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Exploring the impact of initial moisture content on microbial community and flavor generation in Xiaoqu baijiu fermentation

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ABSTRACT

Moisture is essential in microbiota succession and flavor formation during baijiu fermentation. However, it remains unknown how moisture content affects microbiota, metabolism, and their relationship. Here, we compared the difference in volatiles, microbiota characteristics, and potential functions with different initial moisture contents (50 %, 55 %, 60 %, 65 %, 70 %). Results showed that the ratio of ethyl acetate to ethyl lactate and total volatile compounds content increased as the moisture content was elevated from 50 % to 70 %. As increasing moisture content, fermentation system microbiota dominated by *Lactobacillus* was formed more rapidly. *Lactobacillus, Dekkera,* and *Pediococcus* were positively correlated with moisture, promoting the production of propanol, acetic acid, butyric acid, and 2-butanol. The complexity and stability of ecological networks enhanced as moisture content increased ($R^2 = 0.94$, $P = 0.004$). Our study revealed that moisture-drive microbiota was a critical contributor to flavor formation, providing the theoretical basis for moisture control to regulate flavor compounds.

1. Introduction

Solid-state fermentation (SSF) is generally defined as the growth of micro-organisms on (moist) solid material in the absence or near-absence of free water (Pandey, Soccol, & Larroche, 2008). Previous studies have shown that moisture content also affects many aspects of solid-state fermentation. Firstly, moisture content directly affects the thrive or languish of microbial consortia by changing the availability and diffusion of nutrients, the supply of oxygen, the heat dissipation (Patrick Gervais & Molin, 2003). Secondly, moisture content indirectly influences microbial competition and succession. Strains best adapted to the given moisture conditions dominate the ecosystem, steering the flavor profile through their metabolites. Meanwhile, the balance between yeasts and bacteria, influenced by water's availability, contributes to the intricate flavors that develop during fermentation. Finally, moisture content affected the yield and quality of the product produced during solid-state fermentation. Water's unique ability to solubilize a wide range of compounds allows it to act as a conduit for flavor molecules, which influences the extracellular excretion of aroma (Gervais, Belin, Grajek, & Sarrette, 1988), and the separation of flavor compounds. Therefore, maintaining an appropriate moisture content is crucial for successful fermentation and high-quality product production.

As a typical solid-state fermentation, moisture content regulation is a key operation in the fermentation process across all types of baijiu. For example, the moisture content of the fermented grain is adjusted to facilitate normal fermentation before pit fermentation of Nong flavor baijiu, which is called "Daliangshui". Similar operations also exist in Xiasha, Zaosha round in Jiang flavor baijiu. In Xiaoqu baijiu, adding distilled grains with a high moisture content (usually 70 %) also is a way to regulate moisture content. However, the moisture content of different batches of fermented grains can vary significantly (2 %–5 %) due to the lack of water monitoring and control procedures in the actual fermentation process, such as grain moistening, steaming, and distillation. As a result, the yield and quality of base baijiu differ among batches and cellars. In the process of baijiu fermentation, a complex and dynamically changing microbial community plays the core role in the conversion of raw substrates into a diverse array of flavor compounds (Wu, Zhu, Fang, Wijffels, & Xu, 2021). Previous studies also found that moisture content

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was one of the abiotic factors that drive the composition and succession of microbial communities in baijiu fermentation ecosystems (Wu, Du, & Xu, 2023; Zhu, Cheng, Zuo, Huang, & Wang, 2022). However, the role of moisture content in the regulation of microbial community, microbial interaction, and flavor formation is poorly understood in baijiu fermentation, due to the operation restriction. Therefore, it is urgent to understand the internal relationship between moisture content, the dynamic of the microbial community, and the formation of flavor compounds in detail and systematically.

In different types of baijiu, Xiaoqu baijiu is a well-known alcoholic beverage that is highly regarded for its distinctive flavor profile. Moreover, Xiaoqu baijiu commands a broader market presence compared to other types of baijiu because Xiaoqu baijiu is not only a popular commercial baijiu but also a base baijiu for Lujiu (a baijiu-based beverage), and integrated alcoholic beverage (medicinal baijiu, and Chinese cocktails) (Zhang, Yang, Ao, Guo, Su, & Shen, 2017). Meanwhile, the simple fermentation process, short fermentation time, and high yield of Xiaoqu baijiu make it an ideal system for exploratory research. Hence, we used the case of Xiaoqu baijiu to investigate the change patterns of microbial communities and flavor substances under a moisture content gradient. Furthermore, the mechanism underlying the effects of initial moisture content on fermentation parameters, microbial community, and flavor substances was investigated. This research provides some theoretical basis for understanding how moisture affects microbial succession and provides a guiding significance for the improvement and stable production of Xiaoqu baijiu, as well as fermented foods like baijiu.

2. Methods

2.1. Experiment design and sample collection

The fermented grain sample used in this study was sourced from the Fenglin Distillery of the Jing Brand Co., Ltd. in Hubei, China. The initial moisture content of the fermented grains was adjusted by mixing saccharified grains with distilled grains to achieve five different levels (Fig. S1 and Table S1): 70 %, 65 %, 60 %, 55 %, and 50 %, which were labeled as A1-A5. Fermentation commenced after mixing and lasted for 21 days in the medium-sized tanker. In order to make the experiment more scientific, each group dealt with three repetitions, and samples were collected from the upper, middle, and lower layers of the fermentation mixture on days 0 (beginning of fermentation), 5, 10, 15, and 21 (end of fermentation) using a five-point sampling method. Samples collected on the same day were mixed under aseptic conditions. Finally, 73 mixed samples (365 samples) were obtained. The composite samples were divided into two parts: one was frozen in liquid nitrogen and stored at −20 °C for total DNA extraction, while the other was used to determine the fermentation parameters and flavor compounds.

