Contents lists available at SciOpen

Food Science and Human Wellness

journal homepage: https://www.sciopen.com/journal/2097-0765

China Food Publishing Co.

Deciphering microbial dynamics and functional diversity during different rounds of pit fermentation of jiang-flavor Baijiu

Huan Wang[a], Yuxin Cheng[a], Xiaolong You[b], Yongguang Huang[a,*]

[a] College of Liquor and Food Engineering, Key Laboratory of Fermentation Engineering and Biological Pharmacy of Guizhou Province, Guizhou University, Guiyang 550025, China
[b] Guizhou Xijiu Co., Ltd., Zunyi 564622, China

ARTICLE INFO

Article history:
Received 20 December 2023
Received in revised form 12 March 2024
Accepted 1 July 2024

Keywords:
Jiang-flavor Baijiu
Pit fermentation
Biomarkers
Community composition
Function prediction

ABSTRACT

The variation in microbiota during pit fermentation is the main reason for the distinct characteristics of the 7 types of base Baijiu in jiang-flavor Baijiu (JFB) brewing. However, the specific structure, succession, and functional differentiation of microbial communities across different fermentation rounds remain unclear. Therefore, this study compared the differences in microbiota structure, environmental factors driving community assembly, and functional differentiations throughout 1–7 rounds (JC1–JC7) of pit fermentation in JFB production. Results showed that *Lactobacillus* dominated all rounds and complied with declining relative abundance from rounds JC1–JC7. The mould composition was similar in JC3–JC5 while the yeast structure in JC4 was found intermediate between JC3 and JC5. LEfSe analysis unveiled aroma-producing microorganisms as prominent biomarkers in JC1, strong enzyme-producing attributes in JC2, JC6, and JC7 biomarkers, and an enzyme and aroma-producing focus with robust tolerance in JC3–JC5 biomarkers. Acidity mainly regulated the microbial community in the first 4 rounds, with nutrient limitation drove microbial succession from the fifth round onward. Functional predictions underscored enriched amino acid metabolism enzymes in JC6 and JC1, while carbohydrate degradation exhibited predominant enzymatic profiles in JC2, JC6, and JC7. This study laid a foundation for comprehending community composition, succession, and flavor regulatory mechanisms throughout JFB brewing.

1. Introduction

Jiang-flavor Baijiu (JFB), as a typical Baijiu, is carefully blending with the complex and distinct stylistic characteristics of the 7 types of base Baijiu. From the appearance, the variations in style characteristics of 7 rounds can be attributed to the differences in the composition and content of flavor compounds in the base Baijiu for each round[1]. But in essence, the difference of flavor characteristics in 7 rounds is closely related to the characteristics of microbiota because flavor compounds are metabolites of microorganisms. Therefore, revealing the microbial and functional differences in 7 rounds is of great significance to explain the differences in flavor characteristics of 7 rounds of base Baijiu. Among several processes that are closely interfaced with microorganisms, including high-temperature *daqu* making, high-temperature stacking fermentation, and high-temperature pit fermentation, pit fermentation is particularly important. Firstly, pit fermentation commands the lion's share, spanning 7/12 of the entire production timeline. Besides, the brewing environment transitions into an anaerobic environment, thereby fostering the generation of a diverse array of volatile compounds through the intricate succession of the microbiota, encompassing alcohols, aldehydes, acids, esters, and pyrazine[2]. Consequently, the composition and dynamics of the microbial community during pit fermentation

* Corresponding author at: College of Liquor and Food Engineering, Guizhou University, Guiyang 550025, China.
E-mail address: 772566120@qq.com (Y.G. Huang)
Peer review under responsibility of Beijing Academy of Food Science

SciOpen Publishing services by Tsinghua University Press

http://doi.org/10.26599/FSHW.2024.9250344

emerge as a pivotal determinant influencing the eventual flavor structure of JFB.

Recently, researchers have undertaken various research on dynamics and diversity in the pit fermentation of JFB by employing high-throughput sequencing technology. An investigation into the microbial composition during the early stages of JFB fermentation has unveiled that *Lactobacillus*, *Pichia* and *Saccharomyces* were the dominant genera[3]. Further extending the scope, a study comparing the microbial communities and flavor compounds in the upper, middle, and lower layers of fermented grains during the 5th and 6th pit fermentation revealed that heightened ester content in the 5th round was mainly attributed to fungi, whereas the emergence of off-flavors such as *p*-cresol demonstrated associations with specific bacterial and fungal species[4]. Moreover, an investigation into the bacterial community dynamics thought rounds 4 to 7 revealed the dominance of Lactobacillaceae, Thermoactinomycetaceae, Bacillaceae, and Enterobacteriaceae. Notably, Lactobacillaceae exhibited sustained predominance across all rounds investigated[5]. Additionally, a study has researched the evolving composition of microbial community and flavor-related chemicals in the initial 3 rounds of base Baijiu production, identifying that ester synthesis was promoted by the bacteria genus *Lactobacillus* and many low abundances of fungal genera[6]. In addition, the microbial communities of different rounds of pit fermentation in Maotai town were compared, and the common and endemic genera were found[7]. These research results greatly contributed to reveal the microbial structure of JFB fermentation and its influence on the formation of flavor compounds. However, the differences in microbial structure, potential function, and biomarkers among the 7 rounds, as well as the reasons for the variations in microbial succession across these rounds, remain unclear. This lack of clarity persists because many studies only involve comparisons of microbial communities across a few rounds or simplistic analyses focusing on common and unique genera within the 7 rounds. Hence, a more systematic sample composition and deeper data analysis can reveal its micro-ecological structure and fermentation mechanism in more detail and depth.

In this study, the microbial community and diversity characteristics of the fermented grains in the whole cycle (1–7 rounds) of pit fermentation of JFB were analyzed via high-throughput sequencing technology. Subsequently, the biomarkers of each round were identified and the differences of biotic and abiotic factors driving microbial succession were clarified by using multivariate statistical analysis. Finally, PICRUSt II was used to illuminate disparities in functional attributes. By amalgamating these strands of investigation, this study aspires to comprehensively analyze the differences of microbial communities and biomarkers of 7 rounds in the whole fermentation rounds of JFB. At the same time, the flavor differences of 7 rounds of base Baijiu were explained from the point of view of microorganisms. This study also lay a foundation for a comprehensive exploration of the interplay between microbial ecological structures and the intricate flavor dynamics emblematic of Baijiu.

2. Methods

2.1 Sample collection and processing

All samples were collected from the same Baijiu-brewing workshop in Xishui County (28.14 N, 106.18 E), Zunyi, China. Samples were collected during the pit fermentation stage of Baijiu brewing, with samples taken every 5 days from the start to the end of pit fermentation (approximately 30 days). In addition, the fermentation grains samples were selected from different locations (points A, B, and C) (Fig. S1). Under aseptic conditions, the samples from the 3 points (A, B, and C) in the same layer were mixed into 1 sample. A total of 426 samples obtained from 7 rounds were used to form 146 composite samples. The composite samples were divided into 2 portions: one was used for total DNA extraction and the other was used to determine the fermentation parameters. Samples were stored at –20 ℃. The 1–7 rounds were represented as JC1–JC7 respectively.

2.2 Fermentation parameters detection

The moisture of the fermentation grain was monitored *via* a gravimetric method by drying samples to a constant weight at 105 ℃. The total acidity, starch and reducing sugar content of the fermented grains were determined as described by Wang et al.[8].

2.3 Total DNA extraction, amplicon sequencing and bioinformatics analysis

The sample pre-treatment and DNA extraction methods were obtained by Song et al.[9]. For bacteria, the 16S rRNA region was amplified using the universal primer sets 338F and 806R. For fungi, the internal transcribed spacer (ITS) region was amplified with primers ITS3 and ITS4[10]. The purified amplicons were sequenced on the Illumina MiSeq platform (Illumina, San Diego, USA) at Majorbio Bio-Pharm Technology (Shanghai, China).

Raw sequences were processed version 1.8.0 Quantitative Insights into Microbial Ecology (QIIME) (version 1.8.0). The representative bacterial operational taxonomic unit (OTU) sequences were annotated using the Silva 128/16S rRNA database; the representative fungal OTU sequences were compared using the UNITE fungal ITS database (version 8.0). For both bacteria and fungi, a 97% identity threshold was set. The coverage percentage (coverage index), diversity (Shannon index), and richness (Chao index) were calculated at the OTU level.

2.4 Statistical analysis

A one-way ANOVA and Kruskal-Wallis test performed with SPSS (version 19.0) were used to determine the significance of microbial data and statistical significance was indicated by $P < 0.05$. The heatmap was drawn by TBtools (version 1.082). The microbial composition map and correlation heat map were drawn by R (version 3.4.4) with "ggpolt" package and "corrplot" package. LEfSe plots were plotted by R with the "tidyverse" and "ggtree" packages, and the LDA threshold score > 3 was employed to identified biomarkers[11]. All Spearman's rank correlations among the genera used the "Hmisc" package of R and the Mantel test was conducted through "vegan" package in R. The network was created by using the data of high and significant correlation ($P < 0.05$, $|R| > 0.6$) between dominant genera (relative average abundance > 1%) and then the ecological network was divided into modules through the "Modularity" in Gephi (version 9.5). The PICRUSt II was used to predict microbial function.

3. Results and discussion

3.1 Changes of physicochemical properties during pit fermentation of 7 rounds

The dynamic changes in moisture, starch contents, reducing sugar contents, and acidity were examined across 7 rounds. As illustrated in Fig. 1, the change trend of moisture content, acidity, and starch content in fermentation grains during the pit fermentation of each round in JFB was similar, except for reducing sugar content. Nevertheless, variations existed in the alterations of physicochemical properties across 7 rounds. Among these parameters, moisture and acidity exhibited a continual increment throughout each fermentation stage, while the starch content underwent a progressive decrease. In the context of distinct rounds, the starch content exhibited a diminishing trend with successive rounds (ranging from 30.41% to 13.15%), accompanied by an average reduction rate of 5.65%, 5.33%, 3.28%, 0.89%, 1.06%, and 1.04%, respectively. This trend found its origin in the unique brewing process of JFB, wherein sorghum was introduced solely in JC1. The starch content was constantly consumed with the continuous cooking and microbial metabolism[12], aligning with previous research findings[3,13]. The moisture levels and acidity rise in the initial 4 rounds (JC1–JC4) out of the 7, followed by a decline in the subsequent 3 rounds (JC5–JC7). JC4 exhibited the highest mean values for moisture (56.65%) and acidity (4.16 mmol/10 g). Conversely, the alteration trends of reducing sugar varied among 7 rounds. In JC1, reducing sugar content experienced a continuous increase throughout the fermentation process (1.12 to 3.01 g/100 g). For JC2 to JC4, reducing sugar content initially decreased, then increased, and subsequently decreased again. In JC5 to JC7, a consistent decline in reducing sugar content was observed as fermentation progressed (3.39 to 1.91 g/100 g, 1.90 to 1.27 g/100 g, and 1.25 to 0.92 g/100 g, respectively). This phenomenon was attributed to the rapid early-stage degradation of starch content, leading to an accumulation of reducing sugar. In the JC5 to JC7 stages, despite insufficient starch content, microorganisms continued to utilize reducing sugar, a pattern in alignment with previous investigations[13].

Therefore, trends of changes in physicochemical properties within each round exhibit similarities. However, notable disparities emerge in these indicators across 7 rounds. Specifically, starch levels decrease gradually, acidity and moisture initially rise before declining, and the concentration of reducing sugar undergoes intricate fluctuations.

