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ORIGINAL ARTICLE

Identification and reconstruction of the core microbiota in natural fermentation systems: a case study of *jiuyao*

Shuangping Liu[1,2,3,4] · Zhuoyue Zheng[1] · Tiantian Liu[1,2,3,4] · Dongliang Ren[1] · Chen Yang[1] · Bin Qian[3] · Yuezheng Xu[3] · Jian Mao[1,2,3,4]

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Abstract

As a saccharifying and fermenting starter, *jiuyao* is indispensable for *huangjiu* brewing by providing abundant microorganisms and hydrolytic enzymes. However, the current production of *jiuyao* still relies on the preceding year's *jiuyao* inoculation and natural fermentation. Due to the unpredictable and unstable assembly of core microbial community, the quality of *jiuyao* fluctuates across different batches, thus the quality of *huangjiu* suffers subsequently. In this study, we took *jiuyao* as a case study to identifying and reconstructing the core microbiota. Five species, *Pediococcus pentosaceus*, *Saccharomycopsis fibuligera*, *Saccharomyces cerevisiae*, *Mucor indicus*, and *Rhizopus microsporus*, were comprehensively identified as the core microbiota. A synthetic microbiota was constructed through inoculating the core microbiota to artificial *jiuyao*, and further supplemented by the spontaneous inoculation of geography-dependent microorganisms (*Weissella cibaria* and *Rhizopus arrhizus*) from original factory environment during open fermentation. The artificial *jiuyao* ultimately exhibited comparable microbial community and physicochemical indexes with traditional *jiuyao*. Specifically, their gelatinized-starch-hydrolyzing glucoamylase activities were 343.28 ± 32.27 and 340.59 ± 39.80 U/g respectively. Furthermore, *huangjiu* brewed with artificial and traditional *jiuyao* showed similar physicochemical and flavor profiles, with the ester content of the former being 5.32% higher and the content of higher alcohols being 9.64% lower compared to the latter. These results suggested that the rational synthetic core microbiota could substitute preceding year's *jiuyao* and facilitate production to be controllable and tractable. Combined with a specific production environment, it could effectively reproduce the function of *jiuyao* and the terroir flavor of *huangjiu*, providing a scientific guidance for similar fermentation control and optimization.

Keywords Traditional fermention · *Jiuyao* · *Huangjiu* · Core microbiota · Synthetic microbiota · Geography-dependence

Introduction

Brewed foods represented by *baijiu*, *huangjiu*, soy sauce and vinegar, are an important part of traditional Chinese food culture [1]. Among them, *huangjiu* is one of the oldest fermented wines in the world, and is particularly popular in the Yangtze River Delta region of southern China due to its rich nutritional value and unique flavor profile. *Huangjiu* production is a representative process with saccharification and spontaneous fermentation occurring simultaneously. *Jiuyao*, serving as the saccharifying and fermenting starter in *huangjiu* production, not only provides a micro-ecosystem consisting of a variety of microorganisms and enzymes, but provides substrates for the formation of flavor metabolic network [2]. However, like most traditional fermentation, the current production of *jiuyao* remains an empirical traditional craftsmanship. Its natural fermentation totally depends on

✉ Jian Mao
maojian@jiangnan.edu.cn

1 State Key Laboratory of Food Science and Technology, School of Food Science and Technology, National Engineering Research Center of Cereal Fermentation and Food Biomanufacturing, Jiangnan University, Wuxi 214122, Jiangsu, China

2 Shaoxing Key Laboratory of Traditional Fermentation Food and Health, Jiangnan University (Shaoxing) Industrial Technology Research Institute, Shaoxing 312000, Zhejiang, China

3 National Engineering Research Center of Huangjiu, Zhejiang Guyuelongshan Shaoxing Wine Co., Ltd, Shaoxing 312000, Zhejiang, China

4 Jiangsu Provincial Engineering Research Center for Bioactive Product Processing, Jiangnan University, Wuxi 214122, Jiangsu, China

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the inoculation of *muqu* (i.e., the preceding year's *jiuyao*). Once there are defects in the quality of the preceding generation of *jiuyao*, the quality of the subsequent generation of *jiuyao* will also be adversely affected, thereby largely hindering the mechanization, modernization and standardization of *huangjiu* industry [3].

To achieve the controllability of traditional fermentation processes and products, researchers often focus on studying process parameters and microbiota. Among these, identifying the core functional microorganisms associated with key metabolites for biofortification or multi-strain artificial starter construction has been proven effective in Japanese sake, cream cheese, light-aroma *baijiu* and cocoa beans [4–6]. As for *jiuyao*, its physicochemical indexes, microbial community structure, and metabolic characteristics have been extensively studied [7]. And the quality of *jiuyao* has been improved by inoculating strains with high starch-degrading enzyme activities and optimizing fermentation conditions such as temperature, humidity and inoculum [8]. Though there has been a study on combining six species as an artificial starter for *huangjiu* brewing, the identification of core microbiota was not full-scale [9–11].

On the other hand, in open environment, geographical factors including climate, altitude, soil, water, microorganisms and vegetation influence the assembly and succession of microbial communities during natural fermentation processes, and contribute to the terroir characteristics of products, with wine being the most typical example [12, 13]. The fermentation environment is primarily determined by natural geography and gradually modified during long-term production practices, especially environmental microorganisms [14]. However, with the current technical level, it is extremely challenging to reshape the local environmental microbial pools, suggesting that the production of *jiuyao* is inherently geography-dependent. This forms a critical theoretical foundation for the study.

To further investigate the controllable fermentation within a specific environment, *jiuyao* was used as a case study to look into (i) how to clarify the specific composition of the core microbiota, (ii) how to reconstruct a reasonable and effective synthetic microbiota to substitute *muqu*, (iii) how to ensure the appropriate succession and reproducible function of the synthetic core microbiota in complex fermentation process. Here, starting from a taxonomic and functional perspective, a set of executable criteria was proposed for identifying the core microbiota. Then the synthetic core microbiota was constructed and applied to the production of *jiuyao*, seeding yeast, and *huangjiu* (Fig. 1). The entire process was traced and compared with that of the traditional *jiuyao*. Our findings would provide a theoretical foundation for laboratory design and industry application of artificial starters for traditional fermentations.

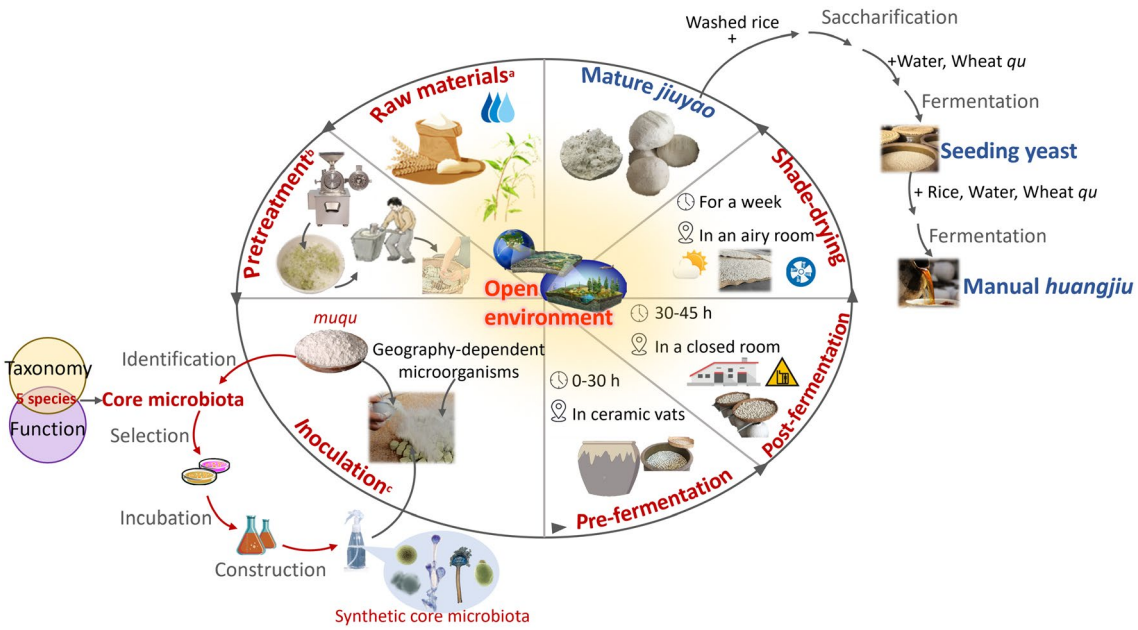


Fig. 1 The production flow chart of *jiuyao*, seeding yeast and manual *huangjiu*. [a]The raw materials of *jiuyao* include early indica rice, *Polygonum hydropiper L.* and water. [b]The pretreatment before inoculation includes grinding the rice and herb, mixing the raw materials, beating to increase viscosity and plasticity, cutting and shaping. [c]The only difference between traditional and artificial *jiuyao* production is the form of microbial inoculation. For traditional *jiuyao*, microorganisms join in the fermentation ecosystem by *muqu* (i. e. the preceding year's *jiuyao*) inoculation. For artificial *jiuyao* in this study, the cells or spores of core microorganisms are mixed and quantitatively sprayed onto the surface of raw materials. Notably, environmental microorganisms are indiscriminately involved in every stage of production, whether it is a traditional or artificial *jiuyao*

Materials and methods

Sample collection

All samples were collected under the guidance of experienced craftsmen from Guyuelongshan Industrial Park in Shaoxing, Zhejiang Province, China. On the one hand, mature *jiuyao* samples for core microbiota identification were collected every summer from 2019 to 2022. On the other hand, the production of *jiuyao* can be divided into three stages, including pre-fermentation in ceramic vats, post-fermentation in a closed room and shade-drying. Correspondingly, process *jiuyao* samples for the function verification of synthetic core microbiota were collected in the summer of 2023 when each stage started. Three biological replicates were used for analysis.