2.2. Fermentation parameters detection

Fermentation parameters were measured, including moisture content, total titratable acidity, starch content, reducing sugar content, amino nitrogen, ammonia, acetic acid, lactic acid, and ethanol. The moisture content of the fermented grain sample was determined via a gravimetric method by drying the samples to a constant weight at 105 °C. Total titratable acidity, starch, and reducing sugar content were determined as described by Wang et al. (Wang, Huang, & Huang, 2021). The amino nitrogen (amino_N) of the fermentation grain was determined using ninhydrin colorimetry. Acetic acid, lactic acid, and ethanol were measured by a high-performance liquid chromatography (HPLC) (Agilent 1260 Infinity) system consisting of a quaternary gradient pump (1260 Iso Pump-G1310B), an autosampler (1260 ALS-C1328B) and a refractive index detector (1260 TCC-G1316A). Separations were performed on a Silgreen Ca/H column operating at 65 °C, the mobile phase was 5 mM sulfuric acid at a flow rate of 0.6 mL min^{-1}, and the running time of the program was 23 min. The pre-treatment of fermented grains was referred to the method of Wang et al. (Wang, Hai, Yan, & Yan, 2017), 5 g samples and 20 mL ultra-pure deionized water were mixed in a 50 mL centrifuge tube and then ultrasonically treated for 30 min, finally centrifuged at 4 °C and 6000*g* for 10 min. Then, the samples were diluted and filtered (0.22 μm RC syringe filters) and 20 μL were injected. The standard curves of each compound concentration were established to quantify compounds. Ammonia was determined using the Nessler reagent spectrophotometry method, which involves reacting ammonia with Nessler reagent to form a yellow–brown complex, the intensity of which can be measured at a specific wavelength using a spectrophotometer, in compliance with the Chinese national standards HJ 535-2009.

2.3. Volatile flavors compounds analysis

The pre-treatment of volatile flavors in fermented grains was referred to as the method of Wu et al. (Wu et al., 2023):10 g samples were diluted by 20 mL 50 % ethanol solution prepared from chromatographic pure ethanol and ultra-pure water. After fully oscillating, these samples were ultrasonically treated for 30 min in the ice bath and then placed at 4 °C for 4 h and filtered (0.22 μm RC syringe filters) to obtain the supernatant. Then, 1 mL supernatant or 1 mL base baijiu (obtained by distillation, and diluted to 50 % ethanol volume with ultrapure water) was put into a 2-mL injection vial, then spiked with an internal standard (10 μL) mixed with 2-methyl-2-butanol (IS1, 16,000 mg/L), amyl acetate (IS2, 16,000 mg/L), and 2-ethyl hexanol (IS3, 16,000 mg/L) for further analysis. The volatile flavors in final pretreated grain samples and base baijiu were analyzed by GC-FID (GC 7890 C; Agilent Technologies, Santa Clara, CA) with Agilent CP-WAX column (50 m × 0.25 mm i.d., 0.25 μm film thickness; J&W Scientific, Folsom, CA) in split mode (30:1). Nitrogen at a constant flow rate of 1 mL/min was used as carrier gas. The injector was held at 250 °C. The oven temperature was maintained at 35 °C for 1 min, then raised to 70 °C at a rate of 3 °C /min, held for 15 min, finally ramped to 190 °C at 3.5 °C /min, and kept at 190 °C for 22 min. Volatile flavor compounds were identified by matching with the retention time of standard compounds at the same chromatographic conditions, and the calibration curves of the corresponding substance evaluated the contents of volatile flavor components.

2.4. Sensory analysis

The sensory analysis method was performed as described by previous study (Sun et al., 2022) with minor modifications. Eight odor descriptors related to the sensory properties of Xiaoqu baijiu were chosen: caramel-like, fruity, sweet, fatty, alcoholic, acidic, grassy, and floral. Aroma profiling was carried out by ten well-trained assessors from the College of Biotechnology, Tianjin University of Science and Technology (four males and six females, aged 25–32). The group members were required to rate the intensity of each attribute on a six-point scale (0 means no odor, 5 means very strongly). Each sample (20 mL) was then transferred into a glass tube and presented in random order. The final results were averaged across the data of all evaluators, and they were then plotted as a spider web diagram.

2.5. Total DNA extraction, amplicon sequencing, and bioinformatics analysis

The sample pre-treatment and DNA extraction methods were obtained by Wang et al. (Wang, Hai, Yan, & Yan, 2017). For bacteria, the 16S rRNA gene was amplified using the universal primer sets 779F (5′-AACMGGATTAGATACCCKG-3′) and 1193R (5′-ACGTCATCCCCACCTTCC-3′). For fungi, the internal transcribed spacer (ITS) region was amplified with primers ITS3F (5′- GCATCGATGAAGAACGCAGC-3′) and ITS4R (5′- TCCTCCGCTTATTGATATGC-3′). The Illumina MiSeq sequencing was performed at Majorbio Bio-Pharm Technology, Shanghai.

Raw sequences were processed using Quantitative Insights into Microbial Ecology (QIIME) (v1.8.0). The representative bacterial operational taxonomic unit (OTU) sequences were annotated using the Silva 132_16 S rRNA database; the representative fungal OTU sequences were compared using the UNITE fungal ITS database (version 8.0). 97 % identity threshold was set to cluster the sequences using UPARSE (version 7.1, https://drive5.com/uparse/).

The α-diversity of OTU richness and the Shannon-Wiener index were evaluated to assess microbial diversity and abundance. The changes in community composition used the Bray-Curtis distance between the samples. A principal coordinate analysis (PCoA) was performed on the distance matrices to visualize the relationships among samples. A similarity analysis (ANOSIM) and a permutational multivariate analysis of variance (ADONIS) were performed to determine significant differences among the sample classifications.

2.6. Statistical analysis

Kruskal-Wallis H test followed by the fdr test was used to determine the significant differences. Spearman correlation analysis was calculated by using the Hmisc package in R software. The correlation coefficient ($|R| > 0.6$, $P < 0.05$) was considered as a valid strong correlation. Gephi software (version 9.5) was used for network visualization. The Mantel test based on was conducted by using a vegan package in R software. The line chart and principal components analysis (PCA) were drawn using Origin 2023. The heatmap was generated using TBtools software. Other figures were plotted by the R package ggplot2 and ccgraph.