3.2 Microbial diversity in 7 rounds

A total of 4 309 003 valid reads were obtained from bacterial 16S rRNA V3–V4 sequences and 4 244 164 high-quality reads were acquired from fungal ITS sequences. The 16S rRNA gene (2 111 OTUs) and ITS (2 402 OTUs) amplicon sequences were clustered at a 97% similarity level. The Coverage estimator of bacteria and fungi across JC1 to JC7 ranged from 98.99% to 99.94% (Table S1), indicating that major bacterial and fungal OTUs had been captured.

The Shannon and Chao index among 7 rounds were compared by Kruskal-Wallis analysis. As shown in Fig. 2, discernible differences were found in microbial diversity (Shannon index) and richness (Chao index) of bacteria and fungi among 7 rounds ($P < 0.01$). Specifically, there was no significant difference in bacterial diversity during the initial 4 rounds. However, the diversity notably increased in the last 3 rounds compared to the first 4 (Fig. 2A). Conversely, the fungal diversity across 7 rounds exhibited cyclical fluctuations, with notably

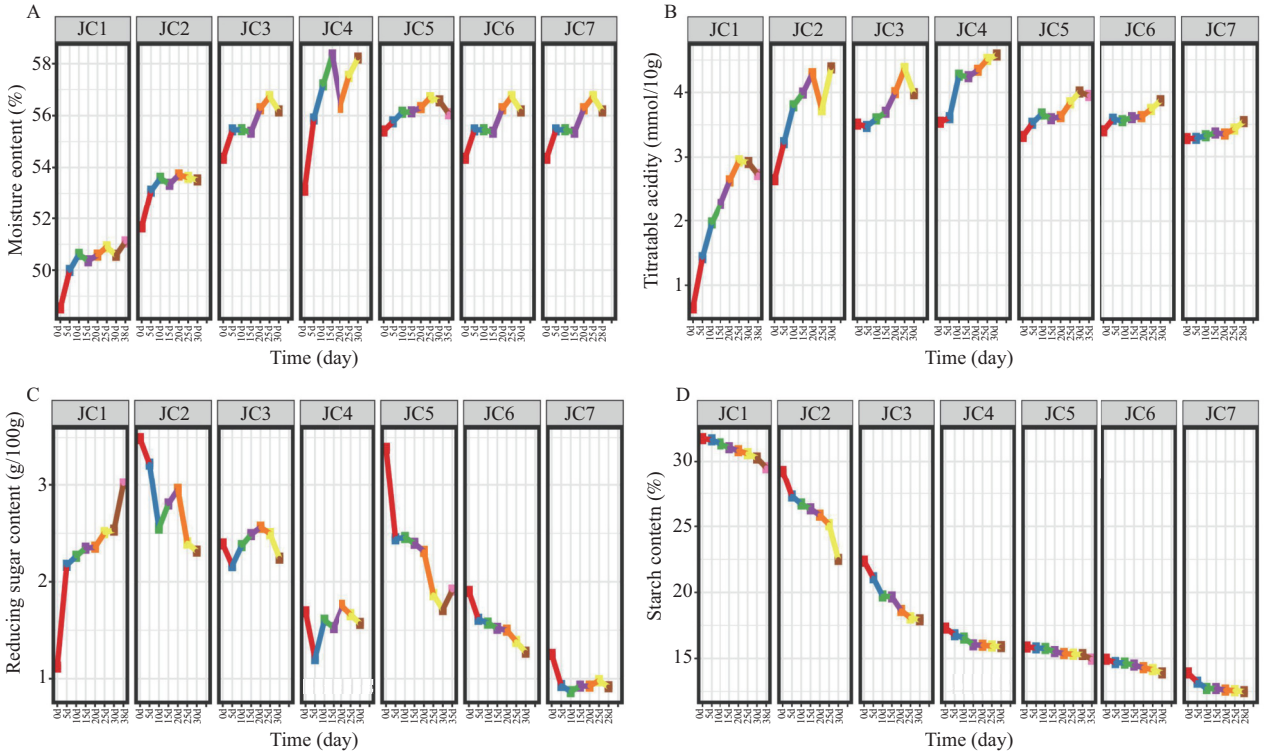

Fig. 1 Changes in the physicochemical properties of fermented grains in 7 rounds of pits fermentation. (A) Moisture content; (B) Acidity; (C) Reducing sugar content; (D) Starch content.

higher Shannon index values in JC2, JC4, and JC6 compared to the other rounds. The richness (Chao index) showed a gradient increasing trend among 7 rounds. The Chao index value in JC1 was significantly lower than in other rounds, followed by no significant difference was observed among JC2 to JC5. Conversely, the Chao index value of JC6 and JC7 markedly exceeded that of the other rounds. In terms of fungi, the richness observed in JC2, JC4, and JC5 significantly exceeded that of the other rounds. This phenomenon could be attributed to the predominance of bacteria originating from high-temperature *daqu*, characterized by their high resistance. In contrast, fungi predominantly derive from the surrounding environment, exhibiting lower resistance[14-15]. These findings collectively underscore that the microbial community in 7 rounds of pit fermentation had distinct characteristics and underwent dynamic changes.

3.3 Microbial community compositions and succession in 7 rounds

For pit fermentation spanning JC1 to JC7, a total of 826 bacterial genera and 683 fungal genera were detected within fermented grains. As shown in Fig. 3A, the trend of bacterial succession observed over 7 rounds, showed a consistent pattern. Throughout each round of fermentation, the microbiome consistently shifted towards dominance by *Lactobacillus*. In the process of Baijiu brewing, *Lactobacillus* was the dominant genus in fermentation, and the rapid increase of its relative abundance implied the stage shift of fermentation[16]. Moreover, *Lactobacillus* was identified as a biomarker for the microbial community succession in the fermentation process of JFB[17]. Hence, *Lactobacillus* can not only be implied the stage shift in fermentation, but can also be used to reveal transitions in 7 rounds in JFB.

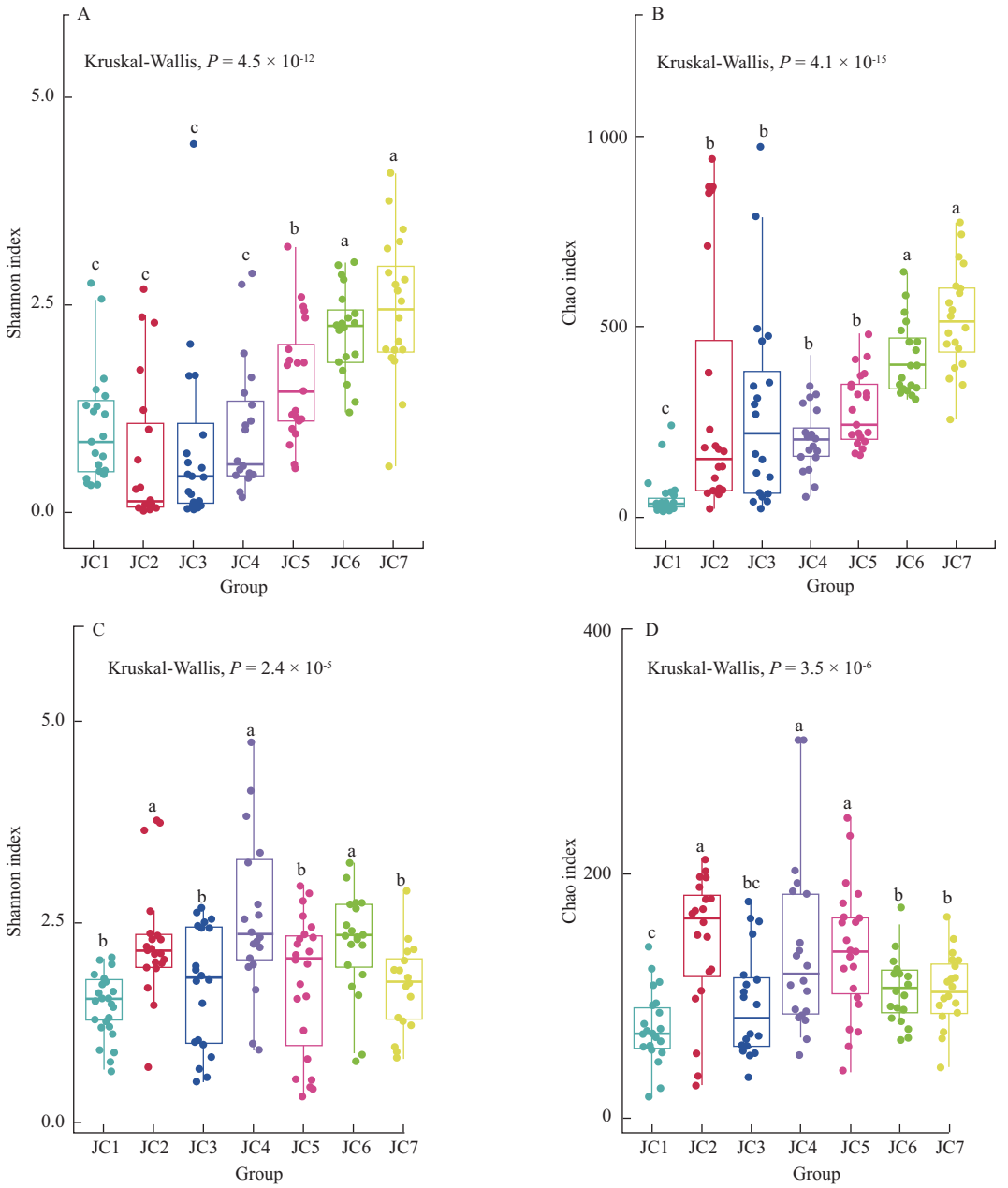

Fig. 2 Box diagram of diversity index of JFB in 7 rounds of pit fermentation. (A) Shannon index of bacteria; (B) Chao index of bacteria; (C) Shannon index of fungi; (D) Chao index of fungi. Different lowercase letters represent significant difference.

