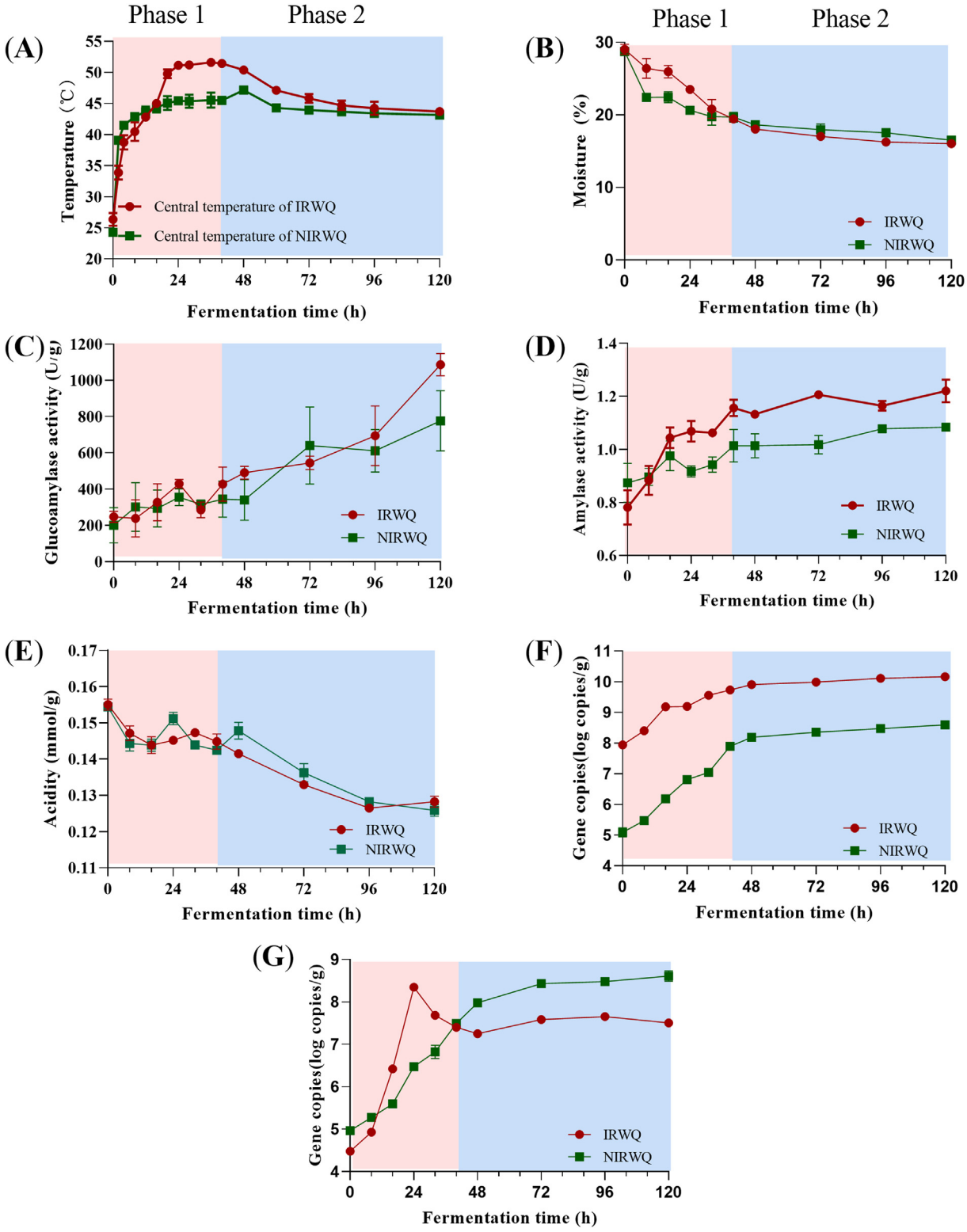

FIG. 2. Dynamics of physicochemical characteristics and biomass during the inoculated raw wheat *Qu* (IRWQ) and non-inoculated raw wheat *Qu* (NIRWQ) fermentation processes. (A) Changes in IRWQ and NIRWQ central temperature, (B) changes of moisture, (C) and (D) Changes of glucoamylase activity and amylase activity, (E) Changes in acidity, (F) and (G) represent the biomass of total bacteria and fungi respectively.

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Saccharopolyspora became the dominant bacterium in NIRWQ. It is shown that the different microbial species contained in the wheat *Qu* and the interaction between different microorganisms can also lead to changes in the abundance of *Saccharopolyspora*. Meanwhile, combined with the previous research results (Fig. 2A and F), bioheat (temperature) and inoculation with *S. rosea* synergistically increased the relative abundance of *Saccharopolyspora*, prompting *Saccharopolyspora* to occupy the dominant microorganism in IRWQ. The relative abundance of *Bacillus* in IRWQ increased dramatically from 0.2% at 0 h to 15% at 24 h, and eventually accounted for 6% at 120 h of fermentation. This may be because the IRWQ produced more bioheat and was more suitable for heat-resistant *Bacillus*. The relative abundance of *Pseudocitrobacter*, which was not identified in IRWQ, varied from 20% to 50% in NIRWQ through fermentation. Interestingly, the relative abundance of *Bacillus* in NIRWQ at 0 h was 9%, which detected only 0.1% at 120 h fermentation.