3. Results and discussion

3.1. Effect of moisture content on volatile flavor compounds

The flavor of baijiu was the key criterion for deciding consumers' preference, and the impact of different moisture contents on the flavor compounds of baijiu was investigated. As shown in Fig. 1A, the total contents of flavor compounds in fermentation grains decreased as the moisture content was elevated from 70 % to 50 %, indicating the moisture content might be essential to flavor formation. In base baijiu, a decrement in the proportion of acids (20.68 %, 20.51 %, 16.76 %, 13.60 %, and 14.68 %, respectively, same below) and alcohols (60.97 %, 58.17 %, 42.99 %, 38.24 %, 35.72 %) was evident within the total flavor compounds as the moisture content decrease. Conversely, there was a rise in the proportion of esters (17.22 %, 19.97 %, 37.84 %, 44.15 %, and 41.42 %) and aldehydes and ketones (1.13 %, 1.36 %, 2.42 %, 4.02 %, and 8.17 %) within the total flavor compounds, with a decrease in

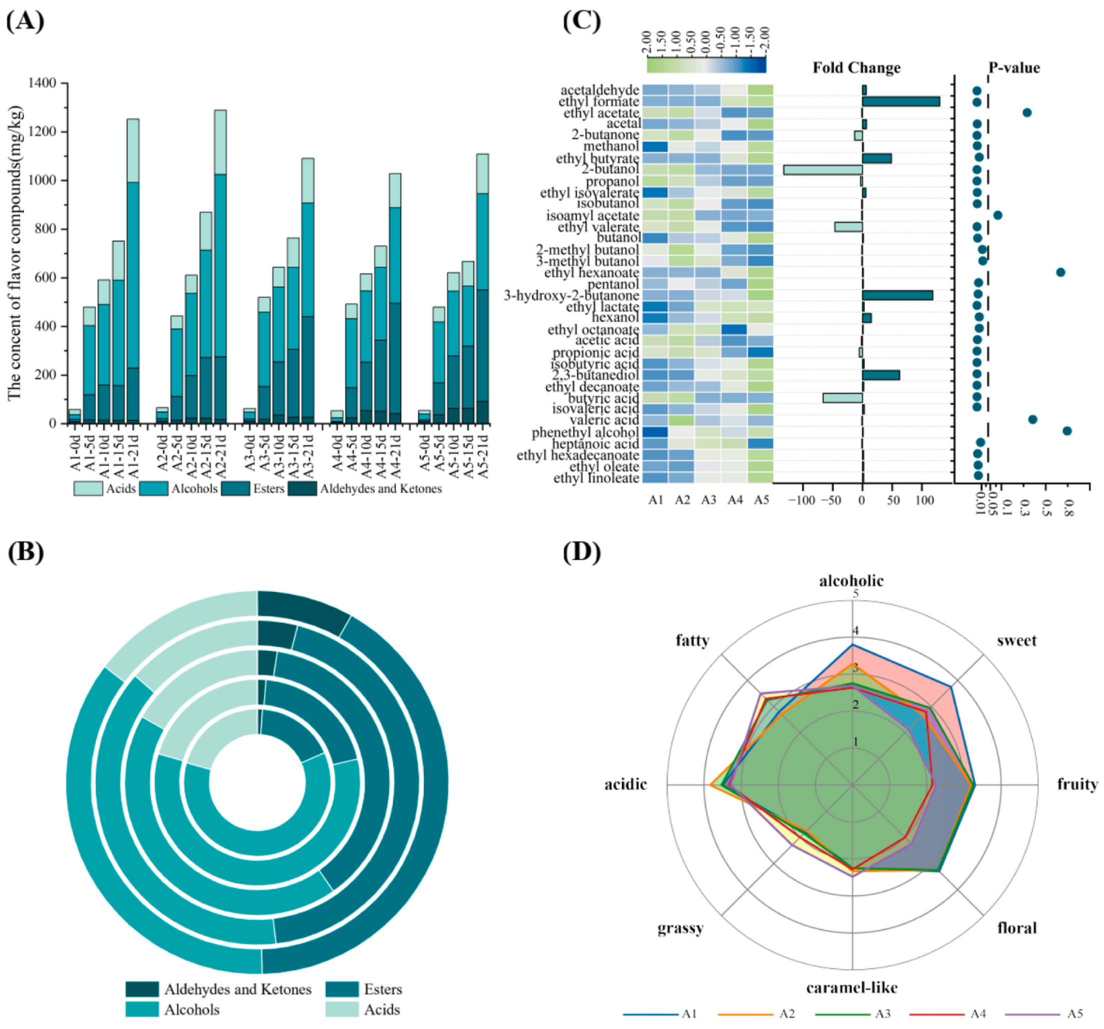

Fig. 1. Volatile flavor profile in different moisture contents, (A) the content of the volatile flavor compounds in fermented grains during the fermentation, (B) the proportion of various flavor substances in base baijiu, (C) heatmap of the volatile flavor compounds of base baijiu, (D) aroma descriptors of base baijiu. Note: The circle layer diagram represents A1 to A5 from the inside to the outside, respectively.

moisture content (Fig. 1B). These findings indicated that alterations in moisture content not only influence the content of flavor compounds but also impact the overall flavor profile and structure of base baijiu.

Specifically, most flavor compounds in base baijiu exhibit significant differences in varying moisture contents ($P < 0.05$), except for ethyl acetate, isoamyl acetate, ethyl hexanoate, valeric acid, and beta-phenethyl alcohol. As shown in Fig. 1C, seven compounds in A1 were significantly higher than in A5, including 2-butanone (13-fold), propanol (3-fold), 2-butanol (132-fold), acetic acid (2-fold), propionic acid (5-fold), butyric acid (66-fold), and ethyl valerate (46-fold). Among them, propanol was one of the primary higher alcohols in baijiu and contributed to the aged taste of baijiu with an appropriate amount (Wang, Sun, Wei, Guo, & Xiao, 2021). 2-butanol and ethyl valerate could contribute to the fruity aroma (Sun et al., 2022). Acetic acid and butyric acid could maintain the taste and enhance the aroma of baijiu (Sun et al., 2022). On the other hand, 15 compounds had a significantly higher content in A5 than in A1, such as acetaldehyde (6-fold), 3-hydroxy-2-butanone (118-fold), 2,3-butanediol (62-fold), hexanol (15-fold), ethyl formate (130-fold), ethyl butyrate (49-fold), ethyl isovalerate (6-fold), ethyl lactate (3-fold), ethyl hexadecanoate (2-fold), ethyl linoleate (3-fold), and ethyl oleate (2-fold). Of which, acetaldehyde had the grassy odor, 3-hydroxy-2-butanone and 2,3-butanediol own the buttery flavor (Sun et al., 2022), and higher fatty acid ethyl ester (ethyl hexadecanoate, ethyl oleate, and ethyl linoleate) contributed fatty aroma (Niu, Yao, Xiao, Xiao, Ma, & Zhu, 2017). These results indicated that some unfavorable aroma substances were enriched as moisture content decreased. Moreover, the ratio of ethyl acetate to ethyl lactate decreased with the decrease of moisture content (0.75, 0.52, 0.24, 0.21, 0.20, respectively) due to the increasing content of ethyl lactate. Research revealed that increasing the ratio of ethyl acetate to ethyl lactate can improve the quality of Xiaoqu baijiu (Sun et al., 2022). Therefore, increasing moisture content can heighten the ratio of ethyl acetate to ethyl lactate, thereby improving the quality of Xiaoqu baijiu.