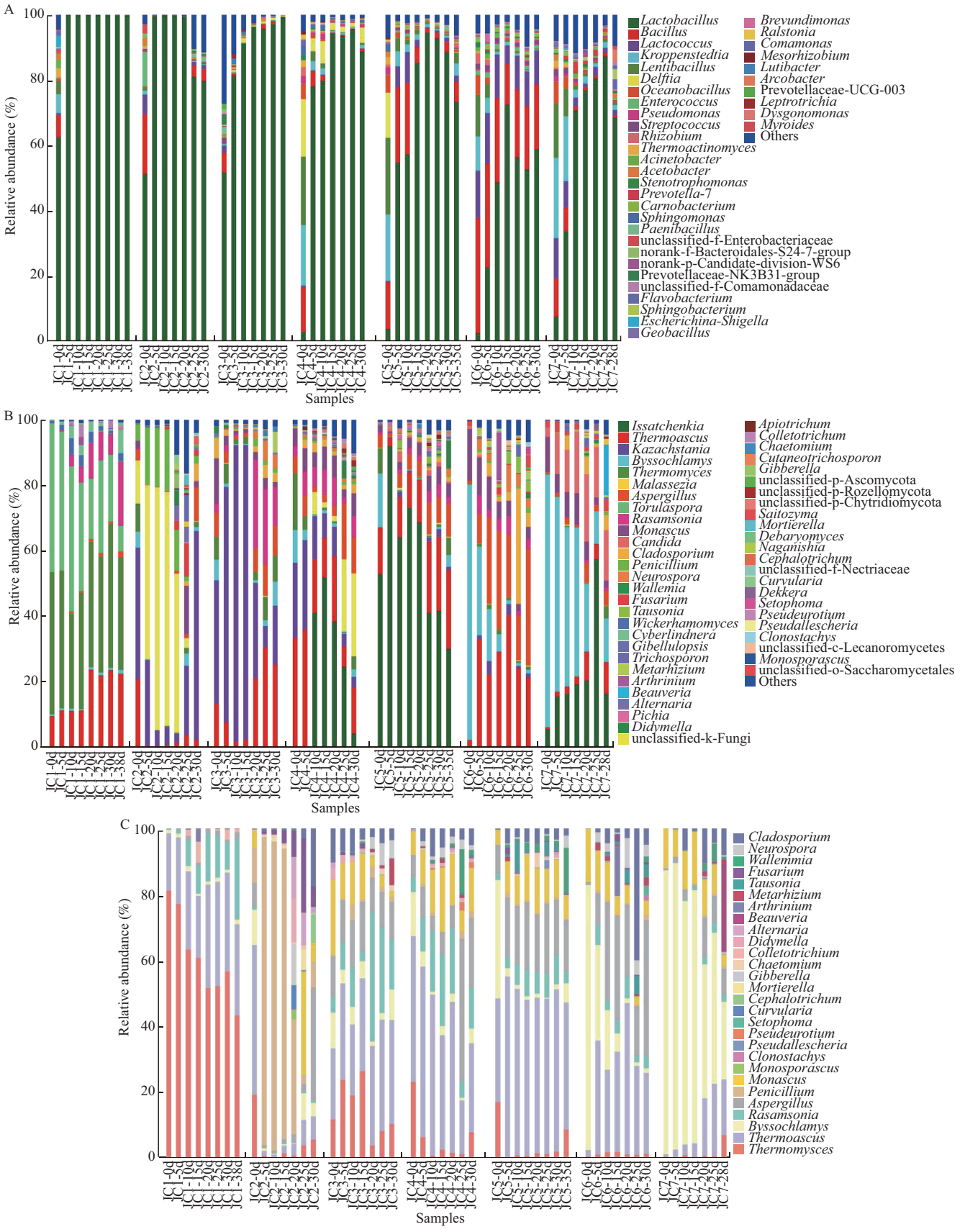

Fig. 3 Identification of short-chain acylcarnitines associated important microbe modules on WGCNA. (A) Clustering dendrograms of microbiota. (B) Heatmap depicted the relationship between the microbiota modules and the significant changed in serum metabolites. (C) Degree topological analysis method of degree provided by cytohubba explored the top 10 important microbes in 2 modules with correspondence with short-chain acylcarnitines. Node colors were from yellow to red, represented the higher rank of the degree index.

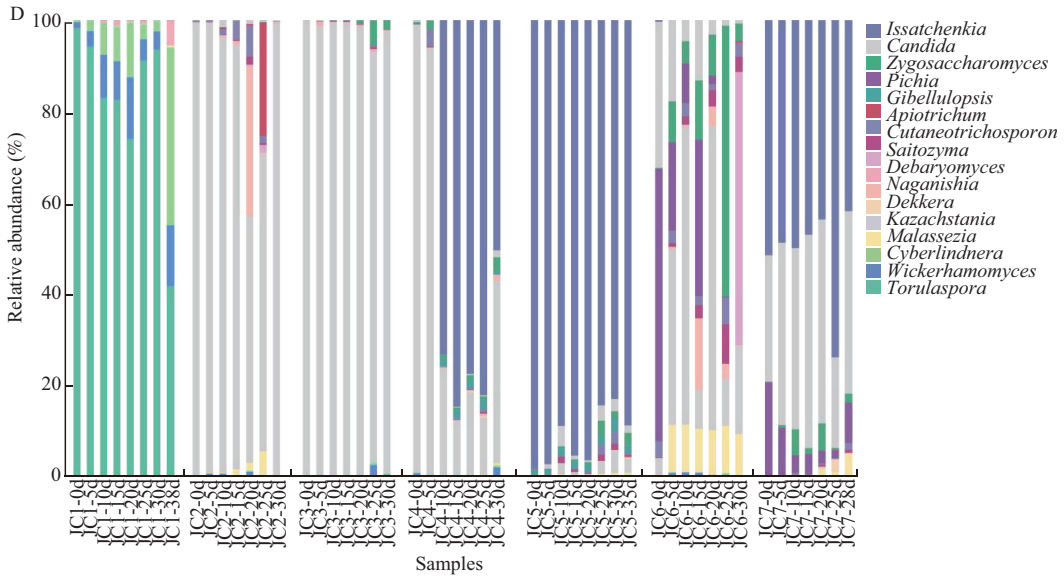

Fig. 3 (*Continued*)

As shown in Fig. 3B, noticeable variations became apparent in the fungal community structure across 7 rounds. Notably, the initial fungal community structure markedly diverged from that present at the end of the last round, suggesting that the initial difference of fungi across varied rounds was mainly from the stacking fermentation process. This result further confirmed that the stacking fermentation of JFB was an effective process to fully enrich fungi[10]. Concurrently, the roles enacted by molds and yeasts within the fungal community were distinct throughout the pit fermentation process. Thus, the microbial communities of fungi were further separated according to function. As shown in Fig. 3C, a shared similarity was observed in the mold structure between JC3, JC4, and JC5, wherein *Thermoascus* (the average abundance was 14.33%, 19.82%, 15.29%, respectively), *Monascus* (3.61%, 5.67%, 3.21%, respectively), and *Aspergillus* (5.60%, 6.64%, 5.80%, respectively) dominated. Furthermore, the mold structure of JC6 shared similarities with JC3 to JC5, as *Thermoascus* (24.41%), *Aspergillus* (19.32%), and *Byssochlamys* (18.54%) were the predominant genera. In contrast, the mold structures of JC1, JC2, and JC7 diverged from this pattern. JC1 was dominated by *Thermomyces* (37.85%) and *Thermoascus* (16.68%), JC2 exhibited *Penicillium* (10.57%) and *Thermoascus* (4.05%) as primary contributors, while JC7 was distinguished by the predominance of *Byssochlamys* (39.26%) and *Monascus* (6.14%). It is generally recognized that the base Baijiu in JC3 to JC5, colloquially known as "Da Huijiu", exhibit superior quality, while those of JC6, or "Xiao Huijiu", possess a roasted or burnt taste, attaining a slightly diminished quality[18]. Previous research also found that some moulds also synthesize flavor compounds[2]. Thus, unlike previous studies, we propose that the distinct mold communities might also be intrinsically linked to the flavor profiles of base Baijiu.

In the pit fermentation process, yeasts play a crucial role as primary functional microorganisms responsible for generating ethanol and flavor compounds[2]. The compositions of yeast were shown in Fig. 3D, wherein the yeast structure within JC2 and JC3 appeared similar, with *Kazachstania* dominating (23.55% and 49.19% respectively). However, unclassified_k_Fungi accounted a substantial proportion (37.72%) within JC2. The yeast composition of JC4 exhibited an intermediate profile, positioned between JC3 (*Kazachstania*, 23.55%) and JC5 (*Issatchenkia*, 57.80%). It was marked by the initial dominance of *Kazachstania* in the early phase, transitioning to *Issatchenkia* in the later stages. In JC6, *Kazachstania* reigned as the predominant yeast, whereas JC7 was characterized by the dominance of *Issatchenkia* (21.37%) and *Candida* (14.09%). JC1 exhibited significant variability in yeast composition, with *Torulaspora* being the most prevalent genus at 30.04%. Therefore, the characteristic yeast structure across 7 rounds was postulated to be the main influencing variations in alcohol production and flavor compounds of base Baijiu.

Furthermore, an analysis was conducted on the distribution characteristics of dominant microorganisms (with an average abundance exceeding 1%) within distinct rounds. Depicted in Fig. 4A, the count of dominant bacteria displayed a general increasing trend among 7 rounds (9, 7, 11, 14, 13, 13, and 20 genera, respectively). This phenomenon predominantly stemmed from the decreasing abundance of *Lactobacillus* during JC1–JC7, because *Lactobacillus* can produce lactic acid, and acetic acid[9] to increase the acidity of the fermented grains, and metabolic bacteriocins produced to antagonize other microorganisms[19]. This result was also consistent with the change of acidity in the physicochemical factor (Fig 1). Furthermore, *Lactobacillus* and *Bacillus* emerged as the dominant bacteria across all rounds, while *Kroppenstedtia* was the dominant bacteria of all rounds, except JC2. This triad of genera collectively constituted the core microbiome for JFB, the result consistent with previous studies[5].

The distribution characteristics of fungi were shown in Fig. 4B, with the dominant fungi counts 11, 21, 14, 18, 19, 21, and 15 in JC1–JC7. Of which, the dominant mold numbers were 7, 15, 13, 10, 12, 17, and 9, while the dominant yeast counts were 4, 4, 1, 4, 7, 3, and 6 in JC1 to JC7, respectively. The results indicated that more dominant molds than dominant yeasts were found in pit fermentation. Core molds included *Aspergillus*, *Byssochlamys*, *Thermoascus*, and *Thermomyces*, dominating in all rounds and showing heat-resistant characteristics. These were also the core molds in stacking fermentation[10].

3.4 Biomarkers of microbial communities in 7 rounds

To gain insight into the microbial characteristics of fermented grain across JC1–JC7, the microbial communities of fermented grains