Alternaria dominated the fungal community in both IRWQ and NIRWQ, with relative abundances ranging from 60% to 83% in IRWQ and 31% to 36% in NIRWQ (Fig. 3C and D). *Rhizomucor* was not detected at 0 h for either IRWQ or NIRWQ, with relative abundances ranging from 2% to 11% in IRWQ and 40–50% for NIRWQ at 24 h fermentation, which formed an antagonistic situation with *Alternaria*. *Cladosporium* (1.3%–9%) and *Sporobolomyces* (1.7%–4.9%) were distributed through the fermentation period of the IRWQ.

Alpha diversity indices were calculated to assess the richness diversity between IRWQ and NIRWQ. In general, the box and whisker plots showed significant differences ($P < 0.01$) in the observed species, Shannon index, and beta diversity between IRWQ and NIRWQ for the bacterial community (Fig. 3E and G). However, the observed species and Shannon index of the fungal community (Fig. 3H and I) are not significantly different ($P > 0.05$) between IRWQ and NIRWQ. The microbial community diversity in

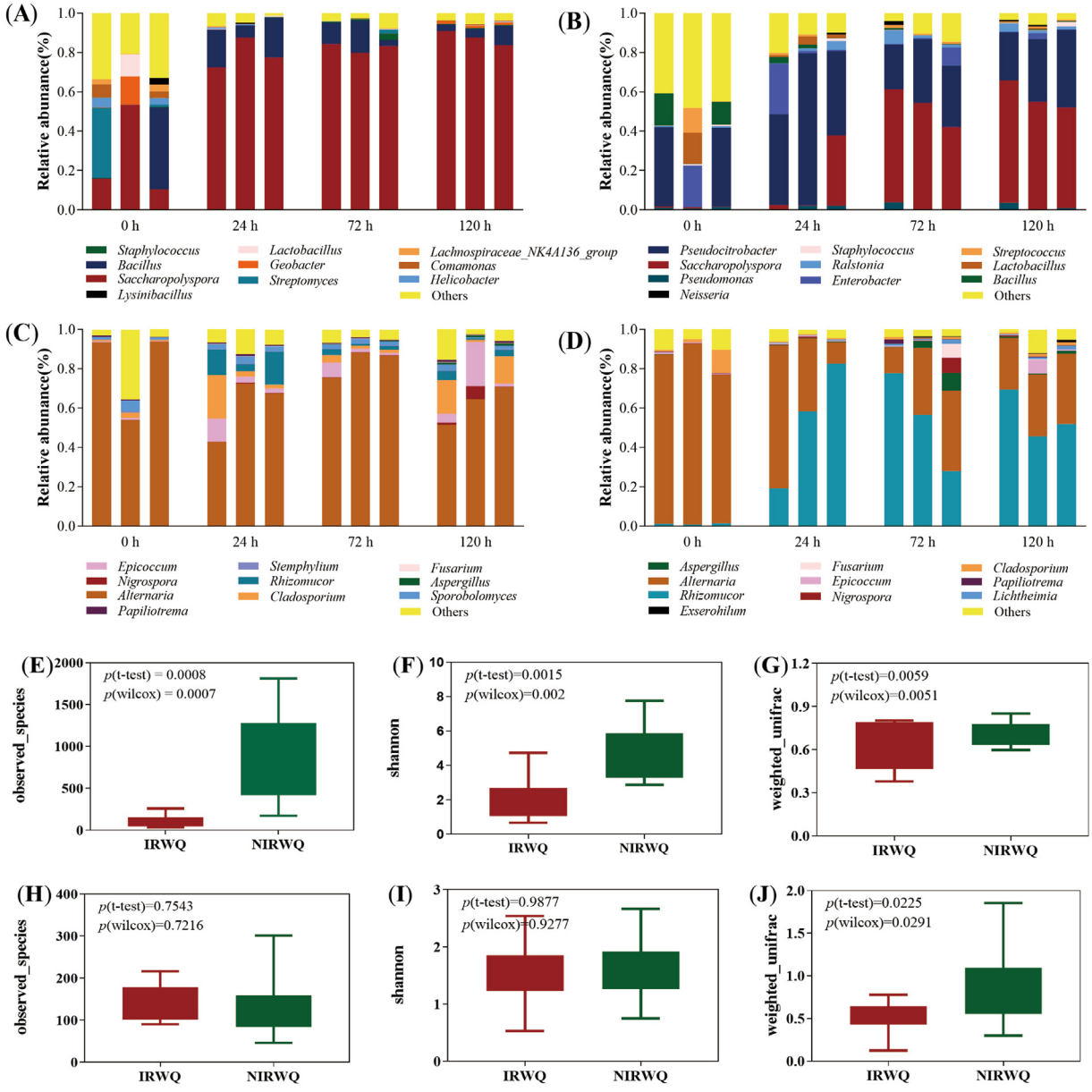

FIG. 3. Relative abundance and species diversity of microbial communities during IRWQ and NIRWQ fermentation. Bacterial genera (A) and (B), and fungi genera (C) and (D) for IRWQ and NIRWQ, respectively. The alpha diversity of the variance in observed species (E), Shannon (F), beta diversity (G) of bacterial communities, and variance in observed species (H), Shannon (I), beta diversity (J) of fungal communities.

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IRWQ was lower than that in NIRWQ, probably because the central temperature of IRWQ was increased from 28°C to 52°C (Fig. 2A), which indicated that microbial metabolism generates bio-heat to increase the core temperature of the wheat *Qu*. The bioheat generated in IRWQ was higher than that in NIRWQ, which led to the inability of most of the microorganisms that were not heat-resistant to survive, and therefore the microbial diversity in IRWQ was lower than that in NIRWQ. In addition, the richness levels of bacteria were higher from 0 h to 8 h during the IRWQ fermentation process (Table S2), although the richness levels of bacteria were sharply lower from 16 h to 120 h. However, the richness levels of fungi increased from 0 to 8 h and then decreased from 16 to 120 h (Table S3). After 24 h, fungi such as *Rhizomucor* and *Alternaria* dominated the fungal community in both IRWQ and NIRWQ, which formed an antagonistic relationship with a small number of microorganisms in wheat *Qu*, resulting in decreased richness levels. The fungal beta diversity analysis showed that there was a significant difference ($P < 0.05$) between IRWQ and NIRWQ (Fig. 3J), which indicated that inoculation of microorganisms affected the intrinsic microbial structure and diversity of wheat *Qu*.