Microbial community analysis of *jiuyao* by amplicon sequencing

Total DNA was extracted by PowerSoil DNA Isolation kit (MO BIO Laboratories, USA). For bacterial community, the 16S rDNA genes were amplified using the universal primers 27F (5′-AGRGTTYGATYMTGGCTCAG-3′) and 1492R (5′-RGYTACCTTGTTACGACTT-3′). For fungal community, ITS sequences were amplified using the primers ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS4R (5′-TCCTCCGCTTATTGATATGC-3′). The amplicon sequencing was performed on the PacBio Sequel platform. All the above-mentioned procedures were completed by Biomarker Technologies Corporation (Beijing, China).

QIIME2 was used for data processing of amplicon sequencing bioinformatics [15]. After quality filtered, denoised, merged and chimera removed, sequences were clustered into Operational Taxonomic Units (OTUs) with a similarity threshold of 0.97 by UCLUST [16]. Subsequently, referring to the Silva database (v138.1) for bacteria and UNITE database (v2022.11) for fungi respectively, the representative sequences generated by clustering were annotated at the species level by Basic Local Alignment Search Tool (BLAST), with a confidence threshold of 0.9.

Biomass analysis of *jiuyao* by qPCR

Total DNA for qPCR was extracted by modified cetyltrimethyl ammonium bromide (CTAB) method [17]. OD_{260}/OD_{280} and OD_{230}/OD_{260} were measured to evaluate the quality and concentration of extracted DNA. The 16S rRNA genes of bacteria were amplified by the forward primer P1 (5′-CCTACGGGAGGCAGCAG-3′) and the reverse primer P2 (5′-ATTACCGCGGCTGCTGG-3′). And 18S rRNA genes of fungi were amplified by Y1 (5′-GCGGTAATTCCAGCTCCAATA-3′) and Y2 (5′-GCCACAAGGACTCAAGGTTAG-3′) [18]. Absolute quantification was based on the comparison of cycle threshold (Ct) with a standard curve generated from amplification of known amounts of the target gene.

Physicochemical index and enzyme activity analysis

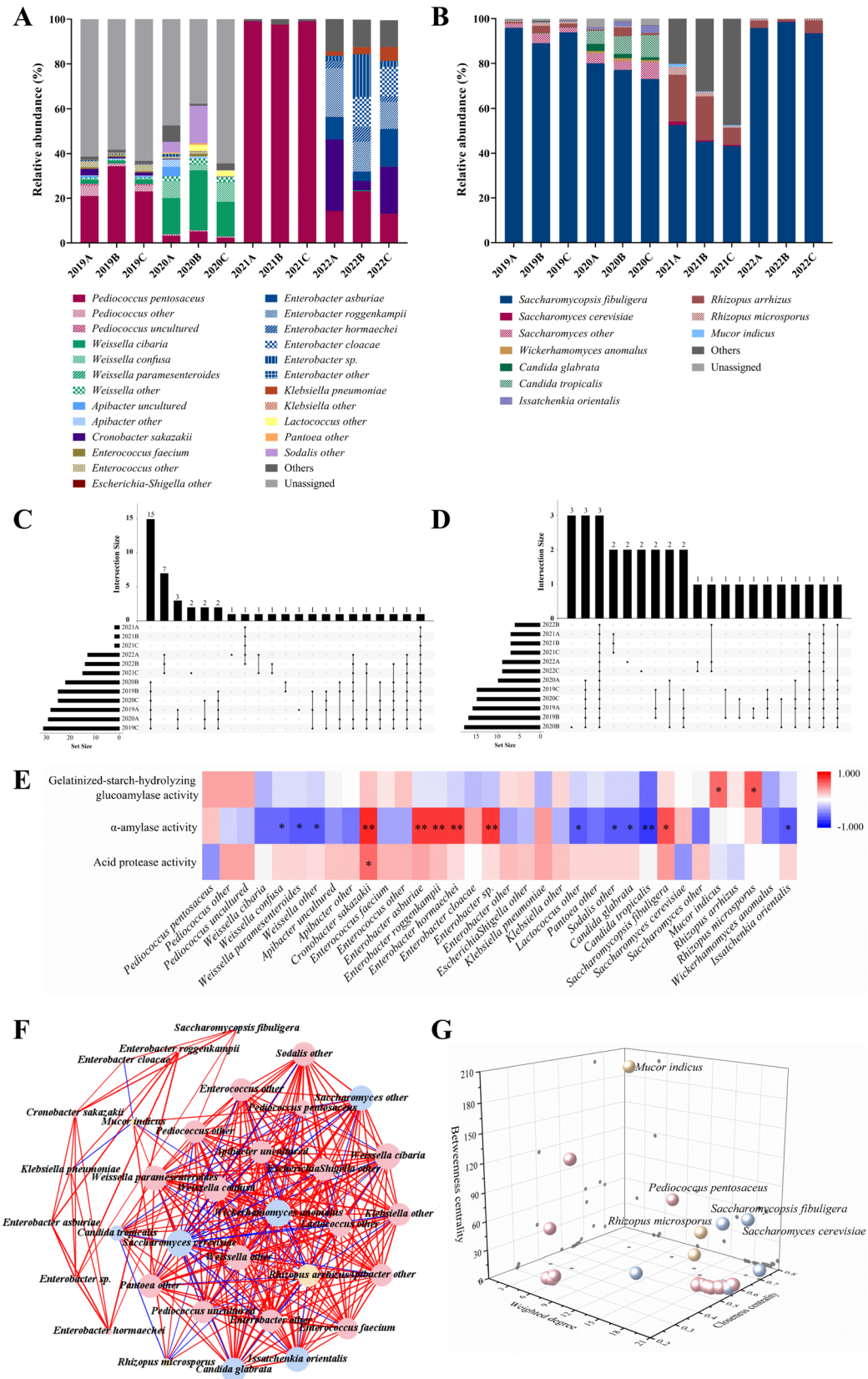
Changes in environmental temperature and humidity during *jiuyao* production were tracked by electronic thermohygrometer. Water content was determined by drying to constant weight. Acidity and ammonium nitrogen were determined through titration method [19]. The content of reducing sugar was determined through 3,5-dinitrosalicylic acid (DNS) assay. The content of alcohol was determined by biosensor analyzer [20]. Enzyme activities including gelatinized-starch-hydrolyzing glucoamylase activity (i.e. glucoamylase), raw-starch-hydrolyzing glucoamylase activity, α-amylase activity, acid protease activity and fermentation activity were measured as previously described [21, 22].

Application of core microbiota in artificial *jiuyao* production at a plant-scale

The production of traditional and artificial *jiuyao* both took place in Guyuelongshan Industrial Park in Shaoxing, Zhejiang Province, China. Their production process depicted in Fig. 1, comprises the following steps: mixing, beating, cutting, shaping, inoculation, fermentation and shade-drying. The only distinction between traditional and artificial *jiuyao* production lies in the form of microbial inoculation. For traditional *jiuyao*, microorganisms join in the fermentation ecosystem by *muqu* inoculation. For artificial *jiuyao* in this study, the inoculum size of each core microorganism was firstly determined based on the microbial biomass and relative abundance in *muqu*. After the microbial cells and spores were cultivated, centrifuged, and washed with sterile water, they were evenly sprayed onto the surface of *jiuyao* in measured amounts. Due to the nature of open fermentation, the succession of the microbiota in *jiuyao*, both traditional and artificial, is greatly dependent on local environmental factors, such as room temperature and humidity, initial microbiota in raw materials and production environment. To ensure successful fermentation, the process parameters are carefully and selectively adjusted under the guidance of experienced craftsmen.