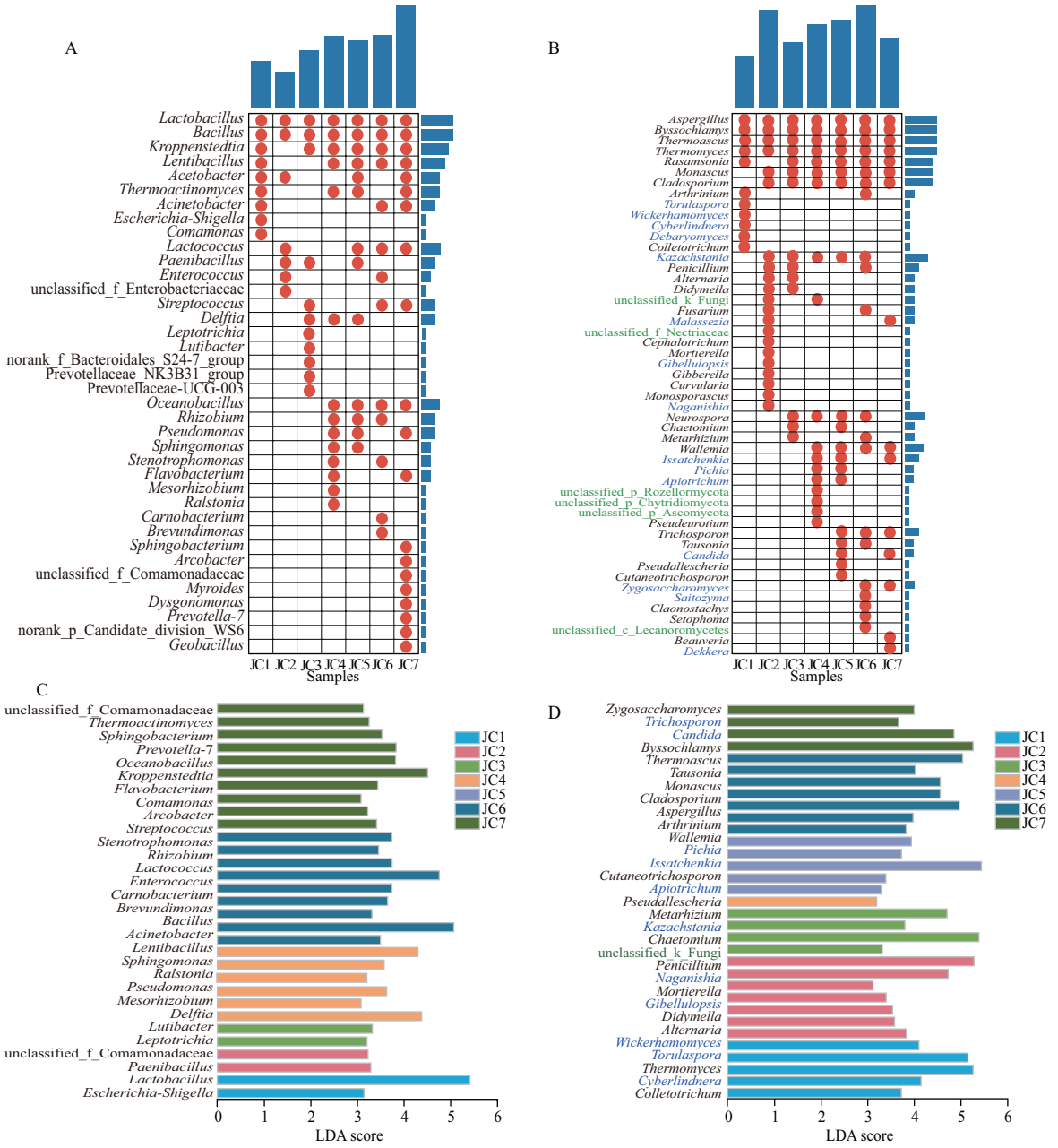

Fig. 4 Distribution of dominant genera and biomarkers in 7 rounds. (A) Distribution characteristic of dominant bacteria genera in 7 rounds. Red dot represents the dominant genus in a certain round, the blue bar chart in the column was the total number of dominant genera in a certain round, and the blue bar chart in the row represent the number of times that a certain genus was identified as the dominant genus in 7 rounds. (B) Distribution characteristic of dominant fungi genera in 7 rounds, molds were marked with black fonts, yeasts were marked with blue fonts, and unclassified genera were marked with green fonts. LEfSe analysis identified (C) bacterial and (D) fungal biomarkers in 7 rounds.

were analyzed using LEfSe analysis to identified the biomarkers of each round. As depicted in Fig. 4C, the biomarkers existed across 7 rounds were different, with 7, 9, 6, 6, 6, 16, and 14 biomarkers identified in each round.

Specifically, the biomarkers of JC1 were *Escherichia-Shigella*, *Lactobacillus*, *Colletotrichum*, *Thermomyces*, *Cyberlindnera*, *Torulaspora*, and *Wickerhamomyces*. Among them, *Escherichia-Shigella* was detected in Xijiu[10] and Langjiu[5], signifying its unique presence within the upper Chishui River environment. *Lactobacillus* was the functional contributor to ethanol, lactic acid, and acetic acid[20]. *Thermomyces*, equipped with the capacity to produce amylases and proteases[20], was also implicated in promoting amino acids degradation and short-chain fatty acids synthesis (such as acetic acid, propionic acid, and butyric acid)[11]. This potentially accounting for the notable rise in acidity within JC1 fermented grains. Meanwhile, *Torulaspora* displayed the ability to enhance ethyl hexanoate, ethyl octanoate and higher alcohols contents[21], while *Wickerhamomyces* played a crucial role in elevating the content of ethyl butyrate, ethyl lactate and ethyl hexanoate[22]. *Cyberlindnera*, an aromatic yeast, could metabolize higher alcohols, ethyl acetate and other flavor substances[23]. These results indicated that the biomarker of JC1 mainly contribute some aroma compounds, such as higher alcohols, acetic acid, ethyl acetate, and ethyl butyrate. These results may explain the previous research findings, wherein JC1 own high

content of higher alcohols and acetic acid, ethyl acetate, and ethyl butyrate[24].

The biomarkers attributed to JC2 comprised *Paenibacillus*, unclassified_f_Enterobacteriaceae, *Alternaria*, *Didymella*, *Gibberella*, *Mortierella*, *Penicillium*, *Naganishia*, and unclassified_k_Fungi. Interestingly, most of JC2 biomarkers exhibited robust enzyme-producing capabilities. *Paenibacillus*, for instance, could produce a diverse variety of enzymes such as amylases, cellulases, hemicellulases, lipases, and pectinases[25]. *Penicillium* could produce protease, cellulase and esterase[26], while *Alternaria* contributed to the synthesis of protease, cellulase, esterase, and pectinase[27]. *Naganishia*, has been reported as an aroma producing yeast, yielding *n*-hexanol, *n*-pentanol and ethyl acetate[28]. Thus, a variety of enzyme-producing microorganisms coupled with few aroma-producing microorganisms characterizes the microbial community of JC2. This profile was largely attributed to the near-exhaustive utilization of crushed fermentation grain in JC1, compelling JC2 to rely extensively on fungal interactions for starch hydrolysis within the uncrushed remnants, thereby yielding fermentable sugars[29]. This result explains the reason of the highest consumption rate of starch in JC2 (7.43) than in other rounds (1.20, 4.44, 1.4, 0.92, 1.04, 1.31). Nevertheless, unclassified_k_Fungi in JC2 need attention, as they could potentially contribute significantly to JC2's flavor or enzyme composition. Advanced methodologies are essential for further elucidation of this aspect.

The biomarkers of JC3 comprised *Leptotrichia*, *Lutibacter*, *Chaetomium*, *Metarhizium*, *Rasamsonia*, and *Kazachstania*. Notably, *Chaetomium* could produce thermostable xylanase, lactase, cellulase, amylase, and glucanase[30]. *Rasamsonia*, characterized as a heat-resistant microorganism, facilitates pH recovery through the conversion of lactate to pyruvate[31]. *Metarhizium* displayed stabilized chitinase activity even under extreme pH and temperature conditions [32]. *Kazachstania*, identified as a robust performer in fermentation comparable to *S. cerevisiae*, and could grow under conditions of high osmolarity, acidity, ethanol, and salt[33]. Besides, *Kazachstania* could metabolize higher alcohols, particularly propanol and isoamyl alcohol[34]. The biomarkers of JC4 encompassed *Delftia*, *Mesorhizobium*, *Pseudomonas*, *Ralstonia*, *Sphingomonas*, and *Pseudeurotium*. Among these, *Delftia* was known to produce β-1,3-glucanases optimally active at 50 °C and pH 4.0[35]. The brewing function of other biomarkers was unknown. The biomarkers associated with JC5 consist of *Lentibacillus*, *Wallemia*, *Apiotrichum*, *Cutaneotrichosporon*, *Issatchenkia*, and *Pichia*. *Lentibacillus* could secret amylase and lipase, coupled with acid metabolism[36]. *Issatchenkia* exhibited high tolerance to acidity during brewing and contributed to the metabolism of lactic acid, creating a favorable environment for the growth of other yeasts[37]. Furthermore, *Issatchenkia* exhibited efficient ethanol production from cellulose and hemicellulose hydrolysate[38]. *Pichia* displayed robust lactate tolerance. Besides, it exhibited the capacity to synthesize a range of compounds, including ethanol, isoamyl alcohol, isobutanol, ethyl acetate, ethyl propionate, and phenylethyl acetate[2]. In conclusion, the biomarkers identified within JC3–JC5 predominantly encompassed enzyme-producing and aroma-generating microorganisms with remarkable tolerance. These functional microorganisms were capable of metabolizing lactic acid and reducing acidity in the environment. This explains the relatively slower rate of acidity increase in JC3–JC5 (0.47, 1.04, 0.63) compared to JC1 (2.08) and JC2 (1.73).

The biomarkers identified in JC6 included *Acinetobacter*, *Bacillus*, *Brevundimonas*, *Carnobacterium*, *Enterococcus*, *Lactococcus*, *Rhizobium*, *Stenotrophomonas*, *Streptococcus*, *Arthrinium*, *Aspergillus*, *Cladosporium*, *Monascus*, *Tausonia*, and *Thermoascus*. Among these genera, *Bacillus*, known for its capacity to metabolize protease and amylase[39], contributed significantly to the formation of flavor[40], such as ethyl caproate[41]. *Aspergillus* could produce a wide array of extracellular enzymes (acid/alkaline proteases and amylase)[42] and pyrazines[40]. *Thermoascus*, characterized by its ability to produce xylanase, heat-resistant alkaline catalase, and other enzymes, played a vital role in influencing the Jiang flavor during fermentation[20,43]. *Monascus* could produce protease and lipase enzyme, which could enhance the production of amino acids and esters[44]. *Cladosporium* could produce various enzymes such as liquefying, cellulose-degrading, proteolytic, and esterifying enzymes. liquefaction, cellulose degradation, proteolysis, and esterification[27]. *Enterococcus* and *Lactococcus* were recognized as contributors to lactic acid production[45-46]. Thus, the biomarkers of JC6 primarily focused on microorganisms that produce enzymes, specifically those responsible for synthesizing proteases and amylases. Given the limited carbon source remaining in JC6, the prevalence of these enzyme-producing microorganisms played a crucial role in maintaining fermentation. Furthermore, the biomarkers in JC6 were closely associated with pyrazine metabolism, which may explain prior findings that higher pyrazine content in base Baijiu from JC6[24].

The biomarkers of JC7 included *Acetobacter*, *Arcobacter*, *Comamonas*, *Flavobacterium*, *Kroppenstedtia*, *Oceanobacillus*, *Prevotella*-7, *Sphingobacterium*, *Thermoactinomyces*, unclassified_f_Comamonadaceae, *Byssochlamys*, *Candida*, *Trichosporon*, and *Zygosaccharomyces*. Among these genera, *Kroppenstedtia*, recognized for its protease production capabilities[47], not only enhanced the levels of acetic acid, lactic acid, and ethyl acetate but also lowered the content of ethyl lactate in Baijiu fermentation[48]. *Byssochlamys* displayed efficient growth even in extremely acidic environmental conditions[49] and demonstrated the ability to secrete xylanase enzymes and produce ethanol[50]. *Oceanobacillus* played an important role in enzyme production during the fermentation of Baijiu, including protease, amylase, cellulase, and esterase[14]. *Candida* could produce esterase, and phenylethyl alcohol[40]. *Zygosaccharomyces* participated in carbohydrate and amino acid metabolism, and could produce ethyl acetate, ethyl phenylacetate, and isoamyl acetate[51]. Consequently, a microbial community primarily engaged in the metabolism of amylase, protease, and cellulase emerged in JC7 following the exhaustion of starch in sorghum. Furthermore, *Zygosaccharomyces* could thrive under high nitrogen conditions[52], and its abundance progressively increased from JC1 to JC7 (Fig. 4D). this result suggested its significant role in influencing the flavor of JFB during the latter stages of fermentation. Future investigations could delve deeper into this aspect.