Functional prediction of microbiota in IRWQ and NIRWQ The metabolic pathways of IRWQ and NIRWQ mainly focus on the metabolism of cofactors and vitamins, energy metabolism, carbohydrate metabolism, amino acid metabolism, xenobiotics biodegradation and metabolism, and lipid metabolism. The 95% confidence interval showed that amino acid metabolism, carbohydrate metabolism, lipid metabolism, xenobiotics biodegradation and metabolism in IRWQ were higher than those in NIRWQ (Fig. S1-A). However, the metabolic potential of NIRWQ in energy metabolism, replication and repair, translation, and the metabolism of cofactors and vitamins was higher than that of IRWQ. The carbohydrate and amino acid metabolic pathways were the ones that differed most noticeably between the two wheat *Qu*, and we investigated the activity of the key enzymes involved in these two metabolic pathways (Table 2). The results showed that IRWQ was higher than NIRWQ in both glucoamylase (1086 U/g vs 776 U/g) and amylase (1.22 U/g vs 1.08 U/g) related to carbohydrate metabolism, with higher amylase and glucoamylase compared to NIRWQ. Also, protease activity associated with amino acid metabolic pathways was higher in IRWQ (10.52 U/g) than in NIRWQ (4.11 U/g). Additionally, PCA plots also showed significant differences in the metabolic pathways annotated to IRWQ and NIRWQ, respectively (Fig. S2). The KEGG metabolic pathway varied depending on the stage of IRWQ fermentation. The metabolic pathways annotated at 0 h–8 h were significantly higher than those at 8 h–120 h, indicating that the metabolic potential of the bacterial community was more robust at this stage. Heat map clustering analysis separated the IRWQ metabolic pathways into two stages, 0 h–8 h and 8 h–120 h (Fig. S1-B), indicating that even the same type of wheat *Qu* has different metabolic functions in different fermentation stages.

Correlations between physicochemical variables and microbial communities In phase 1, temperature ($r = 0.759$, $P = 0.001$) and moisture ($r = 0.732$, $P = 0.006$) correlated extremely significantly with bacterial community succession, and acidity also showed a significant correlation ($r = 0.673$, $P = 0.024$) (Table 1). Meanwhile, temperature ($r = 0.712$, $P = 0.017$) correlated significantly with fungal community succession, but the acidity, moisture, and amylase did not show a correlation ($P > 0.05$) in phase 1. However, temperature correlated significantly ($r = 0.662$, $P = 0.023$) with fungal community succession in phase 2. In short, the temperature is the key driving factor of phase 1 of bacterial and fungal communities, and moisture and acidity are also important factors of bacterial community succession. In phase 2, the temperature is the main driving factor for the fungal community.