Besides, sensory analysis (Fig. 1D) revealed that the total score of A1 was higher than A5, especially in the aroma intensities of sweet, alcohol, fruity, and floral. In contrast, the aroma intensities of grassy and fatty were higher in A5 compared to A1. This result was consistent with the content of base baijiu in different moisture content. Hence, moisture content significantly affects the flavor structure and content of the base baijiu. The regulation of moisture content might be a strategy for precise flavor manipulation.

3.2. Effect of moisture content on fermentation parameters

The utilization and generation of water accompanied the fermentation of Xiaoqu baijiu. As illustrated in Fig. 2A, the moisture content of the fermented grains displayed a dynamic trend throughout the fermentation process, increasing in the first 5 days and maintaining a stable level thereafter. However, the change rate of moisture content increased with decreasing initial moisture content in the first 5 days of fermentation (12.5 %, 11.95 %, 15.12 %, 19.23 %, and 20.96 %, respectively). This suggests that there were variations in microbial growth and metabolism based on the initial moisture content.

To verify the effect of moisture content on fermentation, principal component analysis (PCA) was employed to analyze the dissimilarity matrix of fermentation parameters (moisture content, reducing_sugar, starch, ammonia, amino_N, acidity, lactic acid, ethanol, acetic acid). Results indicated that the left side corresponded to the samples from day 0, while the right side consisted of the samples from days 5–21 (Fig. 2B), indicating the fermentation time was the main factor in PC1 (72.2 %). This pattern can be explained by the flourishing growth of the microbiota during the initial stage, consistent with previous studies (Hu et al., 2021). However, different moisture content played an important role in PC2 (15.4 %). The content of moisture, acetic acid, acidity, and amino_N was higher in A1 and A2, while the content of ethanol was higher in A4 and A5. It is reported that acetic acid has negatively affected the stoichiometry of sugar fermentation by yeast strains (Bellissimi, van Dijken, Pronk, & van Maris, 2009), leading to a lower ethanol content. The lower ethanol content may also be due to the fact that high moisture content promoted heat dissipation and caused low fermentation temperature, consequently, diminishing the production of metabolites (Torija, Rozès, Poblet, Guillamón, & Mas, 2003).

Thus, the change trends of the fermentation parameters were similar among different moisture content, but acetic acid, acidity, amino_N, and ethanol showed significant differences.

3.3. Effect of moisture content on microbial community

3.3.1. Analysis of microbial diversity

Illumina MiSeq sequencing was employed to determine how the moisture content affects the microbial community. A total of 3,765,189 high-quality bacterial sequences and 4,372,817 fungal sequences were clustered into 378 operational taxonomic units (OTUs) and 116 OTUs for bacteria and fungi, respectively, based on the 16S rRNA and ITS amplicons. The coverage index for bacteria and fungi in all samples

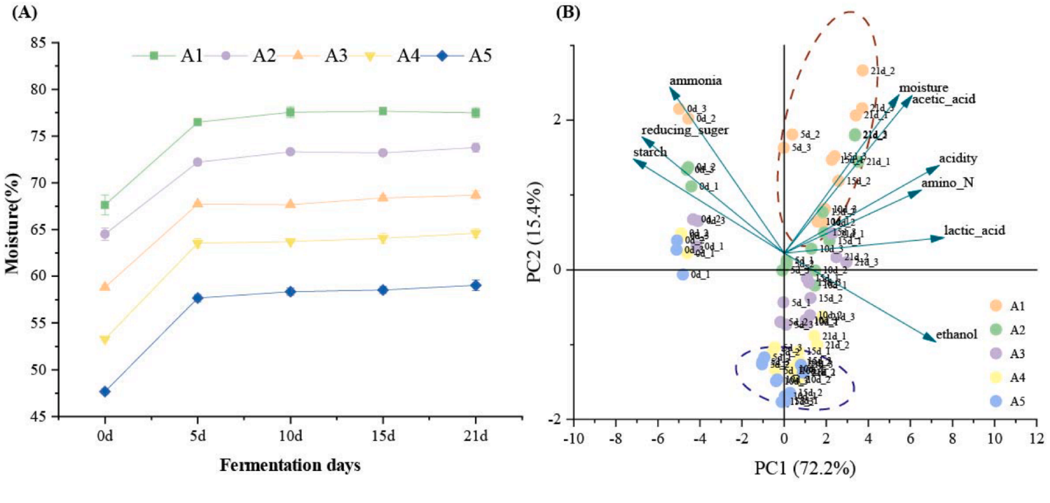

Fig. 2. Dynamics of fermentation parameters of fermented grains with different moisture contents. (A) variation of moisture content during fermentation, (B) PCA of fermentation parameters. Note: the blue dotted frame was the variation of fermentation parameters in A5 at 5-21d, the orange dotted frame was the variation of fermentation parameters in A1 at 5-21d. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ranged from 99.86 % to 99.98 % (Table S2), indicating that the sequencing results provided a comprehensive and accurate reflection of the microbial diversity in the samples. The sequencing depth was sufficient to capture all relevant microbial diversity information in the samples. The evaluation of microbial diversity can be conducted by examining both α diversity and β diversity.

α diversity reflects the complexity of microorganisms within a given sample. The richness (Chao index) and diversity (Shannon index) of microbiota were investigated in different moisture contents. As depicted in Fig. S2A, the diversity of bacteria showed significant differences ($P = 0.002$) in different initial moisture contents. Furthermore, the richness of bacteria significantly decreased ($P = 0.001$) as the moisture content increased (Fig. S2B), indicating that the richness of bacteria differed depending on the initial moisture content. However, no significant differences ($P > 0.05$) in the richness and diversity of fungi were found among the different moisture contents (Fig. S2C and S2D). Thus, while moisture content had minimal effect on fungi, it significantly impacted bacteria. This result can be explained by the fact that bacteria were most sensitive to water activity (Yang, Haoliang, Wang, Qiuhui, & Pei, 2019).

β diversity, a measure of the overall similarity of microbial communities, was assessed in this study. The results indicated that samples with different initial moisture contents exhibited a similar pattern at the beginning of the fermentation process, but gradually differed as fermentation progressed (Fig. S2E). In addition, Table S3 revealed that differences in bacterial community diversity among different moisture contents became more pronounced over time. The changing trend of fungal communities in response to varying moisture levels was more complex, with similarity initially decreasing, then increasing, and then decreasing again (Fig. S2F). This was confirmed by the ANOSIM and Adonis tests (Table S4). Overall, the microbial community of Xiaoqu baijiu exhibited significant differences in response to varying moisture contents.