In summary, the functional microorganisms in 7 rounds of pit fermentation encompass fungi and bacteria originating from daqu (e.g., *Lactobacillus*, *Bacillus*, *Paenibacillus*, etc.). Other bacteria (such as *Escherichia-Shigella*, *Comamonas*, *Leptotrichia*, and Prevotellaceae-UCG-003, etc.) were common environmental bacteria and could occasionally cause human disease. However, their abundance decreased rapidly during fermentation, suggesting that the traditional fermentation process regulates the microbial community,

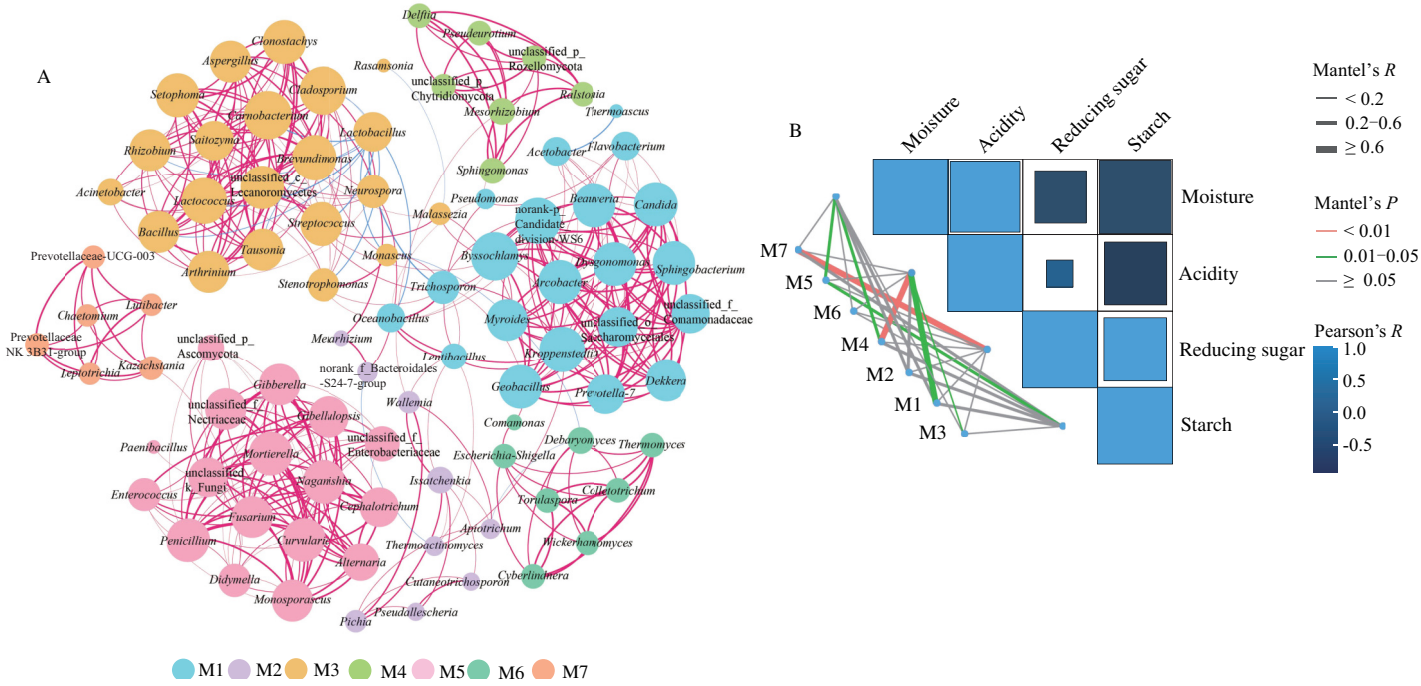

Fig. 5 Correlation analysis chart. (A) Network analysis of dominant microbes in 7 rounds of pit fermentation of JFB; (B) Correlation between each module and each physicochemical factor. The width of the edges corresponds to the *R*-value obtained by the Mantel test, and the color of the edges indicates statistical significance. Correlations between the module and the environmental factor were represented by the color gradient of Pearson's correlation coefficient.

effectively suppressing the growth of harmful microorganisms and ensuring the quality and safety of Baijiu. Additionally, the biomarkers observed in 7 rounds were associated with the specific environment and can explain the differences in wine body style in 7 rounds. Therefore, the biomarkers of 7 rounds play an important role in analysing the flavor differences of 7 rounds of base Baijiu.

3.5 Characteristics of biotic and abiotic factors of dominant microbiome in 7 rounds

Microbial succession is driven by biotic and abiotic factors. In order to analyze the biotic and abiotic driven factors of microbial succession in 7 rounds, the co-occurrence networks of microbial community were established base on the interaction of dominant genera, and the correlation between microbiota and physicochemical factors were calculated. As shown in Fig. 5A, the co-occurrence networks of microbial community could be divided into 7 modules according to the closeness between microorganisms[53], and the distribution characteristics of modules align consistently with the characteristics of dominant genera in 7 rounds. This result indicated that there was a certain relationship between different rounds and modules, and co-occurrence network modules could reflect the characteristics of different rounds.

Specifically, the 2 largest modules were M7 (the nodes accounted for 23.6% of the total nodes) and M6 (23.6%), both containing 21 genera. M7 was primarily characterized by bacteria, encompassing 14 bacterial and 7 fungal members, all of which belonged to dominant genera in JC7. This phenomenon may be attributed to the higher resistance of bacteria compared to fungi[14], as well as the broader range of carbon sources utilized by many bacteria in JC7, such as *Acetobacter*[54], *Arcobacter*[55], *Dysgonomonas*[56], *Lentibacillus*[57], *Geobacillus*[58], and *Pseudomonas*[59]. The Mantel test (Fig. 5B) further demonstrated that M7 was predominantly governed by reducing sugars (R = 0.74, P = 0.001). M6, on the other hand, consisted of 9 bacterial genera and 12 fungal genera, all of which were predominant in JC6. This result suggested that fungi primarily dominated the microbial community in JC6. Additionally, the dominant fungi exhibited positive correlations, implying a synergistic relationship in the catalytic hydrolysis of fermentation substrates. The Mantel test revealed that physicochemical factors did not significantly regulate the microbial community in M6, indicated that the succession of microbial community in JC6 was mainly regulated by biotic factors.

The second largest module, M2 (19.1%), comprised 17 genera (3 bacteria and 14 fungi), all originating from JC2, except for unclassified_p_Ascomycota. Fig. 5A shows that the microorganisms within M2 displayed positive correlations. Among them, some genera (*Paenibacillus*, *Penicillium*, *Alternaria*, *Fusarium,* and *Mortierella*) exhibited robust enzyme-producing capabilities, could degrade carbohydrates to fermentable glucose[25-27,60-61]. This result further indicated that not only the largest number of enzyme-producing fungi were enriched in JC2, but also that microorganisms strengthened carbohydrate degradation through mutual promotion. Similar to M6, the microbial community within M2 exhibited no significant regulation by physicochemical factors. This result indicated that the succession of microbial community in JC2 was also primarily regulated by biotic factors.

Other modules had less members. Among which, M5 (10.11%) had 9 genera (2 bacterial genera and 7 fungal genera), and most of these genera were the dominant genera in JC5 (exception of *Metarhizium* and norank_f_Bacteroidales_S24-7_group). The Mantel test showed that M5 was notably linked to moisture (R = 0.30, P = 0.003 2) and starch (R = 0.36, P = 0.004 8). M1 (8.99%) included 2 bacterial genera and 6 fungal genera, and all of them were the dominant genera of JC1. The Mantel test showed that

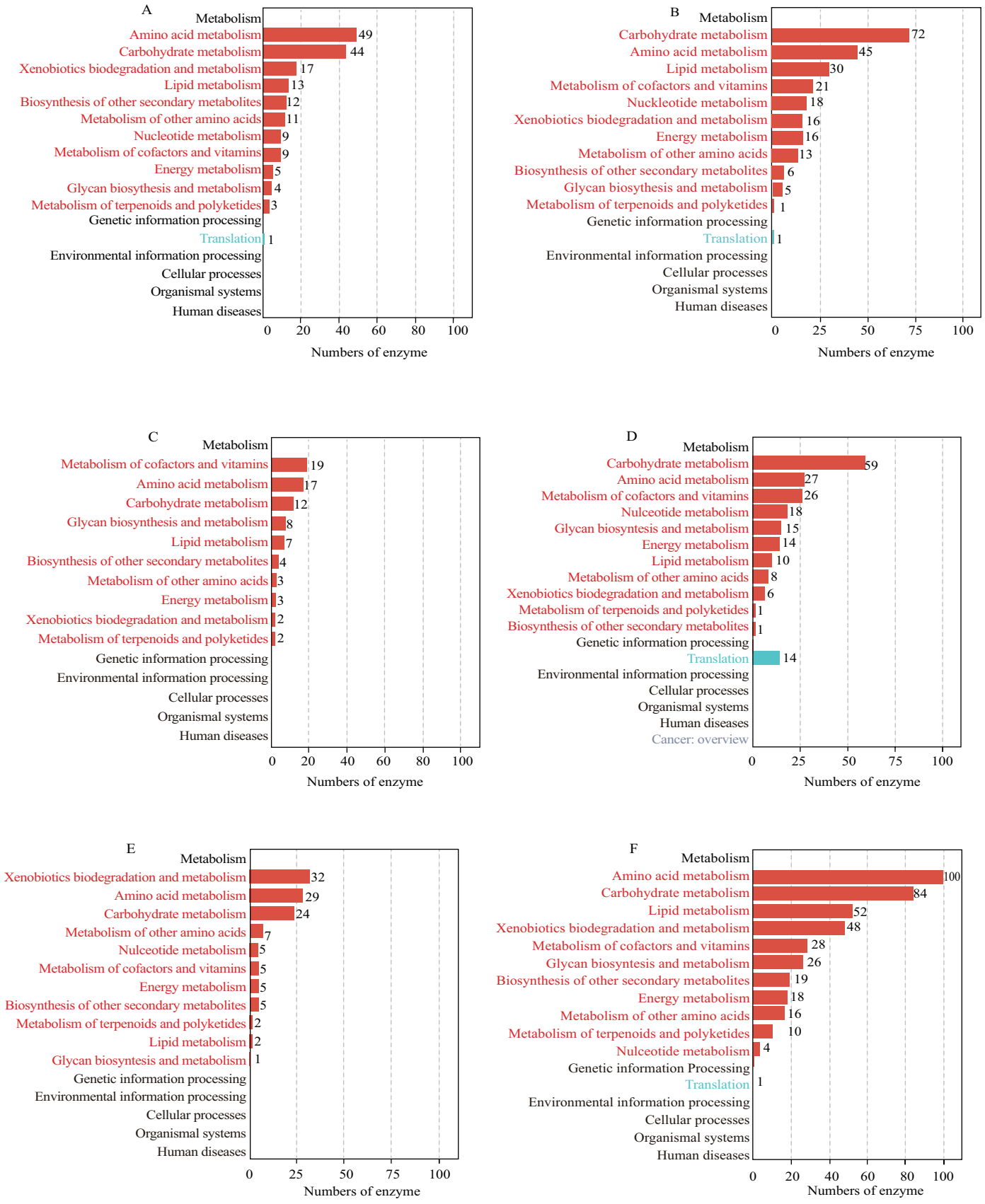

Fig. 6 KEGG pathway annotation of biomarker enzymes in 7 rounds. (A-G) The A-G represent the KEGG pathway annotations of biomarker enzymes of JC1–JC7 respectively. The bold black font represents the primary category of KEGG, and the different colors represent the secondary category in KEGG (Metabolism: red; Genetic information processing: sky blue; Environmental information processing: green; Cellular processes: blue; Organismal systems: orange; Human disease: purple).

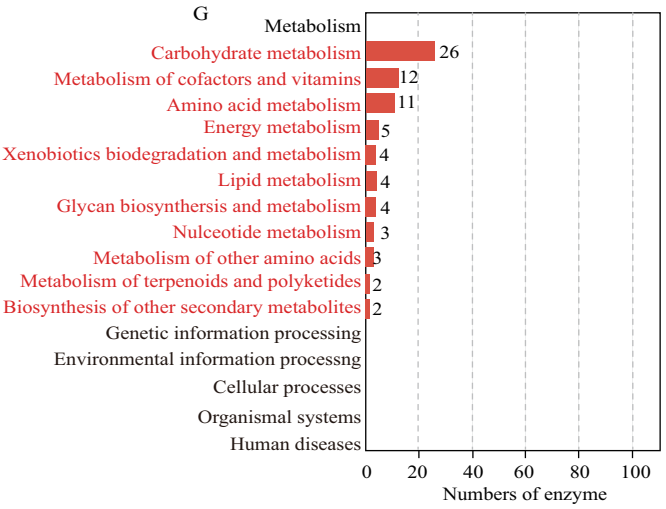

Fig. 6 (*Continued*)

M1 was significantly correlated with acidity (R = 0.81, P = 0.013). The constituents of M4 (7.87%), including 4 bacterial genera and 3 fungal genera, were exclusively sourced from JC4. The Mantel test discerned that M4 exhibited substantial correlation with moisture (R = 0.54, P = 0.021) and acidity (R = 0.6, P = 0.007). M3 (6.74%) contained 4 bacteria and 2 fungi, all of which were from JC3. The Mantel test founded that M3 displayed noteworthy correlation with acidity (R = 0.16, P = 0.041).