Application of *jiuyao* in *huangjiu* production at a lab-scale

The production of manual *huangjiu* was carried out in laboratory and was divided into several steps: rice soaking,


A
Relative abundance (%)
2019A
2019B
2019C
2020A
2020B
2020C
2021A
2021B
2021C
2022A
2022B
2022C
Pediococcus pentosaceus
Pediococcus other
Pediococcus uncultured
Weissella cibaria
Weissella confusa
Weissella paramesenteroides
Weissella other
Apibacter uncultured
Apibacter other
Cronobacter sakazakii
Enterococcus faecium
Enterococcus other
Escherichia-Shigella other
Enterobacter asburiae
Enterobacter roggenkampii
Enterobacter hormaechei
Enterobacter cloacae
Enterobacter sp.
Enterobacter other
Klebsiella pneumoniae
Klebsiella other
Lactococcus other
Pantoea other
Sodalis other
Others
Unassigned
B
Relative abundance (%)
Saccharomycopsis fibuliger
Saccharomyces cerevisiae
Saccharomyces other
Wickerhamomyces anomalus
Candida glabrata
Candida tropicalis
Issatchenkia orientalis
Rhizopus arrhizus
Rhizopus microsporus
Mucor indicus
Others
Unassigned
C
Intersection Size
Set Size
D
Intersection Size
Set Size
E
Gelatinized-starch-hydrolyzing glucoamylase activity
α-amylase activity
Acid protease activity
1.000
-1.000
F
Saccharomycopsis fibuliger
Enterobacter roggenkampii
Enterobacter cloacae
Sodalis other
Enterococcus other
Saccharomyces other
Pediococcus pentosaceus
Cronobacter sakazakii
Mucor indicus
Pediococcus other
Apibacter uncultured
Weissella cibaria
Klebsiella pneumoniae
Weissella paramesenteroides
Escherichia-Shigella other
Weissella confusa
Wickerhamomyces anomalus
Klebsiella other
Enterobacter asburiae
Candida tropicalis
Lactococcus other
Saccharomyces cerevisiae
Weissella other
Rhizopus arrhizus
Enterobacter other
Enterobacter sp.
Pantoea other
Pediococcus uncultured
Enterobacter other
Enterobacter hormaechei
Enterococcus faecium
Issatchenkia orientalis
Rhizopus microsporus
Candida glabrata
G
Mucor indicus
Betweenness centrality
Pediococcus pentosaceus
Saccharomycopsis fibuliger
Rhizopus microsporus
Saccharomyces cerevisiae
Weighted degree
Closeness centrality

◂**Fig. 2** The identification of core microbiota in *jiuyao*. Bacterial (**A**) and fungal (**B**) community structure in *jiuyao*, only microorganisms occurring in more than 6/12 of the samples or with an average relative abundance greater than 1% are shown separately, otherwise they will be grouped into "others". 2019A, 2019B and 2019C refer to three parallel samples of jiuyao of 2019, other years are expressed as the same way; Upset plots of bacteria (**C**) and fungi (**D**), there were one bacterium (*Pediococcus pentosaceus*) and three fungi (*Saccharomycopsis fibuligera*, *Rhizopus arrhizus* and *Rhizopus microsporus*) present in all 12 samples; **E** Heat map based on spearman correlation coefficient between microorganisms and enzyme activities; **F** Microbial co-occurrence network based on spearman correlation coefficient ($|r|>0.5$, $p<0.05$), red and blue edges indicate positive and negative interactions respectively, the size of nodes and the thickness of edges are positively correlated with degree and correlation coefficients respectively; **G** The distribution of microorganisms based on centrality, and the greater the connectivity centrality, closeness centrality and betweenness centrality, the more important the node is

steaming, spontaneous cooling, starters addition, and fermentation. The fermentation was conducted in a 5 L container. A total of 1500 g of rice, 153 g of raw wheat *qu*, 114 g of seeding yeast and 1 L of water were mixed, and then pre-fermented at 28 ℃ for 5 days, followed by post-fermentation at 15 ℃ for 15 days to simulate winter-brewing in practical production. As a hub of yeasts, seeding yeast plays a critical role in manual *huangjiu* production. For its production, a certain mass of glutinous rice was soaked, washed and steamed, then washed with cold boiled water. Then it was mixed with 0.5% (w/w) *jiuyao* powder. The saccharification was carried out at 28 ℃ for 2 days, and the mixture was further mixed with 15% (w/w) raw wheat *qu* and 110% (w/w) water, followed by pre-fermentation at 28 ℃ for 3 days and post-fermentation at 15 ℃ for 12 days.

High Performance Liquid Chromatography (HPLC) was used to analyze the content of organic acids and amino acids in *huangjiu* according to the report by Wang et al. with minor modifications [23]. After *huangjiu* samples were centrifuged, the supernatants were collected. The protein was removed by 10% (w/v) trichloroacetic acid and 0.22 μm microporous membrane. For organic acids, Waters e2695 equipped with a SB-C18 Analytical (4.6 mm × 250 mm, 5 μm) column was uesd, and the detection wavelength was 210 nm. For amino acids, pre-column derivatization and Agilent 1100 equipped with a ODS HYPERSIL (4.6 mm × 250 mm, 5 μm) were used. The speed of flow was set at 1.0 mL/min and the detection wavelength was set at 338 nm and 262 nm.

The extraction of main higher alcohols in *huangjiu* was performed by dispersive liquid–liquid microextraction (DLLME) [24]. 1 mL acetonitrile (disperse solvent), 600 μL dichloromethane (extraction solvent) and 50 μL 4-methyl-2-pentanol solution in ethanol (0.4536 g/L, internal standard) were mixed with 7 mL *huangjiu* samples. Volatile flavor compounds were extracted by headspace solid phase microextraction (HS-SPME) according to the previous reports [25]. 2-octanol (0.1018 mg/L) was taken as the internal standard. Then the quantification of higher alcohols and volatile flavor compounds was performed by gas chromatography-mass spectrometry (GC–MS) with a TG-WAXMS (30 m × 0.25 mm, 0.25 μm) column.

Statistical analysis

One-way analysis of variance (ANOVA) based on Waller-Duncan's test and independent sample t test was carried out using IBM SPSS Statistics 26.0 (IBM Corp., Armonk, NY, USA) to check statistical significance. Spearman correlation coefficient (r) and p values were computed to reveal the relationships between potentially core microorganisms and enzyme activities, as well as the relationships among microorganisms. Origin 2023 64Bit (OriginLab Corp., Northampton, MA, USA) and GraphPad Prism 8 (GraphPad Software, Boston, Massachusetts USA) were used to visualize the microbial community structure, physicochemical indexes, enzyme activities and flavor profiles. A highly correlation coefficient ($|r|>0.5$, $p<0.05$) was filtrated, and co-occurrence network visualization and analysis were performed on Gephi 0.9.7. Orthogonal projections to latent structures discriminant analysis (OPLS-DA) was performed using OmicShare (http://www.omicshare.com/tools). Each experiment was performed in triplicate.

Results and discussion

Identification of core microbiota based on taxonomy and function

The microbial community structures of mature *jiuyao* samples were analyzed by single-molecule sequencing in real time (SMRT sequencing). Before visualization, microorganisms that appeared in less than 6/12 of the samples with an average relative abundance of less than 1% were grouped into the "others" category. As can be seen in Fig. 2A and C *P. pentosaceus* was detected in all 12 samples with an average relative abundance of 36.27%. Bacterial diversity was higher in *jiuyao* of 2019, 2020 and 2022, with lactic acid bacteria represented by *Weissella* and *Pediococcus* dominating the *jiuyao* of 2019 and 2020. Moreover, a large number of *Enterobacteriaceae*, most likely introduced from the environment, were detected in the *jiuyao* of 2022. For fungi (Fig. 2B and D), *S. fibuligera* was detected in every sample with a relative abundance ranging from 43.14% to 98.55%. In addition, *S. cerevisiae*, *M. indicus*, *R. arrhizus*, and *R. microsporus* were present in more than 10/12 samples, despite their low relative abundance. In terms of flavor-related yeasts, such as *Issatchenkia orientalis* and *Wickerhamomyces anomalus* [26], their distribution varied

considerably between samples. Overall, from a taxonomic perspective, we focused primarily on the distribution frequency of microorganisms, rather than their relative abundance, as certain microorganisms with very low abundance can also make a difference to the overall microbial community [27].