3.3.2. Composition and succession of microbial community

The present study used a 97 % sequence similarity threshold for species classification, resulting in the detection of 16 bacterial phyla, 4 fungal phyla, 204 bacterial genera, and 61 fungal genera during the fermentation process. On the whole, members of Firmicutes gradually became more dominant within the bacteriome, whereas the members of Proteobacteria gradually lost their dominance during the fermentation in bacterial phyla (as shown in Fig. S3A). In fungal phyla, the relative abundance of Mucoromycota increased and Ascomycota decreased (as depicted in Fig. S3B). At the genus level, the dominant bacterial genus changed from *Acetobacter* (66.45 %–0.09 %, average relative

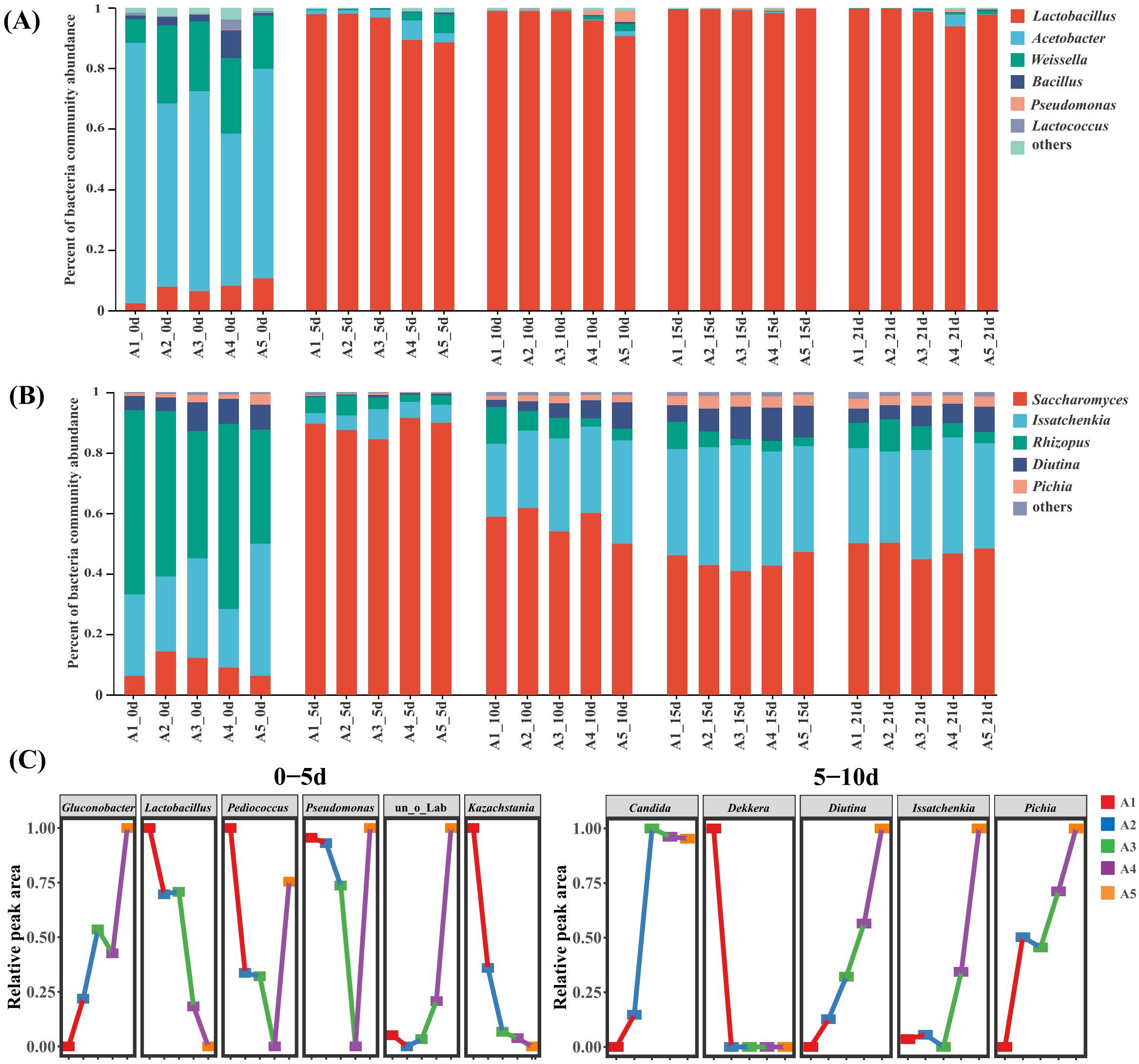

Fig. 3. Diversity of microbial communities during fermentation in different moisture contents. (A) bacterial community at the genus level, (B) fungal community at the genus level, (C) significantly different genera in different moisture contents. Note: un-o-Lab = unclassified_o_Lactobacillales.

abundance, same below) to *Lactobacillus* (7.10 %–99.21 %) as fermentation progresses (Fig. 3A). On the other hand, the dominant fungal genus shifted from *Issatchenkia* (29.59 %–34.33 %) and *Rhizopus* (51.37 %–7.03 %) to *Saccharomyces* (9.57 %–47.92 %) and *Issatchenkia* (29.59 %–34.33 %) during fermentation (Fig. 3B). Similarity trend of dominant microbial succession was found in the fermentation process of Xiaoqu baijiu (Hu et al., 2021). Thus, there was obvious microbial succession in the fermentation process of Xiaoqu baijiu. The bacteria quickly successively formed a flora dominated by *Lactobacillus* within 5 d, while the fungal succession was carried out through the replacement of *Rhizopus*, *Saccharomyces*, and *Issatchenkia*.