Thus, a robust correlation exists between modules in the micro-ecological network and rounds, and closer interactions among dominant genera in the same round. This result may be due to the fact that dominant genera in the same round came into contact with each other, leading to nutritional competition or symbiosis[62], resulting in a stronger correlation among microorganisms within the same round. It is noteworthy that predominantly positive correlations among dominant genera within the same round, which also proved that the dominant genera in the same round promoted fermentation by mutual promotion and homeostatic regulation. Furthermore, acidity mainly regulated the microbial community in the first 4 rounds of fermentation, whereas nutrient limitation was the main cause of microbial succession from the 5th round.

3.6 Characteristic of potentially functional enzymes in 7 rounds

To further analyze the differences of the potentially functions of microbial communities in 7 rounds of pit fermentation, the functions of the dominant microbes were predicted by PICRUSt II. Subsequently, the enzymes with significant differences in 7 rounds were founds. A total of 114, 167, 76, 104, 155, 269, and 77 distinct enzymes were obtained in JC1–JC7, respectively. According to the classification of enzymes at level 2 within the metabolic pathways of KEGG, substantial variations in enzyme profiles were observed across 7 rounds. As shown in Fig. 6, carbohydrate metabolism and amino acid metabolism emerged as prominently active level 2 metabolic pathways in JC1–JC7.

To conduct a more comprehensive analysis of the primary flavor-related metabolic functions within 7 rounds of microbial communities during the pit fermentation, the differential enzymes closely associated with carbohydrate degradation, fermentation, fatty acid metabolism, and the synthesis of flavor-related amino acids were screened out. A total of 117 enzymes were identified and enriched in the aforementioned pathways (Fig. 7A).

It is known that the most crucial aspect of carbohydrate metabolism in the process of Baijiu brewing involved the degradation of macromolecular substances, such as starch, into glucose. This process served as a pivotal source of carbon to facilitate microbial growth. Among 7 rounds, JC2 and JC6 exhibite a greater numbers of carbohydrate-related enzymes compared to other rounds. Specifically, pectinesterase (EC:3.1.1.11), cellulase (EC:3.2.1.4), and chitin deacetylase (EC:3.5.1.41) were most abundant in JC2 while chitinase (EC:3.2.1.14), amylase (EC:3.2.1.1) and glucoamylase (EC:3.2.1.3) exhibited the highest abundance in JC6 (Fig. 7B). Correlation analysis showed that these enzymes were positively related to the dominate fungi of JC2 and JC6 (Fig. S2B). Among these, *Fusarium* was confirmed to have a strong ability to produce cellulase, while both *Penicillium* and *Fusarium* were able to secrete chitinase[63]. Additionally, *Aspergillus* demonstrated the capability to produce amylase[42]. Bacterial-derived chitinase (EC:3.2.1.14) and *β*-xylosidases (EC:3.2.1.37) played a significant role in JC7 (Fig. 7B) and positively related to the dominate bacteria of JC7 (Fig. S2A). These findings suggest that the main functional enzymes of carbohydrate degradation were concentrated mainly in the fungi of JC2 and JC6, while in the bacteria of JC7. And this result was aligning with the trend of enzyme-producing microorganisms among the microbial community biomarkers.

Amino acids play a crucial role as essential nutrients for microbial growth and metabolism. Besides, free amino acids serve as precursors for higher alcohol (*n*-propanol, isobutanol, 2-methylbutanol, and 3-methylbutanol)[64] and acetoin, which was the precursor of pyrazine compounds[65], 2,3-butanediol and 2-butanol[66]. Among 7 rounds, JC6 has the most enzymes related to amino acid metabolism (100), followed by JC1 (49). Specifically, diacetyl reductase ((*R*)-acetoin forming) (EC:1.1.1.303) and diacetyl reductase ((*S*)-acetoin forming) (EC:1.1.1.304) were responsible for acetoin synthesis, while (*R*,*R*)-butanediol dehydrogenase (EC:1.1.1.4) and (*S*,*S*)-butanediol dehydrogenase (EC:1.1.1.76) facilitate the interconversion of acetoin and 2,3-butanediol. Remarkably, JC6 exhibited a higher abundance

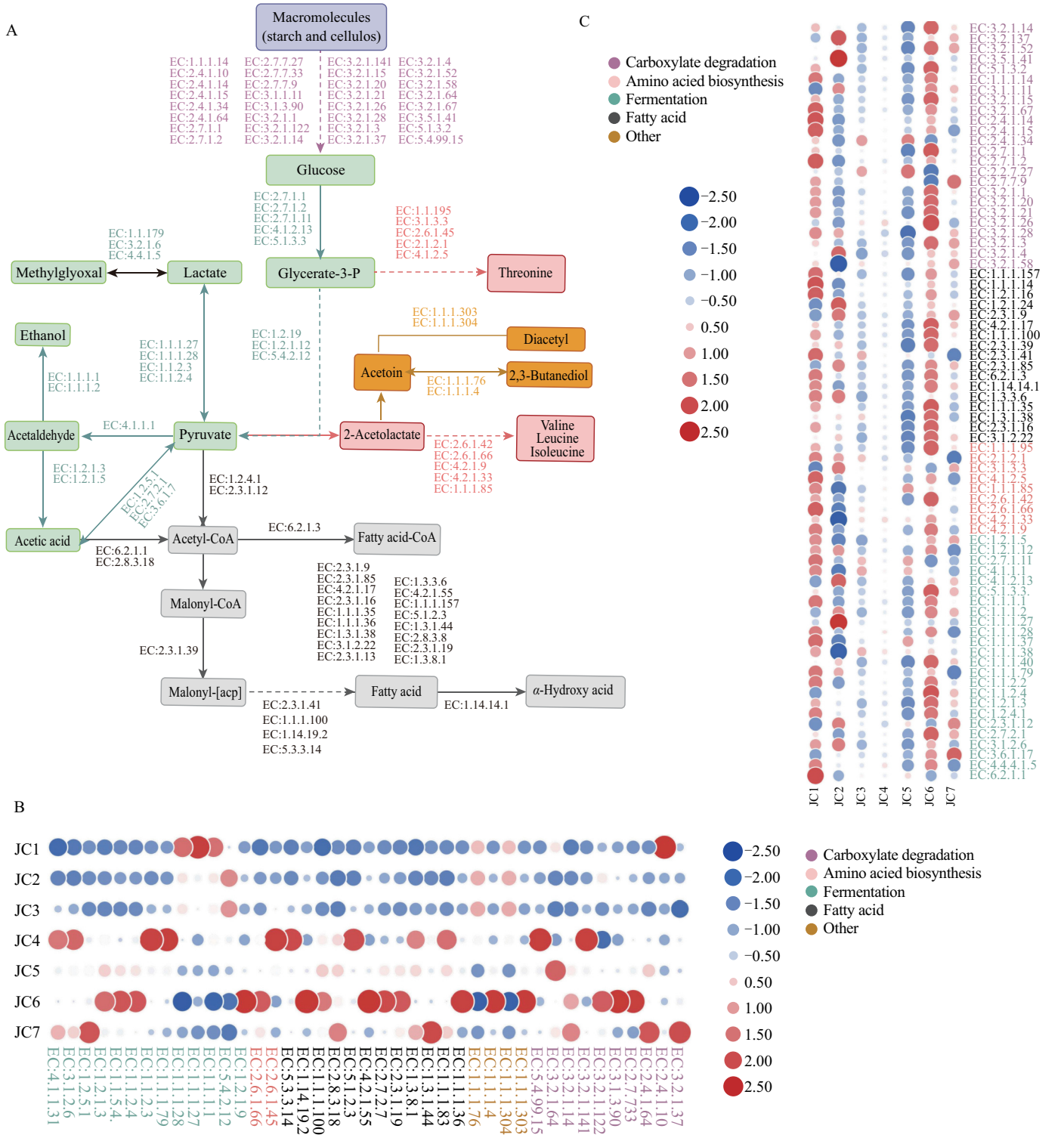

Fig. 7 Heatmap of functional enzyme of microorganisms during 7 rounds of pit fermentation of JFB. (A) Pathways related to flavor substances; (B) Abundance changes of corresponding enzymes in 7 rounds of bacterial communities; (C) Abundance changes of corresponding enzymes in 7 rounds of fungal communities.

of EC:1.1.1.303 and EC:1.1.1.4, whereas JC1–JC4 showed elevated abundance of EC:1.1.1.304 and EC:1.1.1.76. Correlation analysis showed that EC:1.1.1.303 and EC:1.1.1.4 were mainly positively correlated with *Bacillus* while EC:1.1.1.304 and EC:1.1.1.76 were mainly positively correlated with *Lactobacillus* (Fig. S2A). Besides, *Bacillus* has been reported to be the important microorganism for the synthesis of acetoin and pyrazine[67], and *Lactobacillus* could benefit the synthesis of 2-butanol[68]. Previous studies[1,24,69] showed that the content of pyrazines in JC6 was higher and that of 2-butanol in JC1 was higher than other rounds. These results indicating that *Lactobacillus* in the early stage mainly mediated the conversion of acetoin to 2-butanol, while *Bacillus* promoted the synthesis of pyrazines by acetoin in the later stage. For the synthesis of higher alcohols, threonine, valine, isoleucine, and

leucine serve as precursor substances for n-propanol, isobutanol, 2-methylbutanol, and 3-methylbutanol respectively. Among them, enzymes such as EC:1.1.1.95, EC:3.1.3.3, EC:2.6.1.45, EC:2.1.2.1, and EC:4.1.2.5 were involved in threonine synthesis. Fig. 7 shows that their combined abundance was found to be highest in JC1, potentially explaining the elevated content of n-propanol in JC1[24,69]. For the synthesis of valine, isoleucine, and leucine, serval enzymes, including EC:2.6.1.42, EC:2.6.1.66, EC:4.2.1.9, EC:4.2.1.33, and EC:1.1.1.85, played a role. Notably, fungi contribute predominantly to enzymes such as EC:2.6.1.42 and EC:4.2.1.9. Their abundance was highest in JC1 and JC6, aligning with the patterns observed for alcohol dehydrogenase (EC:1.1.1.1). The correlation results showed that the above enzymes were significantly positively correlated with the markers of JC1 and JC6 (*Colletotrichum*, *Thermomyces*, *Torulaspora*, *Arthrinium*, and *Thermoascus*). *Torulaspora* was reported to have the ability to produce higher alcohols[70]. Previous studies[24,69] also indicated higher levels of isobutanol and isoamyl alcohol in JC1 and JC6 compared to other rounds. These findings provided a possible explanation for the reported higher alcohol levels in the base Baijiu of JC1 and the increased pyrazine content in the base Baijiu of JC6[24].