The correlations between environmental variables and the microbial community in IRWQ were shown in Fig. 4. In phase 1, the environmental factors acidity, temperature was significantly positively correlated with *Saccharopolyspora* ($P < 0.01$) (Fig. 4A), and temperature negatively correlated significantly with *Ruminococcus*, *Acinetobacter*, *Staphylococcus*, *Cutibacterium*, *Lysinibacillus*, and *Prevotella* ($P < 0.05$). In phase 2, the environmental factors (temperature, acidity, moisture) were significantly negatively correlated with *Geobacter*, and temperature and moisture are significantly positively correlated with *Bacillus* (Fig. 4B). For fungi, the temperature had a significant positive effect on *Rhizomucor*, *Epicoccum*, *Nigrospora*, and acidity and moisture were significantly negatively correlated with *Rhizomucor*, *Epicoccum*, *Nigrospora* ($P < 0.05$) in phase 1 (Fig. 4C). In phase 2, temperature, acidity, and moisture negatively correlated significantly with *Aspergillus* ($P < 0.05$). Also, temperature and acidity were significantly negatively correlated with *Cladosporium* (Fig. 4D).

Validating the driving effect of temperature on the microbiota To investigate the effect of temperature on microbial community succession, IRWQ after 16 h fermentation at the center temperature of 45.0 ± 0.5°C were moved to 37°C (T37 group) and 50°C (T50 group) for fermentation, and the control group continued to ferment under the original conditions respectively. After 40 h, the center temperatures of wheat *Qu* were 47 ± 0.5°C, and 53.4 ± 0.3°C, respectively. PCoA plots showed that bacterial and fungal microbial communities were separated in IRWQ fermented at 37°C and 50°C, especially the fungal community, indicating that the effect of temperature on the fungal microbial community was greater than that of the bacterial community (Fig. S3). The abundance of *Saccharopolyspora* in the control group (86.7%) was higher than that in the T37 group (55%), which was close to that in the T50 group (78%). The abundance of *Bacillus* in the control group (5.93%) was higher than that in the T37 group (0.46%) and T50 group (0.96%) (Fig. 5A). Fungal microbial communities also differed between the T37 and T50 groups (Fig. 5B). *Alternaria* related to *Aspergillus*, *Epicoccum*, and *Cladosporium* dominated the fungal community in the control group and T50 group. However, *Aspergillus* (43%) dominated the fungal community in the T37 group (Fig. 5B),

TABLE 1. Correlations between physicochemical parameters and microbial communities in different stages of IRWQ fermentation.

Parameters	Phase 1 (0 h–40 h)				Phase 2 (40 h–120 h)			
	Bacteria		Fungi		Bacteria		Fungi	
	r	*p*	r	*p*	r	*p*	r	*p*
Temperature	0.759	0.001	0.712	0.017	0.157	0.129	0.662	0.023
Acidity	0.673	0.024	0.077	0.279	0.120	0.175	0.077	0.266
Glucoamylase	0.019	0.510	0.144	0.197	0.125	0.169	0.144	0.193
Amylase	0.189	0.037	0.152	0.207	0.042	0.580	0.152	0.198
Moisture	0.732	0.006	0.191	0.069	0.657	0.042	0.191	0.076

TABLE 2. Physicochemical parameters and enzymatic activities of IRWQ and NIRWQ.

Parameter	IRWQ	NIRWQ
Moisture (%)	16.03 ± 0.12[a]	16.5 ± 0.21[a]
Acidity (mmol/g dry sample)	0.13 ± 0.01[a]	0.12 ± 0.01[a]
Amylase activity (U/g dry sample)	1.22 ± 0.03[a]	1.08 ± 0.01[b]
Glucoamylase activity (U/g dry sample)	1086.25 ± 49.98[a]	776.44 ± 86.33[b]
Protease activity (U/g dry sample)	10.52 ± 2.45[a]	4.11 ± 1.67[b]

Note: Different letters obtained by ANOVA indicate significant differences at $P < 0.05$ (n = 3).