Moreover, clear turnovers existed in the change rate of relative abundance in some microbiota as the moisture content varied (Fig. 3C). In the first 5 days of fermentation, the bacteria formed the dominant flora with *Lactobacillus*, and the fungal community was dominated by *Saccharomyces*. However, the change rate of relative abundance in

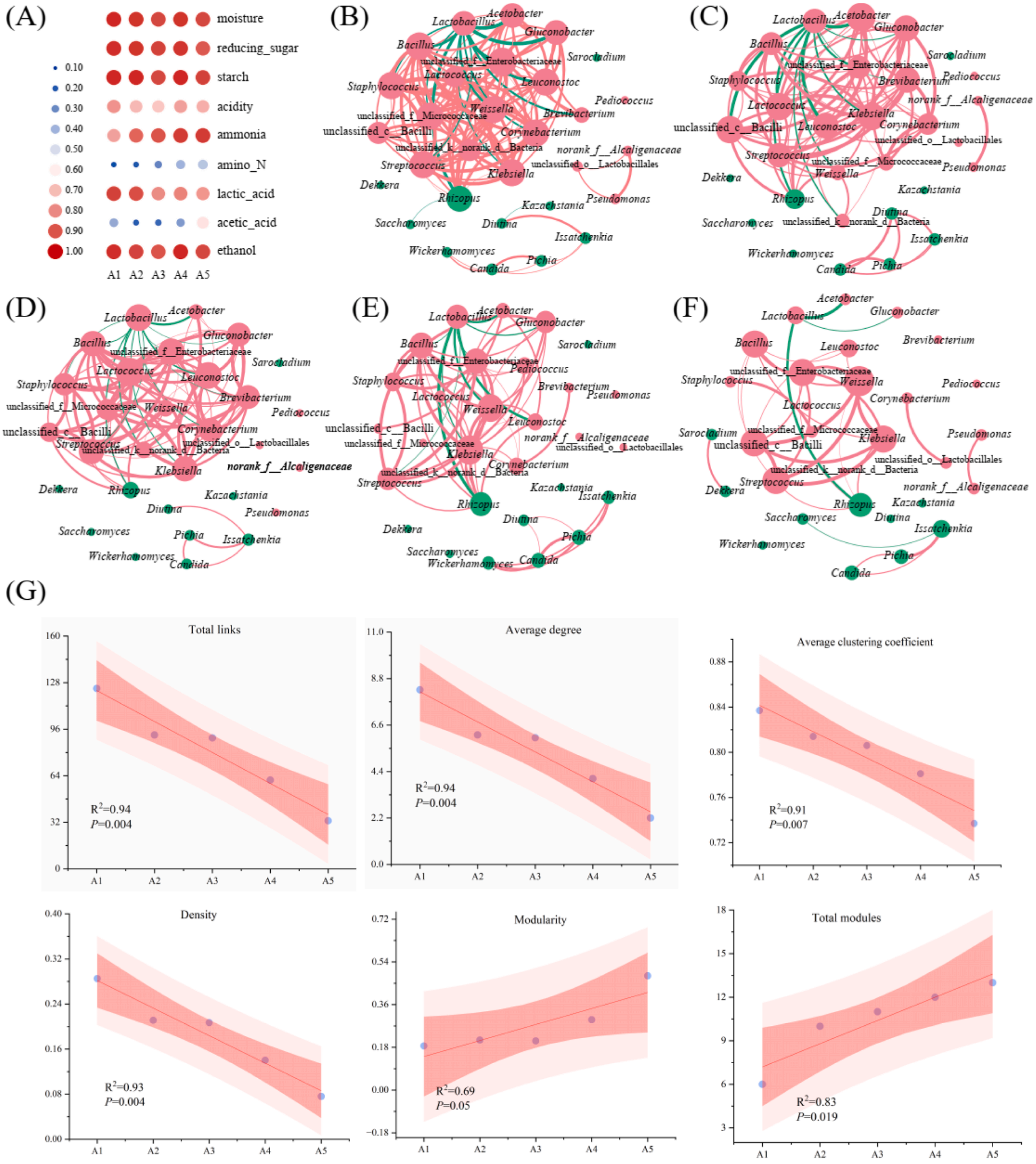

Fig. 4. Characteristic of biotic and abiotic factors in the microbial community at different moisture contents. (A) drivers of the microbiota analyzed by the Mantel test, (B-F). overview of microbial ecological networks in different moisture contents (representing A1-A5 respectively), (G) trend in network topological along with moisture content. Note: In network of microbial community, blue and red balls represented potentially important fungi and potentially important bacteria respectively. The blue and red lines represented negative correlation and positive correlation respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

unclassified_o__Lactobacillales and *Gluconobacter* increased, while the change rate of relative abundance in *Lactobacillus* and *Kazachstania* decreased with the decline of moisture content. Thus, increased moisture content accelerated the formation of *Lactobacillus*-dominated fermentation microbiota. Research showed that *Lactobacillus* was the dominant genus in the later stage of fermentation in baijiu fermentation, and the rapid increase of its relative abundance implied the stage shift of fermentation (Tan, Zhong, Zhao, Du, & Xu, 2019). Hence, moisture content changed the rate of stage shift of fermentation, and the law might also be applicable to the baijiu fermentation system dominated by *Lactobacillus*, such as Daqu light flavor baijiu and Nong flavor baijiu. Besides, *Lactobacillus* could serve as a useful biomarker for the microbial community succession in the fermentation process of Xiaoqu baijiu, as observed in the Nong flavor baijiu fermentation (Tan et al., 2019) and Jiang flavor baijiu fermentation (Zhang et al., 2021). Research showed that *Kazachstania* were able to grow in conditions of high osmolarity, high acidity, and in the presence of organic acids and ethanol, and the performance in fermentation was comparable with the *S. cerevisiae* commercial strain (Korcari, Ricci, Capusoni, & Fortina, 2021). Therefore, more alcohol may be produced by *Kazachstania* as the moisture content increased. In 5-10d, the microbial community, mainly fungi, ushered in the second succession, and the relative abundance of *Saccharomyces* decreased, but non-Saccharomyces increased. As a result, the change rate of relative abundance in *Candida*, *Diutina*, *Issatchenkia*, and *Pichia* increased, while that in *Dekkera* decreased as moisture content decreased. Among these, *Candida*, *Diutina*, *Issatchenkia,* and *Pichia* were recognized as traditional ester-producing yeast strains in baijiu fermentation (Wang, Wu, Nie, Wu, & Xu, 2019; Zha, Sun, Wu, Yin, & Wang, 2018), indicating that the ester production might be heightened with moisture content decrease. This result was consistent with the flavor data depicted in Fig. 1. *Dekkera*, only detected at A1 and A2, has been described as having a higher ethanol yield under oxygen limitation than *S. cerevisiae* (Blomqvist, Eberhard, Schnurer, & Passoth, 2010). Therefore, *Dekkera* may contribute to ethanol when the moisture content is over 60 %. To sum up, the effect of moisture content was not on the overall law of microbial succession, but on the rate of formation of microbiota in the fermentation system.