Acetic acid, ethanol, and lactic acid were pivotal compounds with the highest yield during pit fermentation, playing significant roles in maintaining the fermentation environment and imparting flavor to Baijiu[9]. As shown in Fig.7A, lactic acid could derive from pyruvate and methylglyoxal. Notably, the abundance of lactate dehydrogenase from bacteria (EC:1.1.1.27 and EC:1.1.1.28) decreased across JC1–JC7. Correlation analysis also showed that these two enzymes were significantly positively correlated with *Lactobacillus*. Previous studies have also shown that *Lactobacillus* as a principal lactic acid producer[71]. Focusing on lactate-related enzymes from fungi, the total abundance of these enzymes (EC:1.1.1.27, EC:1.1.1.28, EC:1.1.1.79, EC:3.1.2.6, and EC:4.4.1.5) was highest in JC1, followed by JC2. These outcomes corresponded to the acidity results in Fig.1. Acetaldehyde, a precursor of ethanol and acetic acid, originated from the decarboxylation of pyruvate. As revealed in Figs. 7B and C, pyruvate decarboxylase (EC:4.1.1.1) displayed heightened abundance in JC1, JC3, JC5, and JC4, indicating that a significant potential for alcohol production in these rounds. However, alcohol dehydrogenase (EC:1.1.1.1 and EC:1.1.1.2), which was provided by both bacteria and fungi, displayed high abundance in JC1, JC6, and JC7. This was inconsistent with the conclusion that the alcohol yields were the highest in JC3–JC5[18]. This discrepancy may stem from acetaldehyde dehydrogenase catalyzing higher aldehydes to generate higher alcohols. Acetic acid forms from acetaldehyde *via* acetaldehyde dehydrogenase. bacterial-derived acetaldehyde dehydrogenase (EC:1.2.1.3) was primarily abundant in JC4–JC7, while fungal-derived acetaldehyde dehydrogenase (EC: 1.2.1.3 and EC:1.2.1.5) were notable in JC6 and JC1. Additionally, pyruvate could yield acetate through other pathways (EC: 1.2.5.1, EC:2.7.2.1, and EC:3.6.1.7), with these enzymes being more prevalent in JC6 and JC7. Thus, the enzymes that generate acetate showed the highest abundance in JC6, and this was inconsistent with the content of acetic acid in the literature[24]. This incongruity may stem from EC:1.2.1.3, the most prevalent enzyme, also engaging in fatty acid degradation and amino acid metabolism.

Fatty acid was an important taste substance in Baijiu and fatty acid CoA served as the vital precursor for esters formation[72]. A total of 28 enzymes related to fatty acid synthesis were screened out in 7 rounds. Among these, Acetyl-CoA, the important precursor of fatty acid and fatty acid CoA, could be generated from pyruvate and acetate to through EC:6.2.1.1, EC:2.8.3.18, EC:1.2.4.1, and EC:2.3.1.12. Their abundance was high in JC1, and JC2, in accordance with previous research that highlighted the elevated levels of total ethyl esters, particularly ethyl acetate[24]. The enzyme EC:1.1.1.100 was involved in fatty acid elongation, exhibiting increased abundance in bacteria across JC1–JC6 while diminishing in JC7. This result was consistent with the variation in acid content observed in 7 rounds of JFB[68].

4. Conclusion

In summary, this study revealed the similarities and differences in the structure and potential function of microbial communities, and identified biomarkers and driving factors in 1–7 rounds of the pit fermentation process. Results showed that the bacterial structure across 7 rounds was dominated by *Lactobacillus*, while the fungal community structure among 7 rounds showed discernible dissimilarities. The driving factors of the microbial community were dissimilarities among 7 rounds. Acidity mainly regulated the microbial community in the first 4 rounds of fermentation, whereas nutrient limitation was the main cause of microbial succession from the 5th round. Pertinently, functional predictive analysis underscored the enrichment of amino acid metabolism-related enzymes within JC6, further emphasizing the roles of functional enzymes within JC2, JC6, and JC7 in carbohydrate degradation during the fermentation process. Overall, this study enhanced our comprehension of microbial community structure, succession, biomarkers identification, and metabolic attributes across 7 rounds, providing a pivotal foundation for the precise regulation and enhancement of pit fermentation, ultimately producing high-quality JFB.

Declaration of interest

The authors declared that there is no conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://doi.org/10.26599/FSHW.2024.9250344.

References

[1] Q. Liu, D. He, Y. Ma, et al., Sensory profile and the contribution of key aroma compounds in jiang-flavor rounded-base Baijiu produced in the Chishui river basin, LWT-Food Sci. Technol. 189 (2023) 115474. https://doi.org/10.1016/j.lwt.2023.115474.

[2] L. Wang, Research trends in jiang-flavor baijiu fermentation: from fermentation microecology to environmental ecology, J. Food Sci. 87 (2022) 1362-1374. https://doi.org/10.1111/1750-3841.16092.

[3] F. Hao, Y. Tan, X. Lv, et al., Microbial community succession and its environment driving factors during initial fermentation of Maotai-flavor Baijiu, Front. Microbiol. 12 (2021) 669201. http://doi.org/10.3389/fmicb.2021.669201.

[4] W. Wang, Y. Xu, H. Huang, et al., Correlation between microbial communities and flavor compounds during the fifth and sixth rounds of sauce-flavor Baijiu fermentation, Food Res. Int. 150 (2021) 110741. http://doi.org/10.1016/j.foodres.2021.110741.

[5] L. Wang, K. Zhong, A. Luo, et al., Dynamic changes of volatile compounds and bacterial diversity during fourth to seventh rounds of Chinese soy sauce aroma liquor, Food Sci. Nutr. 9 (2021) 3500-3511. http://doi.org/10.1002/fsn3.2291.

[6] Y. Xu, M. Wu, J. Niu, et al., Characteristics and correlation of the microbial communities and flavor compounds during the first three rounds of fermentation in Chinese sauce-flavor Baijiu, Foods 12 (2023) 207. http://doi.org/10.3390/foods12010207.

[7] L. Wang, Analysis of microbial community structure characteristics of fermented grains of pit fermentation of Maotai flavor Baijiu, Guizhou University, 2021. http://doi.org/10.27047/d.cnki.ggudu.2021.002441.

[8] M. Wang, Q. Zhao, C. Su, et al., Analysis of the microbial community structure during brewing of Sichuan Xiaoqu Baijiu, J. Am. Soc. Brew. Chem. 77 (2019) 210-219. http://doi.org/10.1080/03610470.2019.1605033.

[9] Z. Song, H. Du, Y. Zhang, et al., Unraveling core functional microbiota in traditional solid-state fermentation by high-throughput amplicons and metatranscriptomics sequencing, Front. Microbiol. 8 (2017) 1294. http://doi.org/10.3389/fmicb.2017.01294.

[10] H. Wang, Y. Huang, Y. Huang, Microbiome diversity and evolution in stacking fermentation during different rounds of jiang-flavoured Baijiu brewing, LWT-Food Sci. Technol. 143 (2021) 111119. http://doi.org/10.1016/j.lwt.2021.111119.

[11] C. Zhu, Y. Cheng, Q. Shi, et al., Metagenomic analyses reveal microbial communities and functional differences between Daqu from seven provinces, Food Res. Int. 172 (2023) 113076. http://doi.org/10.1016/j.foodres.2023.113076.

[12] J. Niu, S. Yang, Y. Shen, et al., What are the main factors that affect the flavor of sauce-aroma Baijiu, Foods 11 (2022) 3534. http://doi.org/10.3390/foods11213534.

[13] L. Yang, C. Xian, P. Li, et al., The spatio-temporal diversity and succession of microbial community and its environment driving factors during stacking fermentation of Maotai-flavor baijiu, Food Res. Int. 169 (2023) 112892. http://doi.org/10.1016/j.foodres.2023.112892.

[14] M. Wang, J. Yang, Q. Zhao, et al., Research progress on flavor compounds and microorganisms of Maotai Flavor Baijiu, J. Food Sci. 84 (2019) 6-18. http://doi.org/10.1111/1750-3841.14409.

[15] H. Du, X. Wang, Y. Zhang, et al., Exploring the impacts of raw materials and environments on the microbiota in Chinese Daqu starter, Int. J. Food Microbiol. 297 (2019) 32-40. https://doi.org/10.1016/j.ijfoodmicro.2019.02.020.

[16] Y. Tan, H. Zhong, D. Zhao, et al., Succession rate of microbial community causes flavor difference in strong-aroma Baijiu making process, Int. J. Food Microbiol. 311 (2019) 108350. https://doi.org/10.1016/j.ijfoodmicro.2019.108350.

[17] H. Zhang, L. Wang, H. Wang, et al., Effects of initial temperature on microbial community succession rate and volatile flavors during Baijiu fermentation process, Food Res. Int. 141 (2021) 109887. https://doi.org/10.1016/j.foodres.2020.109887.

[18] Y. Dai, Z. Tian, W. Meng, et al., Microbial diversity and physicochemical characteristics of the Maotai-flavored liquor fermentation process, J. Nanosci. Nanotechno. 20 (2020) 4097-4109. https://doi.org/10.1166/jnn.2020.17522.

[19] C. Wu, J. Huang, R. Zhou, Genomics of lactic acid bacteria: current status and potential applications, Crit. Rev. Microbiol. 43 (2017) 393-404. https://doi.org/10.1080/1040841X.2016.1179623.

[20] P. Liu, L. Miao, Multiple batches of fermentation promote the formation of functional microbiota in Chinese miscellaneous-flavor Baijiu fermentation, Front. Microbiol. 11 (2020) 75. https://doi.org/10.3389/fmicb.2020.00075.

[21] T. Fernandes, F. Silva-sousa, F. Pereira, et al., Biotechnological importance of *Torulaspora delbrueckii*: from the obscurity to the spotlight, J. Fungi 7 (2021) 712. https://doi.org/10.3390/jof7090712.

[22] J. Liu, Y. Chen, G. Fu, et al., Improvement of the flavor of major ethyl ester compounds during Chinese Te-flavor Baijiu brewing by *Wickerhamomyces anomalus*, Food Biosci. 50 (2022) 102022. https://doi.org/10.1016/j.fbio.2022.102022.

[23] Z. Li, S. Wu, X. Long, et al., Isolation, identification and characteristics of aroma-producing yeast for rice wine, Food and Fermentation Industries 47 (2021) 43-50. https://doi.org/10.13995/j.cnki.11-1802/ts.025463.

[24] S. Shen, J. Liu, R Luo, et al., Analysis of the influence of microbial community structure on flavor composition of jiang-flavor liquor in different batches of pre-pit fermented grains, Fermentation 8 (2022) 671. https://doi.org/10.3390/fermentation8120671.

[25] E. Grady, J. Macdonald, L. Liu, et al., Current knowledge and perspectives of *Paenibacillus*: a review, Microb. Cell Fact. 15 (2016) 203. https://doi.org/10.1186/s12934-016-0603-7.

[26] J. Xu, S. Qiu, B. Hu, et al., Research on functions of the mold in Maotai-flavor liquor brewing, Liquor Making 42 (2015) 32-38. http://doi.org/10.3969/j.issn.1002-8110.2015.05.011.

[27] J. Sun, W. Liu, W. Qi, et al., Composition of mold communities and functional enzyme activities in Maotai-flavor liquor fermented grains, Journal of Chinese Institute of Food Science and Technology 13 (2013) 9. https://doi.org/10.16429/j.1009-7848.2013.08.026.

[28] X. Wang, Q. Ge, Y. Niu, et al., A *Cryptococcus albicans* strain *Naganishia albida*YC22 with high production of aroma substances and its application, CN202110088808.X,2021.

[29] Y. Xu, K. Ji, 15-Moutai (Maotai) production and sensory properties, Alcoholic Beverages 25 (2012) 315-330. https://doi.org/10.1533/9780857095176.3.315.

[30] H. Li, J. Chen, A. Li, et al., Purification and partial characterization of β-1,3-glucanase from *Chaetomium thermophilum*, World J. Microb. Biot. 23 (2007) 1297-1303. https://doi.org/10.1007/s11274-007-9366-y.