Based on correlation analysis, the core microbiota was further explored and identified from a functional perspective. During the fermentation of *jiuyao*, microorganisms metabolize to produce abundant enzymes, thus determining the quality of *jiuyao*. The gelatinized-starch-hydrolyzing glucoamylase, α-amylase and acid protease activities of *jiuyao* were listed in Table 1, suggesting that enzyme activities varied greatly between samples, which was a result of unstandardized manual process. The heat map (Fig. 2E) showed that there were significant positive correlations between gelatinized-starch-hydrolyzing glucoamylase activity and *M. indicus* ($\rho = 0.577$, $p = 0.049$) as well as *R. microsporus* ($\rho = 0.601$, $p = 0.039$). Some *Enterobacteriaceae*, *S. fibuligera*, *S. cerevisiae* and *R. arrhizus* were also slightly positively correlated with gelatinized-starch-hydrolyzing glucoamylase activity. In addition, *S. fibuligera* ($\rho = 0.678$, $p = 0.015$) and *Enterobacteriaceae* represented by *Cronobacter sakazakii* ($\rho = 0.804$, $p = 0.002$) and *Enterobacter* significantly contributed to α-amylase activity. As for acid protease activity, most bacteria and yeasts here were positively correlated with it. However, considering that *Enterobacteriaceae* are pathogenic and were only observed in the samples of 2022, they were excluded from the core microbiota.

The microbial co-occurrence network based on spearman correlation coefficient ($|r| > 0.5$, $p < 0.05$) included 34 effective nodes and 288 edges (Fig. 2F), which could reveal potential microbial interactions, where the key nodes often represent species who play an important role in maintaining the stability of microbial community [28, 29]. As can be seen in Fig. 2G, the core microbiota was identified based on the weighted degree, closeness centrality and betweenness centrality of nodes [30, 31]. *S. cerevisiae* demonstrated a closeness centrality of 0.70, indicating its central role within the network and the interactions associated with it were rarely dependent on other intermediate nodes. And *M. indicus* acted as a bridge between nodes, with the highest betweenness centrality of 200.50. Notably, nodes with high betweenness centrality do not necessarily have a high connectivity degree [32].

Combining the distribution of microorganisms in 12 samples, their contribution to the enzyme activities of *jiuyao*, as well as their role in the stabilization of microbial communities, *P. pentosaceus*, *S. fibuligera*, *S. cerevisiae*, *M. indicus* and *R. microsporus* were identified as the potential core microbiota in *jiuyao*. Specifically, *M. indicus*, *R. microsporus* along with *S. fibuligera* supply available small-molecule substrates for the growth of other microorganisms by secreting hydrolases [33], and *S. cerevisiae* is accountable for ethanol production. In addition, *P. pentosaceus*, as a lactic acid bacteria, can not only create an acidic fermentation microenvironment to prevent the colonization of miscellaneous organisms, but is also conducive to the formation of flavor substances [34]. To conclude, each of the aforementioned five microorganisms performs its unique duties and is indispensable for the establishment of *jiuyao* micro-ecosystem.

Construction of synthetic microbiota for *jiuyao* production

In the production of traditional *jiuyao*, the preceding year's *jiuyao* was utilized as *muqu*. Specifically, microorganisms in mature *jiuyao* of 2022 were inoculated onto the surface of new *jiuyao* of 2023. To increase the comparability between traditional and artificial *jiuyao*, we determined the size of each core microorganism in the synthetic microbiota based on their biomass and relative abundance in *jiuyao* of 2022. The concentration ranges of bacteria and fungi standards used in this procedure were 3.76×10^2 to 6.34×10^6 and 1.54×10^3 to 9.06×10^6 copies/μL respectively (Supplementary Fig. 1). The linear fits of the standard curves all met the requirement, and the amplification efficiencies were all within the range of 90–110%, indicating that both standard curves could be used for quantitative analysis of the bacterial and fungal biomass in *jiuyao*.

To clarify the absolute content of each core microorganism in *muqu*, the biomass was further calculated based on the results of SMRT sequencing and qPCR (Table 2). Correspondingly, bacteria, yeasts and mold spores were

Table 1 Enzyme activities of mature *jiuyao* in four years

	2019A[a]	2019B	2019C	2020A	2020B	2020C	2021A	2021B	2021C	2022A	2022B	2022C
Gelatinized-starch-hydrolyzing glucoamylase activity (U/g)	411	459.94	480.51	111.53	108.79	136.59	239.66	172.31	307.01	424.13	395.76	448.63
α-amylase activity (U/g)	1.57	1.5	1.34	0.51	0.32	0.58	1.05	0.87	1.23	1.76	1.69	2.02
Acid protease activity (U/g)	137.91	175.74	165.01	131.08	132.55	133.01	39.48	27.57	51.39	202.57	197.36	181.84

[a]2019A, 2019B and 2019C refer to three parallel samples of *jiuyao* of 2019, other years are expressed as the same way

Table 2 The composition of synthetic microbiota for artificial *jiuyao* production

	Biomass[a] (CFU/g *muqu*)	Strain	Average Relative Abundance[b] (%)	Calculated Inoculum[c] (CFU/kg rice flour)	Inoculum size[d] (μL/kg rice flour)
Bacteria	5.62×10^8	*Pediococcus pentosaceus*	16.83	2.65×10^9	1000
Fungi	3.61×10^7	*Saccharomycopsis fibuligera*	95.95	9.70×10^8	1000
		Saccharomyces cerevisiae	0.08	8.46×10^5	1
		Mucor indicus	0.02	2.20×10^5	49
		Rhizopus microsporus	0.44	4.45×10^6	1000

[a]The biomass was determined according to the standard curves obtained by qPCR

[b]The average relative abundance of microorganism was determined by amplicon sequencing of mature *jiuyao* of 2022

[c]The raw material for traditional *jiuyao* usually includes 1 kg early indica rice flour, 8.25 g *Polygonum hydropiper* L., 555 mL water and 28 g *muqu*. The microbial inoculum was determined based on the addition of *muqu*, biomass and relative abundance. For instance, the calculated inoculum of *Pediococcus pentosaceus* (2.65×10^9 CFU/kg rice flour) = *muqu* addition (28 g/kg rice flour) × bacterial biomass (5.62×10^8 CFU/g *muqu*) × average relative abundance (16.83%)

[d]Bacteria, yeast and mold spores were diluted to 2.65×10^9, 9.70×10^8 and 4.45×10^6 CFU/mL respectively. And the microbial inoculum size was determined based on calculated inoculum and concentration after dilution

respectively diluted to the specific concentration and then mixed by volume. A total volume of 3050 μL of mixed microbial suspension was obtained and sprayed as the starter for 1 kg of indica rice flour.

Once the above mixed microbial suspension was sprayed into the *jiuyao* system, it indicated that the core microbiota had entered a fully open and non-sterile environment. A SourceTracker analysis on *daqu* microbiota revealed that the fungal communities predominantly originated from tools and indoor floors, whereas raw materials are the main sources of bacterial communities [35]. We therefore hypothesized that the assembly and succession of microbial community during *jiuyao* fermentation are inevitably affected by endogenous and exogenous environmental factors, such as temperature and humidity in the workshop, bioheat, and the interactions between microorganisms originating from raw materials, tools, air and inoculated species [3]. Moreover (Supplementary Fig. 2), given the unpredictable insitu metabolic activity, unrecognized microbial interactions, and unsustainable function stability of the synthetic microbiota based on "bottom-up" assembly in *jiuyao* system, we integrated the concept of "top-down" self-assembly, allowing geography-dependent climate, water, soil, microbiota and among other factors to help establish the *jiuyao* micro-ecosystem [36, 37].

Comparison of microbial diversity and biomass during *jiuyao* production

Similar to traditional *jiuyao*, the production of artificial *jiuyao* can also be divided into three stages: pre-fermentation, post-fermentation and shade-drying. Considering the microbial colonization and succession in *jiuyao* is a relatively long process, we compared the microbial communities in traditional and artificial *jiuyao* at the beginning of pre-fermentation (S1), the end of pre-fermentation (S2), the end of post-fermentation (S3), and the end of shade-drying (S4).

As shown in Fig. 3A, the initial bacterial communities of both traditional and artificial *jiuyao* were dominated by *P. pentosaceus*, the relative abundance of which was 93.01% ± 3.96% and 88.59 ± 5.32% respectively. And there was a trace level of *Enterobacteriaceae*, most likely introduced from raw materials, tools and environment. As fermentation developed, the bacterial diversity increased significantly due to the colonization of environmental microorganisms, with *Enterobacter* taking up a large proportion. At the end of post-fermentation, *W. cibaria* was detected in all samples, potentially introduced via the bamboo baskets used in the post-fermentation period. *W. cibaria*, as an emerging lactic acid bacteria with promising health benefits, has been widely found in *daqu*, kimchi, fermented batter and fermented sausages [38–41]. When shade-drying ended, the relative abundances of *P. pentosaceus* in traditional and artificial *jiuyao* were 16.83 ± 1.84% and 13.32 ± 1.65% respectively, showing no statistically significant difference. And differences in bacterial community were mainly reflected in the composition and content of other lactic acid bacteria and *Enterobacteriaceae*.