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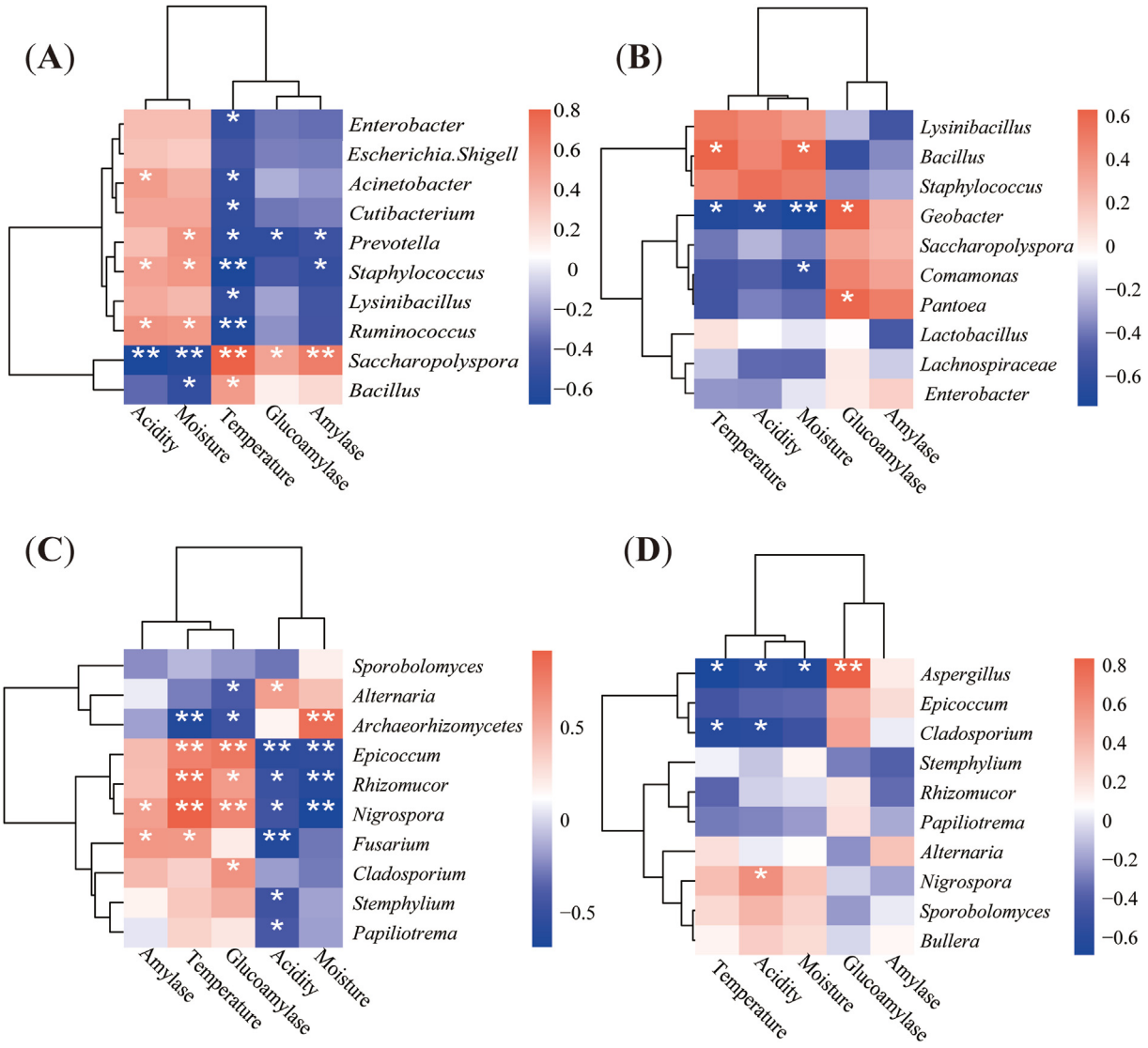

FIG. 4. Correlation between microorganisms and physicochemical variables in the fermentation of IRWQ by Spearman. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. (A) and (B) represent Phase 1 and 2 of bacterial community change, (C) and (D) represent phase 1 and 2 of fungal community change.

which indicated that bio-heat had the greatest effect on *Aspergillus* in the fungal community.

After 120 h of fermentation, the moisture content of IRWQ in the T37 group (18.8 ± 0.08%) was significantly ($P < 0.05$) higher than that in the T50 group (15.3 ± 0.25%), and the main reason for that is that the high temperature (50°C) evaporates some water from IRWQ (Fig. 5C). Glucoamylase, amylase and titratable acidity were significantly ($P < 0.05$) different in the T37 and T50 groups, indicating that bioheat plays a key role in the physicochemical parameters of wheat *Qu* (Fig. 5C and D). There are significant differences in bacterial and fungal biomass among different temperature treatment groups. The bacterial biomass of the control group (10.16 ± 0.02 log copies g^{-1}) was significantly ($P < 0.05$) higher than that of the T37 group (9.89 ± 0.08 log copies g^{-1}) and the T50 group (9.99 ± 0.01 log copies g^{-1}) (Fig. 5E). In contrast, the biomass of fungi in the control group (7.51 ± 0.04 log copies g^{-1}) was significantly ($P < 0.01$) lower than that in the T37 group (7.67 ± 0.02 log copies g^{-1}), but not significantly different ($P > 0.05$) from that of the T50 group (Fig. 5F).

Application of IRWQ in *huangjiu* fermentation In this study, we applied IRWQ to *huangjiu* fermentation to investigate its performance and the results are shown in Table 3. Although the contents of alcohol, total acid, and amino nitrogen in the IRWQ group were lower than those in the control group (Table 3), these physicochemical indicators met the standards of the Chinese *huangjiu* national standard (GB/T 13662-2018). The total amino acid content of the IRWQ group was lower than that of the control group (1.45 g/L vs 2.02 g/L), but the bitter amino acids including Leu, Ile, Val, His, Arg, Phe, and Lys were higher in the control group than in the IRWQ group (0.93 g/L vs 0.68 g/L, an increase of 27%). These results showed that *huangjiu* fermented with IRWQ can relieve the bitterness and increase the comfort of mechanized *huangjiu*.