3.3.3. The biotic factors and abiotic factors of microbial community

Microbial community succession occurs as a result of microbial adaptation to biotic factors (microorganisms) and abiotic factors (fermentation parameters). The dynamic changes of biotic factors and abiotic factors with moisture content were unraveled in this study. As shown in Fig. 4A, the relationship between fermentation parameters and microbial community in different moisture content systems exhibited differences. Particularly, as the moisture content was elevated from 70 % to 50 %, the correlation between lactic acid and microbial community decreased (r = 0.926, 0.907, 0.796, 0.810, 0.775, respectively), while the correlation between ammonia and microbial community (r = 0.7511, 0.8699, 0.9021, 0.938, 0.9389, respectively), the correlation between amino_N and microbial community (r = 0.1728, 0.1945, 0.2905, 0.4009, 0.4432, respectively) increased. Thus, the driving force of lactic acid was weakened, while that of the nitrogen sources was strengthened with the reduced moisture content.

The interaction among microorganisms shapes microbial diversity and functions, and changes in the ecological network structure composed of microbial interaction can affect ecosystem function and stability (Wagg, Schlaeppi, Banerjee, Kuramae, & van der Heijden, 2019). Therefore, five microbial ecological networks were constructed to unravel the changes in microbial interactions with different moisture content (Fig. 4B-F). Results showed that the co-occurrence relationships among microorganisms underwent profound changes in the transition from high moisture content (70 %) to low moisture content (50 %). The link between *Lactobacillus* and other bacteria weakened with the decreasing moisture, and most bacteria were significantly negatively correlated with *Lactobacillus* (Fig. 4B-F). It was reported that *Lactobacillus* are important microorganisms for successful food fermentation which could produce bacteriocin to inhibit the growth of other microorganisms (Alvarez-Sieiro, Montalbán-López, Mu, & Kuipers, 2016). Meanwhile, *Lactobacillus* could produce lactic acid, and acetic acid (Angelis et al., 2016; Laakso et al., 2011) to maintain the acidic fermentation environment, which could regulate the equilibrium relationship among microorganisms (Zhang, Du, Wu, & Xu, 2015). This result explained the reason why the richness of bacteria significantly decreased ($P = 0.001$) as the moisture content increased (Fig. S2C). In addition, the total number of links ($R^2 = 0.94$, $P = 0.004$), average degree ($R^2 = 0.94$, $P = 0.004$), average clustering coefficient ($R^2 = 0.91$, $P = 0.007$), density ($R^2 = 0.93$, $P = 0.004$) decreased significantly with the reducing moisture content (Fig. 4G), revealed that the complexity of the microbial network decreased sharply and the associations between microorganisms tended to be simple. According to the conclusion that stability is positively correlated with complexity (Yuan et al., 2021), we found reducing moisture content decreased the complexity of the microbial network and then weakened the stability of the ecological networks. Modularity is an important property of ecological networks, which can indicate spatial compartmentalization, nutritional resource partition, and ecological niche differentiation (Dai et al., 2022). As shown in Fig. 4G, the modularity ($R^2 = 0.69$, $P = 0.05$) and the total number of modules ($R^2 = 0.83$, $P = 0.019$) exhibited a significantly downward trend with increasing moisture content, indicating that functional modules increased with the decrease of moisture content. This result was consistent with other studies which found that high salinity stress decayed the complexity of the network (Li et al., 2023). Collectively, the complexity and stability of ecological networks were positively correlation with moisture content, suggesting interdependence between moisture conditions and microbial community dynamics.

3.3.4. Prediction of metabolic characteristics of microbial community

Disparities in the succession of microbial communities can lead to substantial variations in metabolic flux. The functional profiles of the microbial community were predicted using PICRUSt II. As depicted in Fig. 5A, all pathways were categorized into four major groups and 32 subcategories. Subsequently, specific pathway selection was conducted through Kruskal-Wallis analysis, focusing on two classes within Biosynthesis (amino acid biosynthesis, fatty acid, and lipid biosynthesis), three classes within Degradation (alcohol degradation, amino acid degradation, fatty acid and lipid degradation), and five classes within Generation of Precursor Metabolites and Energy (electron transfer chains, fermentation, glycolysis, pentose phosphate pathways, TCA cycle). Results revealed significant differences in 0, 10, 64, 72, and 79 pathways among bacteria and 0, 2, 3, 5, and 2 pathways among fungi at 0d, 5d, 10d, 15d, and 21d, respectively (Fig. 5B), and these findings corroborate previous results demonstrating moisture content mainly affects the bacterial community.

In the biosynthesis class, differential expression patterns were found in 33 bacterial pathways (2, 26, 30, and 32, respectively, the same below) and four fungal pathways (0, 0, 2, and 2) for amino acid synthesis, as well as 10 bacterial pathways (0, 7, 10, and 1) and four fungal pathways (2, 3, 3, and 2) for fatty acid and lipid biosynthesis, throughout the 5–21-d period. In the degradation category, six differential pathways (2, 5, 6, and 6) of bacteria and one differential pathway of fungi (0, 0, 1, and 0) involved in amino acid degradation, along with four bacterial pathways related to alcohol degradation (0, 4, 4, and 2), were identified. The class pertaining to the generation of precursor metabolites and energy revealed 13 differential pathways related to fermentation (4, 9, 8, and 1). These results indicated that the adjustment of moisture content significantly affected the function of the microbial community, and significant differential pathways were mainly concentrated in synthesis, especially amino acid synthesis.