Figure 3B illustrates the succession of fungal community at the species level. In both traditional and artificial *jiuyao*, a fungal community composed of *S. fibuligera*, *S. cerevisiae*, *M. indicus*, *R. arrhizus* and *R. microsporus* can be found at each stage, among which *S. fibuligera* was always absolutely dominant. For artificial *jiuyao*, the relative abundance of *S. fibuligera* ranged between 93.13 and 98.96%, and for traditional *jiuyao*, it ranged between 91.40 and 99.53%. While other microorganisms, such as *Candida tropicalis* and *Fusarium*, were present with a very low relative abundance at the beginning of the fermentation, and

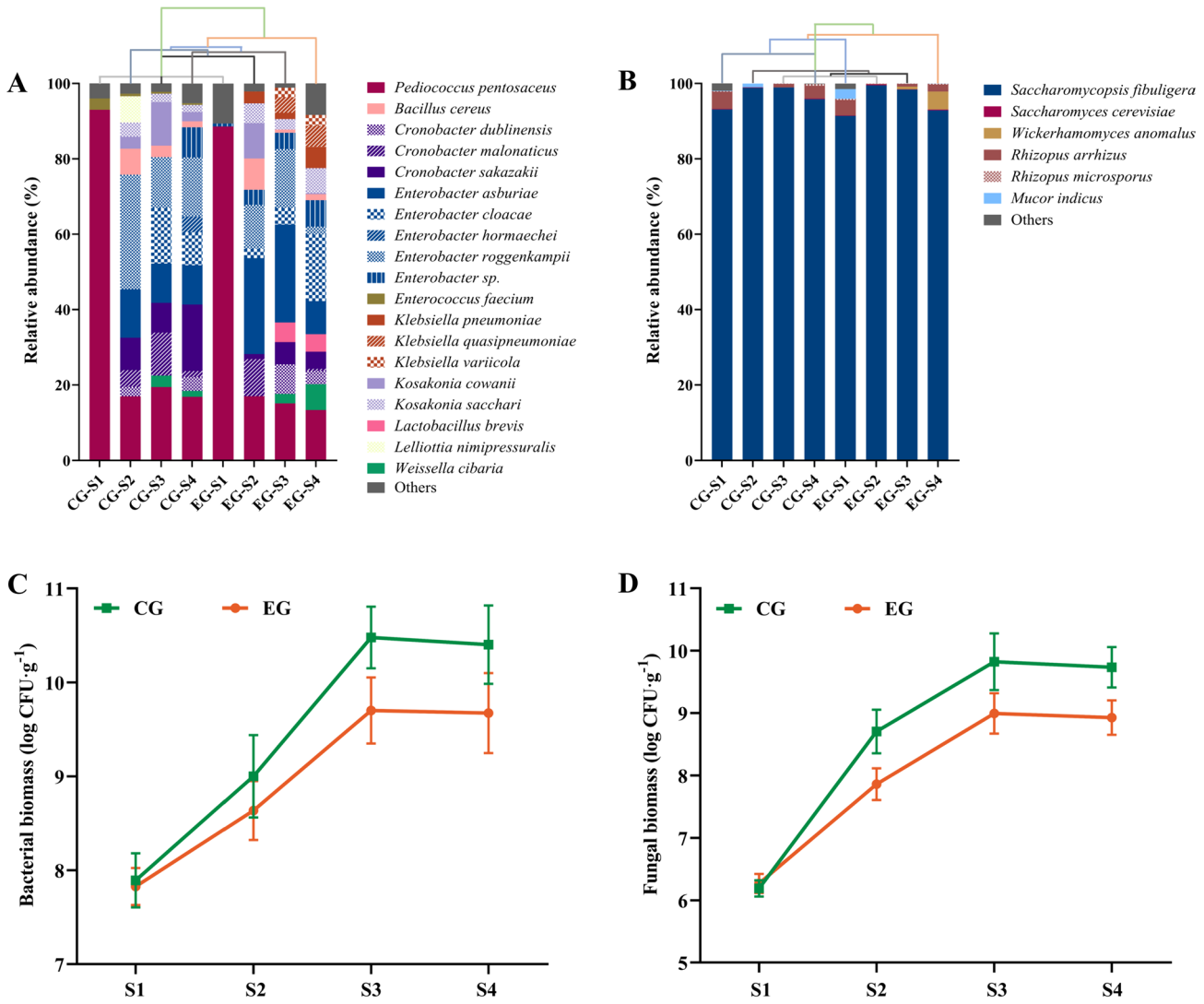


Fig. 3 Comparison of microbial diversity and biomass during *jiuyao* production. Bacterial (**A**) and fungal (**B**) community structure in traditional and artificial *jiuyao* of different production stages; Dynamic changes of bacterial (**C**) and fungal (**D**) biomass during *jiuyao* production. CG and EG refer to traditional *jiuyao* and artificial *jiuyao* respectively. S1, S2, S3 and S4 refer to the beginning of the pre-fermentation, the end of the pre-fermentation, the end of the post-fermentation, and the end of the shade-drying

the internal environment containing ethanol, organic acids, sesquiterpenes and flavonoids filtered them out later [42]. Given the fact that we had never artificially inoculated *R. arrhizus* to artificial *jiuyao*, it can be speculated that *R. arrhizus* spontaneously entered from the workshop environment and participated in the self-assembly of the microbial community throughout the whole fermentation process of *jiuyao*, which was identified as the core microorganism in a previous study [9].

The changes in biomass (Fig. 3C and D) suggested that microbial growth and multiplication mainly occurred in the pre-fermentation and post-fermentation periods, and that microorganisms were less active and basically dormant or even dead during the shade-drying period due to the reduction in the water content. Moreover, it is noteworthy that the change trend of biomass during both traditional and artificial *jiuyao* fermentation was consistent. The biomass of artificial *jiuyao* was lower than that of traditional *jiuyao* when production finished, but there was no statistically significant difference.

Given the specific production environment, it can be inferred that the assembly of the microbial community in *jiuyao* was dominated by the deterministic process [43]. Despite the initial inoculation of different microorganisms, their fermentation processes were both affected by interspecies competition and environmental filtration. Reflected in the ultimate microbial community structure, difference between traditional and artificial *jiuyao* was found in the

composition of non-core microbiota, whereas the core microbiota was substantially identical in terms of composition and relative abundance, which theoretically ensured the saccharification and fermentation function of *jiuyao* [44].

Comparison of physicochemical indexes and enzyme activities during *jiuyao* production

Considering the properties of open fermentation, the quality of *jiuyao* is inevitably affected by environmental factors, and the surroundings will also receive the feedback from fermentation process, therefore monitoring environmental changes is crucial to ensure product quality [45]. Here the changes in microenvironmental temperature and humidity during the main fermentation process (i.e., pre-fermentation and post-fermentation) of traditional (Fig. 4A) and artificial (Fig. 4B) *jiuyao* were tracked. The microenvironmental temperature and humidity of the two showed the same change trend, first increasing and then decreasing during both the pre-fermentation and post-fermentation periods. Concretely speaking, the pre-fermentation was carried out in ceramic vats covered with lids and sacks, and the warm and wet environment was in favor of the rapid growth of filamentous fungi, thus the temperature and humidity reached the first peak (traditional *jiuyao*: the 17th hour, 34.5 °C, 92.7%; artificial *jiuyao*: the 23rd hour, 34.7 °C, 93.6%). Then the sacks and lids were successively removed, and the temperature and humidity subsequently dropped, where the temperature returning to room temperature was one of the signs of the end of the pre-fermentation. The semi-finished *jiuyao* were transferred to a closed workshop for post-fermentation, and the temperature and humidity reached the second peak due to the vigorous growth and metabolism of bacteria (traditional *jiuyao*: the 33rd hour, 37.7 °C, 84.9%; artificial *jiuyao*: the 35th hour, 38.1 °C, 84.5%). During this period, *jiuyao* was turned over every two hours to ensure that the temperature did not exceed 38 °C, and the windows and doors were opened to ventilate in the later period of post-fermentation. However, the fermentation of the artificial *jiuyao* took place with a slight delay compared to traditional *jiuyao*, with the first peak of artificial *jiuyao* occurring six hours later than that of traditional *jiuyao*, but the interval between the two peaks of artificial *jiuyao* was shorter than that of the traditional *jiuyao*.