Significantly, the content of total higher alcohols was significantly lower ($P < 0.05$) in the IRWQ group than in the control group (488 mg/L vs 528 mg/L, decreased by 8%), suggesting that IRWQ-fermented *huangjiu* can reduce the content of higher alcohols and increase drinking comfort. Principal component analysis (PCA)

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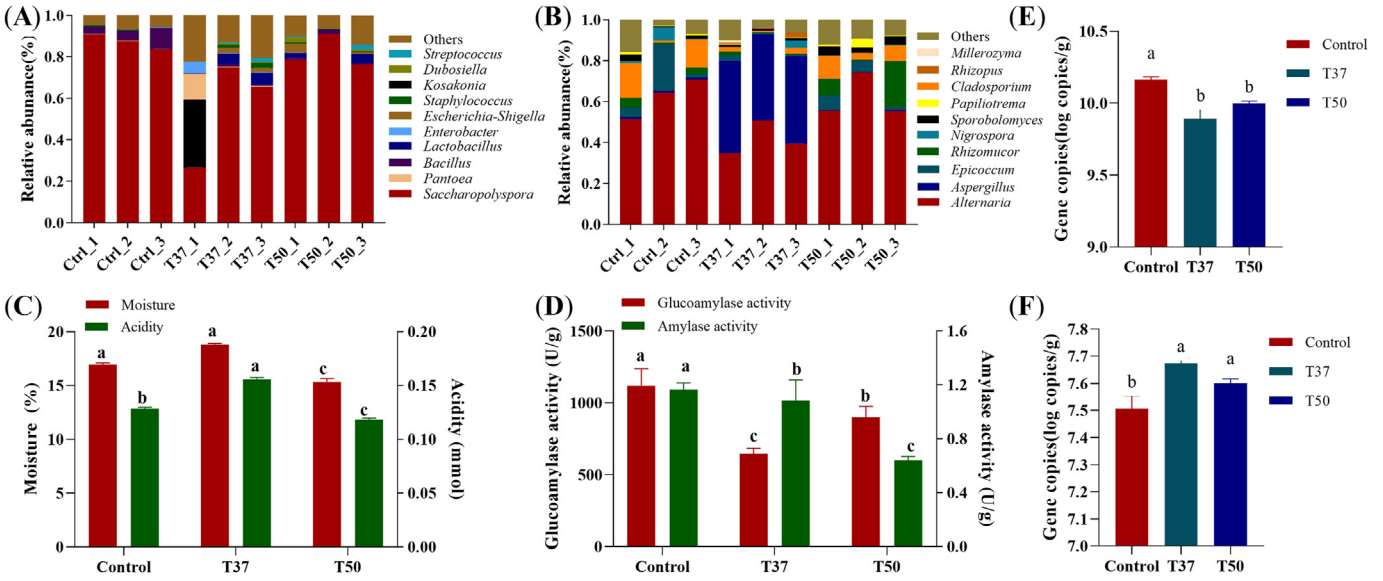

FIG. 5. Effect of temperature on the composition of the bacterial community (A), fungal community (B), acidity and moisture content (C), glucoamylase and amylase activities (D), and the total bacterial biomass (E), fungal biomass (F) within IRWQ after 120 h of fermentation. IRWQ samples after 16 h of fermentation (control group, 45 ± 0.5°C) were transferred to 37°C (T37 group) and 50°C (T50 group) for fermentation, respectively. Data are the mean ± SD of measurements. Bars with the different letter are significantly different ($P \leq 0.05$).

mapping was performed using the concentrations of flavor compounds (except higher alcohols) to investigate the effect of IRWQ on the flavor of *huangjiu* (Fig. 6). The variability of PC1 and PC2 was 58.6% and 13.9%, respectively, which together accounted for 72.5% of the total variability. The major esters on the left side of the PCA plot including ethyl lactate, isoamyl acetate, and ethyl isovalerate are major contributors to the IRWQ. The major esters are distributed on the right side of the PCA plot include ethyl acetate, ethyl caproate, ethyl formate, and ethyl butyrate were the major contributors to the control group. Total ester content in the control group and IRWQ group in the current study were 70.55 mg/L and 60.25 mg/L (Table 3), respectively, and they all contributed to the fruity and floral aromas of *huangjiu*. The above studies show that the *huangjiu* fermented by IRWQ has the advantage of reducing the content of bitter amino acids and higher alcohols.