[31] Y. Yang, S. Wang, Z. Lu, et al., Metagenomics unveils microbial roles involved in metabolic network of flavor development in medium-temperature daqu starter, Food Res. Int. 140 (2021) 110037. https://doi.org/10.1016/j.foodres.2020.110037.

[32] R. St-Leger, R. Cooper, A. Charnley, Characterization of chitinase and chitobiase produced by the entomopathogenic fungus *Metarhizium anisopliae*, J. Invertebr. Pathol. 58 (1991) 415-426. https://doi.org/10.1016/0022-2011(91)90188-V.

[33] D. Korcari, G. Ricci, C. Capusoni, et al., Physiological performance of *Kazachstania* unispora in sourdough environments, World J. Microbiol Biotechnol. 37 (2021) 88. https://doi.org/10.1007/s11274-021-03027-0.

[34] I. Jood, J. Hoff, M. Setati, Evaluating fermentation characteristics of Kazachstania spp. and their potential influence on wine quality, World J. Microb. Biot. 33 (2017) 129. https://doi.org/10.1007/s11274-017-2299-1.

[35] V. Blättel, M. Larisika, P. Pfeiffer, et al., Beta-1,3-glucanase from *Delftia tsuruhatensis* strain MV01 and its potential application in vinification, Appl. Environ. Microb. 77 (2011) 983-990. https://doi.org/10.1128/aem.01943-10.

[36] J. Wang, D. Ma, Y. Wang, et al., *Lentibacillus amyloliquefaciens*sp. nov., a halophilic bacterium isolated from saline sediment sample, Antonie van Leeuwenhoek. 109 (2016) 171-178. https://doi.org/10.1007/s10482-015-0618-9.

[37] N. Deng, H. Du, Y. Xu, Cooperative response of *Pichia kudriavzevii* and *Saccharomyces cerevisiae* to lactic acid stress in Baijiu fermentation, J. Agric. Food Chem. 68 (2020) 4903-4911. https://doi.org/10.1021/acs.jafc.9b08052.

[38] T. Guan, X. Wu, R. Hou, et al., Application of *Clostridium butyricum*, *Rummeliibacillus suwonensis*, and *Issatchenkia orientalis* for Nongxiangxing baijiu fermentation: improves the microbial communities and flavor of upper fermented grain, Food Res. Int. 169 (2023) 112885. https://doi.org/10.1016/j.foodres.2023.112885.

[39] X. Wu, Diversity and dynamics of yeasts and bacteria during the solid state fermentative process contributing to Chinese Maotai-flavor liquor making, Wuxi, Jiangnan University, 2013.

[40] Y. Jin, D. Li, M. Ai, et al., Correlation between volatile profiles and microbial communities: a metabonomic approach to study Jiang-flavor liquor daqu, Food Res. Int. 121 (2019) 422-432. https://doi.org/10.1016/j.foodres.2019.03.021.

[41] C. Zhao, X. Yan, S. Yang, et al., Screening of *Bacillus* strains from Luzhou-flavor liquor making for high-yield ethyl hexanoate and low-yield propanol, LWT-Food Sci. Technol. 77 (2017) 60-66. https://doi.org/10.1016/j.lwt.2016.11.035.

[42] Y. Huang, Q. Wu, Y. Xu, Isolation and identification of a black *Aspergillus* strain and the effect of its novel protease on the aroma of Moutai-flavoured liquor, J. I. Brewing. 120 (2014) 268-276. http://doi.org/ 10.1002/jib.135.

[43] S. Mcclendon, T. Batth, C. Petzold, et al., *Thermoascus aurantiacus* is a promising source of enzymes for biomass deconstruction under thermophilic conditions, Biotechnol. Biofuels 5 (2012) 54. https://doi.org/10.1186/1754-6834-5-54.

[44] S. Zhang, T. Wang, Y. Zhang, et al., Effects of *Monascus* on proteolysis, lipolysis, and volatile compounds of camembert-type cheese during ripening, Foods 11 (2022) 1662. https://doi.org/10.3390/foods11111662.

[45] Y. Wee, L. Reddy, H.W. Ryu, Fermentative production of *L*(+)-lactic acid from starch hydrolyzate and corn steep liquor as inexpensive nutrients by batch culture of *Enterococcus faecalis* RKY1, J. Chem. Technol. Biot. 83 (2008) 1387-1393. https://doi.org/10.1002/jctb.1953.

[46] H. Onyeaka, O. Nwabor, Chapter 11-lactic acid bacteria and bacteriocins as biopreservatives, in Food preservation and safety of natural products, Academic Press, 2022, pp: 147-162. https://doi.org/10.1016/B978-0-323-85700-0.00012-5.

[47] X. Yu, H. Feng, L. Zhai, et al., Dynamic changes of *Thermoactinomyces* and their functional genes in high temperature Daqu of Sesame-flavored Baijiu, Food and Fermentation Industries 45 (2019) 71-77. https://doi.org/10.13995/j.cnki.11-1802/ts.019959.

[48] P. Wang, Q. Wu, X. Jiang, et al., *Bacillus licheniformis* affects the microbial community and metabolic profile in the spontaneous fermentation of Daqu starter for Chinese liquor making, Int. J. Food Microbiol. 250 (2017) 59-67. http://doi.org/10.1016/j.ijfoodmicro.2017.03.010.

[49] J. Houbraken, R. Samson, J. Frisvad, *Byssochlamys*: significance of heat resistance and mycotoxin production, Adv. Exp. Med. Biol. 571 (2006) 211. https://doi.org/10.1007/0-387-28391-9_14.

[50] A. Galanopoulou, I. Haimala, D. Georgiadou, et al., Characterization of the highly efficient acid-stable xylanase and *β*-xylosidase system from the fungus *Byssochlamys spectabilis* athum 8891 (*Paecilomyces variotii* athum 8891), J. Fungi 7 (2021) 430. https://doi.org/10.3390/jof7060430.

[51] V. Rojas, J. Gil, F. Piñaga, et al., Studies on acetate ester production by non-*Saccharomyces* wine yeasts, Int. J. Food Microbiol. 70 (2001) 283-289. https://doi.org/10.1016/s0168-1605(01)00552-9.

[52] J. Wei, J. Lu, Y. Nie, et al., Amino acids drive the deterministic assembly process of fungal community and affect the flavor metabolites in Baijiu fermentation, Microbiol. Spectr. 11 (2023) e0264022. https://doi.org/10.1128/spectrum.02640-22.

[53] S. Ma, H. Luo, D. Zhao, et al., Environmental factors and interactions among microorganisms drive microbial community succession during fermentation of Nongxiangxing daqu, Bioresour. Technol. 345 (2022) 126549. https://doi.org/10.1016/j.biortech.2021.126549.

[54] X. Li, F. Huang, X. Chang, et al., The status and analysis of the nutritional characteristics of acetic acid bacteria, Food Res. Dev. 39 (2018) 219-224. https://doi.org/10.3969/j.issn.1005-6521.2018.15.042.

[55] S. Gao, Z. Li, Y. Hou, et al., Effects of different carbon sources on the efficiency of sulfur-oxidizing denitrifying microorganisms, Environ. Res. 204 (2022) 111946. https://doi.org/10.1016/j.envres.2021.111946.

[56] X. Sun, J. Wei, J. Li, et al., Whole-genome analysis of the dominant bacterium *Dysgonomonas macrotermitis* in the hindgut of *Macrotermes barneyi*, Acta Microbiol. Sin. 58 (2018) 995-1003. https://doi.org/10.13343/j.cnki.wsxb.20170340.

[57] J. Wang, Y. Li, Z. Ruan, et al., Complete genome sequence of strain *Lentibacillus amyloliquefaciens* LAM0015T isolated from saline sediment, J. Biotechnol. 220 (2016) 88-89. https://doi.org/10.1016/j.jbiotec.2016.01.019.

[58] A. Hussein, B. Lisowska, D. Leak, Chapter 1-the genus *Geobacillus* and their biotechnological potential, in: Advances in applied microbiology, Academic Press, 2015, pp. 1-48. https://doi.org/10.1016/bs.aambs.2015.03.001.

[59] F. Lv, Y. Zhan, W. Lu, et al., Regulation of hierarchical carbon substrate utilization, nitrogen fixation, and root colonization by the Hfq/Crc/CrcZY genes in *Pseudomonas stutzeri*, iScience 25 (2022) 105663. https://doi.org/10.1016/j.isci.2022.105663.

[60] S. Wang, Q. Wang, W. Xiang, et al., Purification and characterization of the thermostable protease from *Fusarium oxysporim* M1, Microbiology China 48 (2021) 1604-1615. https://doi.org/10.13344/j.microbiol.china.200741.

[61] Q. Ning, L. Chen, F. Li, et al., Effects of mortierella on nutrient availability and straw decomposition in soil, Acta Pedologica Sinica 59 (2022) 206-217. https://doi.org/10.11766/trxb202006020213.

[62] Q. Wu, Y. Kong, et al., Flavor profile of Chinese liquor is altered by interactions of intrinsic and extrinsic microbes, Appl. Environ. Microbiol. 82 (2016) 422-430. http://10.1128/AEM.02518-15.

[63] P.V. Suresh, P.Z. Sakhare, N.M. Sachindra, et al., Extracellular chitin deacetylase production in solid state fermentation by native soil isolates of *Penicillium monoverticillium* and *Fusarium oxysporum*, J. Food Sci. Technol. 51 (2014) 1594-1599. https://doi.org/10.1007/s13197-012-0676-1.

[64] L. Hazelwood, J. Daran, M. Van, et al., The Ehrlich pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae* metabolism, Appl. Environ. Microbiol. 74 (2008) 2259-2266. https://doi.org/10.1128/AEM.02625-07.

[65] C. Shu, Pyrazine formation from amino acids and reducing sugars, a pathway other than Strecker degradation, J. Agr. Food Chem. 46 (1998) 1515-1517. https://doi.org/10.1021/jf970999i.

[66] D. Cui, Y. Wei, L. Lin, et al., Increasing yield of 2,3,5,6-tetramethylpyrazine in baijiu through *Saccharomyces cerevisiae* metabolic engineering, Front. Microbiol. 11 (2020) 596306. https://doi.org/10.3389/fmicb.2020.596306.

[67] H. Wang, C. Sun, S. Yang, et al., Exploring the impact of initial moisture content on microbial community and flavor generation in Xiaoqu baijiu fermentation, Food Chem.: X 20 (2023) 100981. https://doi.org/10.1016/j.fochx.2023.100981.

[68] L. Li, Y. Ma, Y. Huang, et al., Analysis of volatile flavor compounds in base liquor for maotai-flavor Baijiu during mechanized fermentation, Food Science 42 (2021) 199-206. http://doi.org/10.7506/spkx1002-6630-20200530-366.

[69] N. Xie, Y. Shi, J. Li, et al., Research progress in biological production of chiral acetoin, Guangxi Sciences 24 (2017) 33-39. https://doi.org/10.13656/j.cnki.gxkx.20170119.003.

[70] S. Benito, The impact of *Torulaspora delbrueckii* yeast in winemaking, Appl. Microbiol. Biotechnol. 102 (2018) 3081-3094. https://doi.org/10.1007/s00253-018-8849-0.

[71] Y. Zhang, Community structure and function of lactic acid bacteria during Maotai-flavor liquor fermentation, Wuxi, Jiangnan University, 2015.

[72] W. Shi, J. Li, Y. Chen et al., Enhancement of C6–C10 fatty acid ethyl esters production in *Saccharomyces cerevisiae* CA by metabolic engineering, LWT-Food Sci. Technol. 145 (2021) 111496. https://doi.org/10.1016/j.lwt.2021.111496.