The physicochemical indexes of *jiuyao* are important for evaluating its quality. As illustrated in Fig. 5A, the bio-heat generated by microbial metabolism promoted water evaporation, and the water content of both groups of *jiuyao* decreased progressively from about 41% at the beginning of pre-fermentation to about 20% at the end of post-fermentation. After shade-drying, the water contents of traditional and artificial *jiuyao* decreased to $12.74 \pm 0.15\%$ and $12.70 \pm 0.08\%$ respectively, where most microbial metabolism was inhibited, which was conducive to the long-term storage of *jiuyao*. For acidity (Fig. 5B), it showed an increasing and then decreasing trend. The heat and moisture insulating environment in the pre-fermentation period was favorable to the growth and reproduction of acid-producing microorganisms [46], and the rapid accumulation of organic acids led to a rise in acidity, which could not only provide a microenvironment for the fermentation of *jiuyao* by inhibiting the growth of undesirable microorganisms, but increase the flavor substances in the brewing process of *huangjiu* [47]. However, due to the volatilization and esterification, the total acid in traditional and artificial *jiuyao* finally fell back to 9.16 ± 1.36 and 9.90 ± 1.82 g/kg respectively.

Enzyme activities are the most intuitive indicators for evaluating the performance of *jiuyao* as a saccharifying and fermenting starter. Gelatinized-starch-hydrolyzing glucoamylase (Fig. 5C), raw-starch-hydrolyzing glucoamylase (Fig. 5D) and α-amylase (Fig. 5E) are collectively known as amylase, and the synergy of the three is crucial

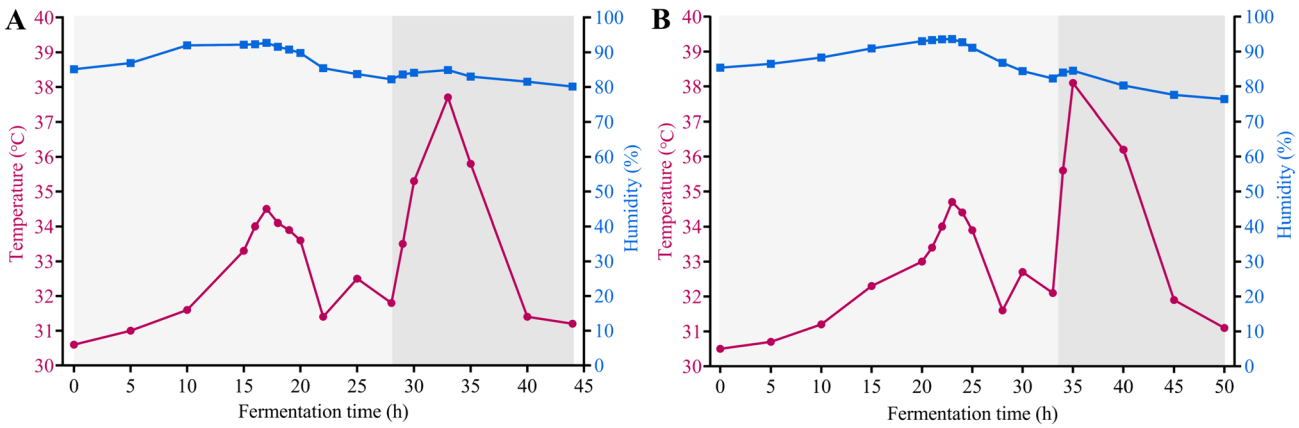


Fig. 4 Changes in microenvironmental temperature and humidity during the main fermentation process of traditional (**A**) and artificial *jiuyao* (**B**)

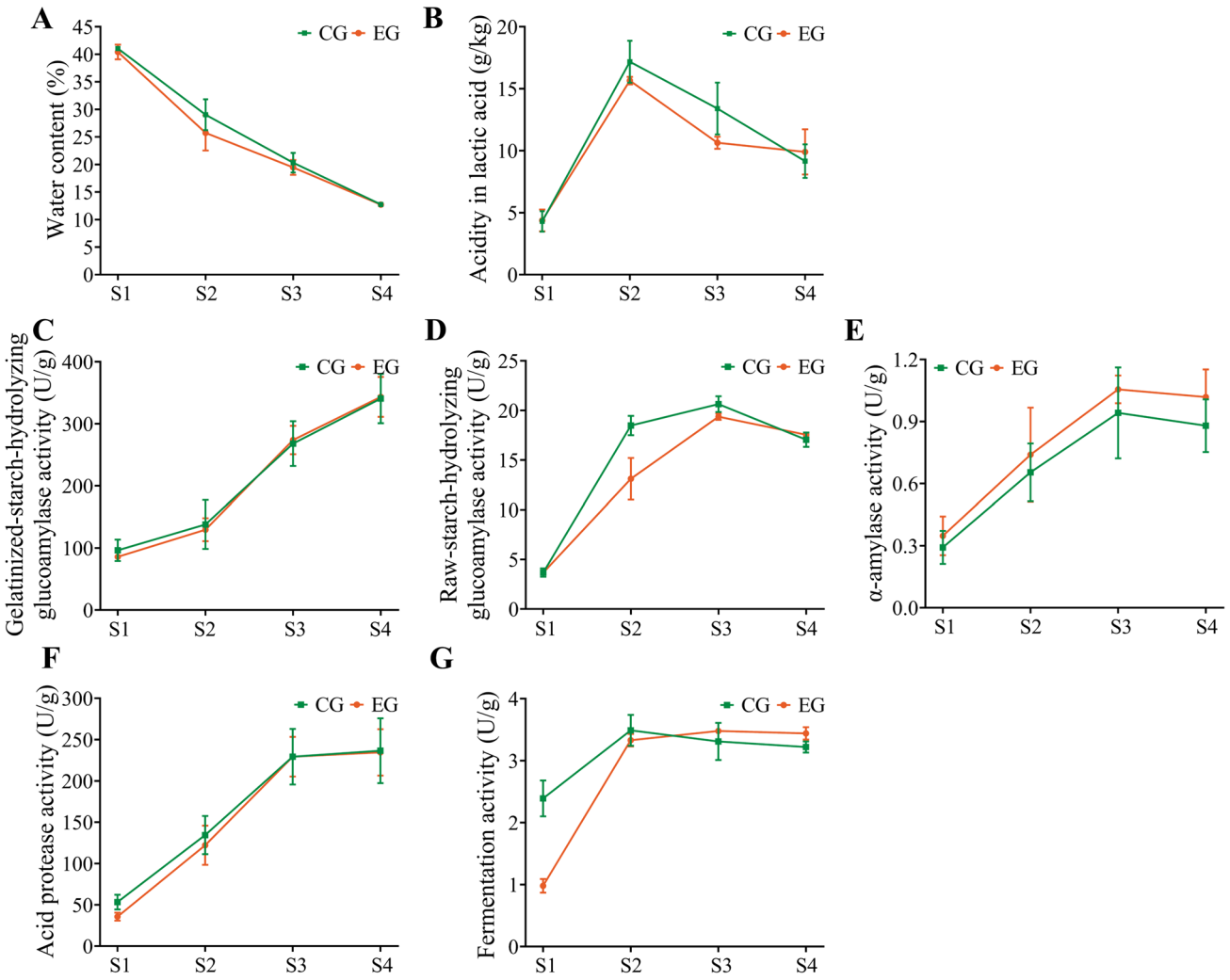


Fig. 5 Changes in water content (**A**), acidity (**B**), gelatinized-starch-hydrolyzing glucoamylase activity (**C**), raw-starch-hydrolyzing glucoamylase activity (**D**), α-amylase activity (**E**), acid protease activity (**F**) and fermentation activity (**G**) during the production process of traditional (CG) and artificial *jiuyao* (EG)

to improve raw material utilization and alcohol yield [48]. Gelatinized-starch-hydrolyzing glucoamylase continued to accumulate throughout the production period and its activity could reach 340.59 ± 39.80 and 343.28 ± 32.27 U/g for traditional and artificial *jiuyao* when shade-drying ended respectively. Whereas the accumulation of raw-starch-hydrolyzing glucoamylase and α-amylase mainly occurred during the main fermentation period and were partially inactive during the shade-drying period. Acid protease activity also rose steadily in both traditional and artificial *jiuyao* before shade-drying (Fig. 5F), the hydrolysis products of which including amino acids and polypeptides, are not only essential nutrients for microorganisms, but key precursors for flavor formation in *huangjiu* brewing [49]. Fermentation activity refers to the ability to ferment sugar to alcohol. As can be seen in Fig. 5G, the fermentation activity of *jiuyao* increased rapidly during the pre-fermentation period, and remained stable afterward. Although the fermentation activity of artificial *jiuyao* was far lower than that of the traditional one at the beginning of fermentation, the situation reversed at the end of shade-drying (3.22 ± 0.09 U/g for traditional *jiuyao* and 3.44 ± 0.10 U/g for artificial *jiuyao*).

In general, all indexes of both traditional and artificial *jiuyao* showed almost the same change trend during production, and there were no significant differences in any of the indexes except that the fermentation activity of artificial *jiuyao* was significantly higher than that of traditional *jiuyao* ($p = 0.048$) when shade-drying ended. To summarize, both traditional and artificial *jiuyao* complied with the standard of a saccharifying and fermenting starter for manual *huangjiu* production, further supporting the rationality and validity

of the aforementioned identification and construction of the core microbiota.