DISCUSSION

Saccharopolyspora belongs to Actinomycetes, which can produce enzymes, vitamins, and cellulose degradation promoting factors and is a kind of safe biological resource bacteria (30). Previous studies have confirmed that *Saccharopolyspora* is the main dominant microorganism in wheat *Qu* (10,31). Metagenomic sequencing shows that the abundance of *Saccharopolyspora* during *huangjiu* fermentation ranged from 8.26% to 54.77%, indicating that the main contribution of *Saccharopolyspora* in *huangjiu* comes from wheat *Qu* (11). In this study, *S. rosea*, with high glucoamylase and amylase activity, was selected and inoculated into raw wheat *Qu* for biofortification, and the abundance of *Saccharopolyspora* in IRWQ (87%) was significantly higher than the those *Saccharopolyspora* in NIRWQ (55%) (Fig. 3A and B). Analysis of the composition of the dominant microbial community in wheat *Qu* found that *Bacillus* appeared in IRWQ, while *Pseudocitrobacter* appeared in NIRWQ. This may be because the IRWQ produced more bioheat and was more suitable for heat-resistant *Bacillus*. For fungi (Fig. 3C and D), *Alternaria* is the dominant microorganism in IRWQ, while *Rhizomucor* is the dominant microorganism in NIRWQ. Non-inoculation of *S. rosea* seemed to promote the growth of *Rhizomucor*. Previous studies have also confirmed that *Rhizomucor* is the dominant microorganism in wheat *Qu*, which was consistent with the results of this study (32). The amino acids in wheat *Qu* may be derived from the degradation of proteins or peptides originating from the raw material as well as from microbial synthesis (33), and the relative abundance of amino acids predicted in this study was higher in IRWQ than in NIRWQ (Fig. S1), suggesting that high temperatures in wheat *Qu* (52°C) facilitates protein degradation for more amino acid synthesis compared to NIRWQ (47°C) (18).

Wheat *Qu* fermentation is in an open work environment. Thus, environmental conditions are critical in the fermentation of wheat *Qu*, and have a significant impact on the type and number of microorganisms (34,35). Previous studies have confirmed that temperature, acidity, and moisture can affect the microbial composition of *Daqu* (17,36,37). In this study, we found that acidity was significantly negatively correlated with *Saccharopolyspora*, while temperature was significantly positively correlated with *Saccharopolyspora* ($P < 0.01$) (Fig. 4A). Furthermore, studies have shown that bio-heat (19), acidity, humidity, ethanol, and temperature are the main factors driving the evolution of microorganisms in *baijiu Daqu* (17,18). Peak-temperature of *Daqu* had a greater impact on the fungal community than the bacterial community in *Jiupei* (38). In this study, we only investigated the effects including temperature, moisture, acidity,

TABLE 3. Physicochemical parameters and flavor content of *huangjiu* fermented by IRWQ.

	Control (factory wheat *Qu*)	IRWQ
Alcohol content (%vol)	16.02 ± 0.02^a	15.73 ± 0.05^b
Reducing sugar (g/L)	8.51 ± 0.21^b	16.91 ± 0.21^a
Total acid (g/L)	4.73 ± 0.08^a	4.1 ± 0.15^b
Amino nitrogen (g/L)	1 ± 0.03^a	0.69 ± 0.03^b
Amino acid (g/L)	2.02 ± 0.11^a	1.45 ± 0.06^b
Bitter amino acid (g/L)	0.93 ± 0.05^a	0.68 ± 0.04^b
Total higher alcohols (mg/L)	528 ± 14.46^a	488.42 ± 11.32^b
Esters (mg/L)	70.55 ± 5.33^a	60.25 ± 3.84^b
Phenols (mg/L)	34.49 ± 1.15^a	27.19 ± 0.95^b
Aldehydes (mg/L)	0.81 ± 0.02^a	0.83 ± 0.01^a

Different letters obtained by ANOVA indicate significant differences at $P < 0.05$ (n = 3).