Comparison of physicochemical indexes during seeding yeast production

The microorganisms in seeding yeast originate predominantly from *jiuyao*, followed by wheat *qu*, other raw materials and the environment. The fermentation of seeding yeast is essentially a process of enriching functional microorganisms, especially yeasts. The quality of seeding yeast can comprehensively reflect the quality of *jiuyao*, and meanwhile partly determine the quality of manual *huangjiu*. To further evaluate and compare the application effect of traditional and artificial *jiuyao*, we tracked the changes in physicochemical indexes during seeding yeast fermentation (Fig. 6), finding that the two groups showed a similar change trend in each physicochemical index.

The seeding yeast production process can be divided into three stages, namely saccharification, pre-fermentation and post-fermentation. The first 48 h after mixing washed rice and *jiuyao* is called the saccharification period, when the amylase and protease introduced by *jiuyao* played a hydrolyzing role, and the contents of alcohol (Fig. 6A), reducing sugar (Fig. 6B), acid (Fig. 6C) and ammonium nitrogen (Fig. 6D) of fermentation broth all increased. During this, due to the hydrolysis of starch being much more intense than the consumption of reducing sugar, reducing sugar accumulated rapidly and reached the peak of the whole fermentation process at the 48th hour (112.91 ± 4.03 g/L in CG and 106.71 ± 10.55 g/L in EG). When the fermentation broth reached a certain height, water and wheat *qu* were added to the fermentation system, and the fermentation consequently shifted from a solid to a semi-solid state. With the rapid growth and reproduction of yeasts, especially *S. cerevisiae*, massive reducing sugar were fermented into alcohol, and the alcohol contents of the two groups were both higher than 16% (v/v) at the end of pre-fermentation. Additionally, the acidity and ammonium nitrogen content decreased sharply within the first 24 h of pre-fermentation (the dilution

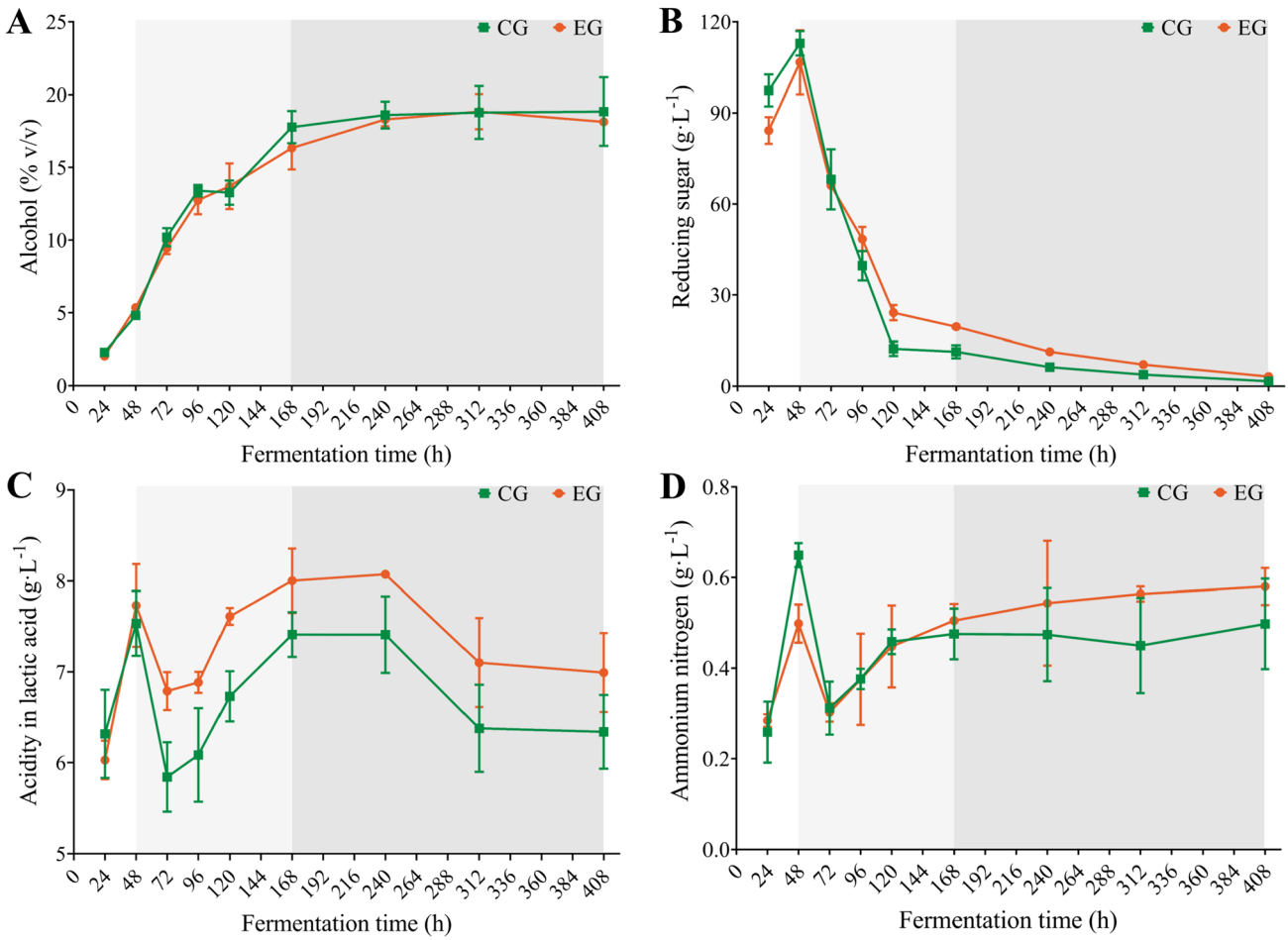


Fig. 6 Changes in the content of alcohol (**A**), reducing sugar (**B**), acid (**C**) and ammonium nitrogen (**D**) during the fermentation of seeding yeast inoculated with traditional (CG) and artificial *jiuyao* (EG) respectively

effect of the water might be responsible), and then gradually increased, which further promoted the colonization of yeasts and the formation of flavor substances. Post-fermentation was performed at a relatively low temperature, at which the microbial metabolism was weakened and the physicochemical indexes were stable. Notably, the acidity was slightly decreased due to the involvement of certain organic acids in the synthesis of esters.

To summarize, the fermentation performances of seeding yeasts inoculated with traditional and artificial *jiuyao* were at the same level, with no significant difference in any physicochemical indexes when fermentation finished. These findings confirmed that the artificial *jiuyao* based on the core microbiota inoculation was almost identical to traditional *jiuyao* from the perspective of the production practice.

Comparison of physicochemical indexes and flavor characteristics of *huangjiu*

To further elucidate that there was no difference between traditional and artificial *jiuyao*, the physicochemical indexes and flavor characteristics of *huangjiu* were analyzed and compared.

As shown in Table 3, there was no significant difference in the contents of alcohol, reducing sugar, acid and ammonium nitrogen between the two groups of *huangjiu* when fermentation finished, and both met the requirements of GB/T 17946–2008. In the production of all alcoholic beverages, the conversion from fermentable sugars to alcohol is always the key process, thus the alcohol content can most intuitively reflect whether the fermentation process is normal. Even though a variety of microorganisms have the ability to produce alcohol and influence the sensory properties of the final product, *S. cerevisiae* always dominates [50]. As in the case of the seeding yeasts, the alcohol of *huangjiu* brewed with artificial *jiuyao* was slightly lower than that brewed with traditional *jiuyao*, while the reducing sugar was the opposite, which may be related to the lower biomass of *S. cerevisiae* and higher amylase activity in the artificial *jiuyao*. Furthermore, the average ammonium nitrogen of both groups of *huangjiu* exceeded 0.6 g/L, mainly benefited by the hydrolysis of rice protein by protease, and yeast autolysis [51, 52].