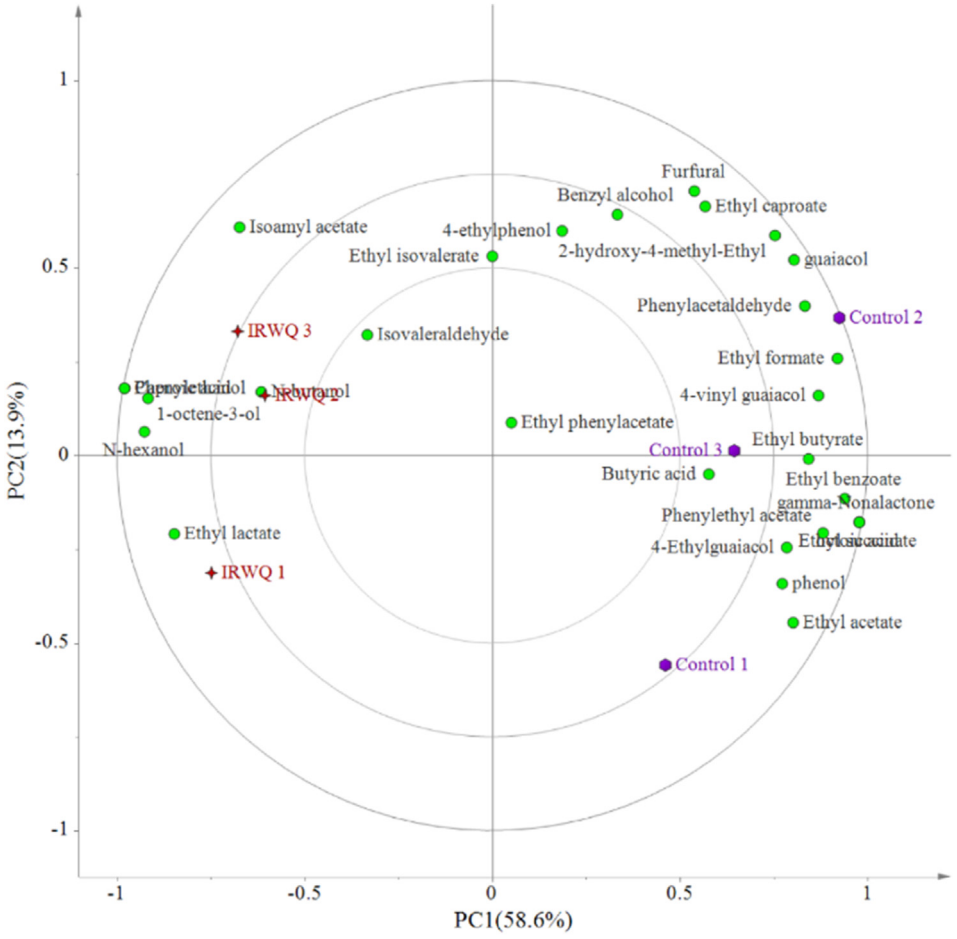

FIG. 6. Principal component analysis of volatile flavor components of *huangjiu*.

glucoamylase, and amylase on the composition and succession of microbial communities in wheat *Qu*, but the interaction between the various physicochemical variables on wheat *Qu* needs to be further investigated in depth.

Endogenous bio-heat inhibits the growth of most microorganisms and enriches a few heat-resistant microorganisms such as *Bacillus* and *Saccharopolyspora* (19). In this study, and the descending trend of Shannon index values and the change of the abundance of *Saccharopolyspora* also supports this view (Table S2, Fig. 3A and B). Bio-heat and inoculation with *S. rosea* synergistically increased the relative abundance and biomass of *Saccharopolyspora* in wheat *Qu*, prompting *Saccharopolyspora* to occupy the dominant microorganism in wheat *Qu* (Fig. 3A). We artificially varied the fermentation temperature of IRWQ and found that the temperature in the center of IRWQ in the T37 group was 47 $\pm$ 0.5°C, which was correlated with the decrease of relative abundances of thermotolerant taxa, including *Bacillus* and *Saccharopolyspora* (Fig. 5A). The results confirmed that the bio-heat produced within wheat *Qu* was associated with the composition and function of wheat *Qu* microbiota. In addition, the abundance of the dominant microorganism *Saccharopolyspora* in the T37 group was significantly lower than that of the control group, and the corresponding glucoamylase and amylase activity was also significantly lower than that of the control group, indicating that there was an association between *Saccharopolyspora* and the production of glucoamylase and amylase in IRWQ. Furthermore, previous studies have found that *Saccharopolyspora* is rich in enzymes associated with the hydrolysis of starch and the synthesis of esters, alcohols, and acids in the fermentation of *huangjiu* (11). In addition to the factors investigated in this study, other environmental factors such as oxygen concentration, carbon dioxide concentration, and the ratio of the two are also environmental factors that affect the microflora in wheat *Qu* fermentation, which need to be further studied.

Huangjiu fermentation is a simultaneous process of saccharification and fermentation, and wheat *Qu* provides the various enzymes and microorganisms that drive the fermentation of *huangjiu* (39). Amino acids are the main nutritional components of *huangjiu*, as well as the precursors for the synthesis of flavor substances such as alcohols and esters (40). Previous studies have also shown that the bitterness (the main source of bitter amino acids) in mechanized *huangjiu* is heavier than in traditional manual *huangjiu*, which has affected the comfort of drinking of mechanized *huangjiu* (2). We applied IRWQ to *huangjiu* fermentation and found that the bitter amino acids were higher in the control group than in the IRWQ group (0.93 g/L vs 0.68 g/L, an increase of 27%). Higher alcohols are the most important flavor compounds in alcoholic beverages, but high levels of higher alcohols can be linked to intoxication and hangovers since they accelerate the intoxicating effects of ethanol (41). In this study, we applied IRWQ to ferment *huangjiu* and found that it reduced the content of higher alcohols by 8% compared to the control, suggesting that IRWQ-fermented *huangjiu* can reduce the content of higher alcohols and increase drinking comfort. Therefore, the IRWQ can be used alone or in combination with other *huangjiu* wheat *Qus* to improve the drinking comfort of *huangjiu*, and promote the development of the *huangjiu* industry.

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