Table 3 The physicochemical indexes of manual *huangjiu* fermented by seeding yeast inoculated with traditional (CG) and artificial *jiuyao* (EG) respectively

	Alcohol (% v/v)	Reducing sugar (g/L)	Acidity in lactic acid (g/L)	Ammonium nitrogen (g/L)
CG	16.37 ± 0.51^a	9.71 ± 0.32^a	6.81 ± 0.46^a	0.61 ± 0.07^a
EG	15.97 ± 0.40^a	10.36 ± 0.98^a	7.00 ± 0.19^a	0.63 ± 0.03^a

The same letter in the same column indicates no significant difference among groups ($p > 0.05$)

The flavor of Shaoxing *huangjiu* is primarily manifested in two aspects: taste and aroma characteristics. Organic acids and free amino acids determine the taste characteristics, while volatile flavor compounds determine the aroma characteristics to a large extent. Organic acids, mainly from raw materials (soaked rice and seeding yeast), microbial metabolism, as well as alcohol and aldehydes oxidation, are essential to the sensory, nutritional and safety attributes of *hungjiu* [53, 54]. As shown in Fig. 7A, lactic acid and acetic acid constituted more than 70% of the total organic acids in *huangjiu* and might play a role in buffering the taste. The total amount of organic acids in *huangjiu* brewed with artificial *jiuyao* (6.71 ± 0.35 g/L) was slightly lower than that brewed with traditional *jiuyao* (7.73 ± 0.66 g/L), but there was no significant difference. Instead, the difference between the two groups of *huangjiu* was mainly reflected in the content of lactic acid and succinic acid, which might result in the slightly weaker mellow aftertaste of *huangjiu* brewed with artificial *jiuyao*. Additionally, free amino acids in *huangjiu* are not only the nitrogen sources for brewing microorganisms, but key precursors for the synthesis of a variety of flavor substances [49]. There is the widest variety and richest content of amino acids in *huangjiu* among all alcoholic beverages, with more than 20 kinds of amino acids detected, including all essential amino acids [55]. As can be seen in Fig. 7B, the total amount of amino acids in *huangjiu* brewed with traditional and artificial *jiuyao* fluctuated between 3.62–4.11 and 3.58–4.49 g/L respectively, with 17 kinds of amino acids identified (the multi-element mixed standard solution for HPLC contained only 17 kinds of amino acids, implying that there might be more kinds of amino acids in *huangjiu* yet to be characterized and quantified). Given that the content of each of the above-mentioned components was not significantly different, it can be inferred that the balance of bitter, sweet, sour, astringent and umami flavors overlapped between the two groups of *huangjiu*.

Alcohols, aldehydes, acids, esters and phenols make up the skeleton of the *huangjiu*'s volatile flavor (Supplementary Table 1). In this study, 41 volatile flavor compounds were detected and found to exist in all six samples, including 8 alcohols, 8 aldehydes, 6 acids, 15 esters and 4 phenols (Fig. 7E). As shown in Fig. 7C, there was no significant difference in the total amount of volatile flavor compounds between two groups of *huangjiu* (585.56 ± 30.55 mg/L in CG and 496.73 ± 103.17 mg/L in EG), further suggesting that artificial *jiuyao* has not significantly adverse effect on the flavor formation of *huangjiu*. The difference between the two groups mainly emerged in the content of alcohols, especially phenethyl alcohol and 3-methyl-1-butanol. Phenethyl alcohol and 3-methyl-1-butanol, together with 1-propanol and 2-methyl-1-propanol, constitute the main

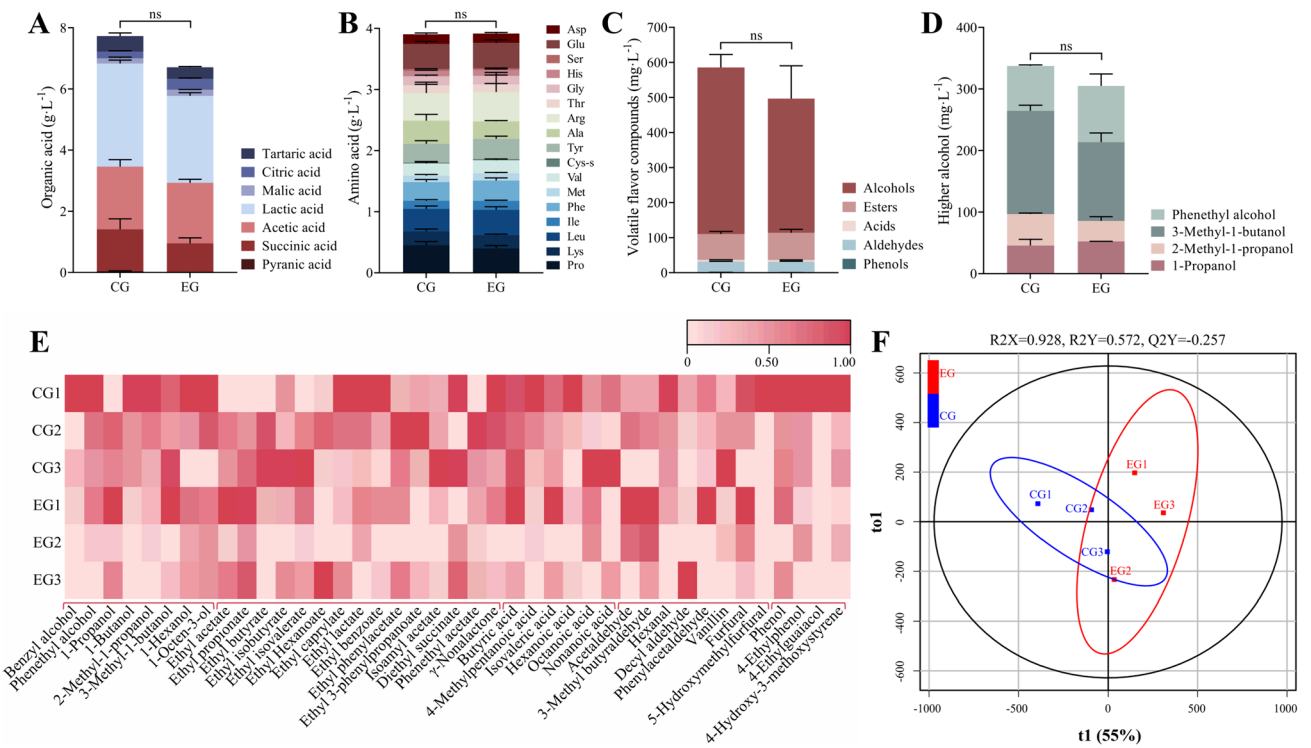


Fig. 7 Comparison of the content of organic acid (**A**), amino acid (**B**), total volatile flavor compound (**C**), and main higher alcohols (**D**) of *huangjiu* brewed with traditional (CG) and artificial *jiuyao* (EG). The content of volatile flavor compounds after min–max normalization (**E**) and the OPLS-DA score plot (**F**)

body of higher alcohols, an appropriate amount of which can make *huangjiu* harmonious and mellow, but instead an excessive amount of higher alcohols will increase the off-flavors and damage the after-drinking comfort [56, 57]. As shown in Fig. 7D, the total amount of higher alcohols in *huangjiu* brewed with traditional and artificial *jiuyao* were 337.41 ± 1.00 and 304.88 ± 41.29 mg/L respectively, suggesting that both groups of *huangjiu* could provide a good sensory experience while meeting health needs. Additionally, the ethyl esters represented by ethyl acetate and ethyl lactate contributed to the bright fruity aroma of Shaoxing *huangjiu* [25, 58]. As a supervised discriminant analysis method, OPLS-DA was applied to predict the classification of samples and screen the differential volatile flavor compounds between groups (Fig. 7F). The R2X and R2Y were both greater than 0.5, suggesting that the explanation ratios of the constructed model were pretty good. The separation of samples was not obvious on the x-axis in the scores plot, indicating that it was hard to differentiate the volatile flavor profiles of *huangjiu* whether brewed with artificial or traditional *jiuyao*. Furthermore, no differential volatile flavor compounds were found according to the criteria of VIP > 1 and $p < 0.05$, which further confirmed the similarities in the volatile flavor profiles of the two groups of *huangjiu*.

Conclusions

In this study, *P. pentosaceus*, *S. fibuligera*, *S. cerevisiae*, *M. indicus* and *R. microsporus* were comprehensively identified to be the core microbiota in *jiuyao* from both taxonomic and functional perspectives. And top-down and bottom-up approaches were integrated to construct the synthetic microbiota, whose application effects in *jiuyao*, seeding yeast and *huangjiu* proved that a tractable fermentation system with function and flavor reproducibility was successfully obtained. The nature of open fermentation allowed geography-dependent factors to make a difference in the self-assembly of the core microbiota, which also guaranteed, to a certain extent, the quality of artificial *jiuyao* and the terroir flavor of Shaoxing *huangjiu*. In conclusion, this study presented a method to identify the core microbiota in a natural fermentation system and to construct a synthetic microbiota for industrial application. Future research on microbial biogeography might extend the explanation of stability and terroir characteristics of traditional fermented foods.

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Data availability The data that support the findings of this study are available on request from the corresponding author, Jian Mao, upon reasonable request.

Declarations

Conflict of 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.

Ethical approval and consent to participate Not applicable.

Consent for publication The work described has not been submitted elsewhere for publication, in whole or in part, and all of the authors participated in the work and have agreed to the content of the manuscript.

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