Specifically, the highest moisture content (A1) increased the abundance of THRESYN-PWY (super pathway of ʟ-threonine biosynthesis)

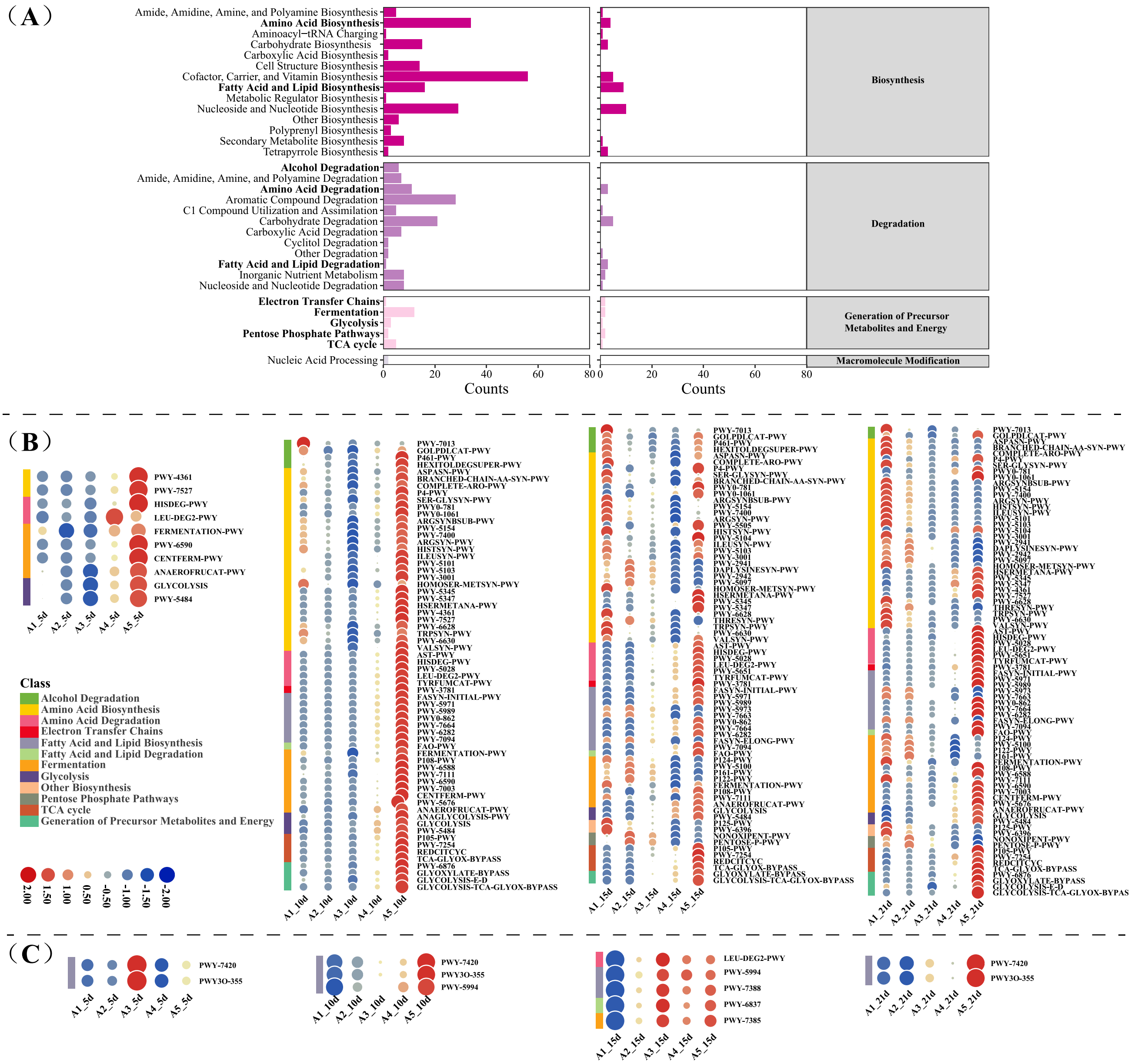

Fig. 5. Differential pathways of microbial community in different moisture contents. A. an overview of metabolic pathways; B. significant differential pathways of bacteria; C. significant differential pathways of fungi. Note: The larger and redder the circle, the higher the abundance. The colors of the bars on the left represent the different classifications of metabolic pathways.

within the class of amino acid synthesis during the 5-21d period, and the abundance of PWY-7013 (L-1,2-propanediol degradation) in the class of alcohol degradation at 10–15 d. It has been reported that the threonine pathway played a central role in propanol formation, providing an explanation for the elevated content of propanol observed in A1. Besides, PWY-7013 is related to the production of propionic acid or propanol (Selwet, 2020). The elevated abundance of the PWY-7013 pathway in A1 may serve as an additional explanation for the higher content of propanol observed under high moisture conditions. Besides, P124-PWY (Bifidobacterium shunt), PWY-5100 (pyruvate fermentation to acetate and lactate II), P161-PWY (acetylene degradation), and P122-PWY (hetero-lactic fermentation) in the class of generation of precursor metabolites and energy displayed the highest abundance in A1. These pathways were involved in acetate production (Fig. S4), aligning with the observation that the content of acetic acid in A1 was twice as high as that in A5 (Fig. 1).

Conversely, the lowest moisture content (A5) heightened the abundance of FASYN-INITIAL-PWY (super pathway of fatty acid biosynthesis initiation), PWY-5971 (palmitate biosynthesis II), PWY-7664 (oleate biosynthesis IV - anaerobic), PWY-6282 (palmitoleate biosynthesis I) in the class of fatty acid and lipid biosynthesis. In the class of fatty acid and lipid degradation, an increased abundance was observed in the FAO-PWY (fatty acid β-oxidation I) pathway. Additionally, pathways related to fermentation, namely CENTFERM-PWY (pyruvate fermentation to butanoate), PWY-5676 (acetyl-CoA fermentation to butanoate II), PWY-7003 (glycerol degradation to butanol), and ANAEROFRUCAT-PWY (homolactic fermentation), exhibited heightened abundances in A5. Previous research has shown that higher fatty acid ethyl esters, such as ethyl oleate, ethyl linoleate, and ethyl hexadecanoate, were produced from corresponding higher fatty acids and ethanol (Ding, Wu, Huang, & Zhou, 2015). The products of β-oxidation, namely acetyl-CoA and ATP, play a crucial role in providing energy for microbial proliferation and acting as precursor substances for ester formation (Kumari, 2018). Thus, high concentrations of ethyl hexadecanoate, ethyl oleate, and ethyl linoleate were found in A5 (Fig. 1). Meanwhile, the productions of CENTFERM-PW, PWY-5676, PWY-700, and ANAEROFRUCAT-PWY were lactic acid and butyric acid, which were the precursors of ethyl lactate and ethyl butanoate (Wang, Wang, Sun, Tan, Zheng, & Zhu, 2022). Consequently, the content of butanol, ethyl butyrate, and ethyl lactate was high in A5 (Fig. 1). Besides, in the class of generation of precursor metabolites and energy, it was observed that the TCA cycle and PWY-3781 (aerobic respiration I) displayed higher abundances in A5 at 10–21 d, indicating that microorganisms under low moisture conditions remained active in aerobic respiration. Additionally, the glycolysis pathway exhibited higher abundance in A5, while the Pentose Phosphate Pathways revealed increased abundance in A1. This disparity suggests differences in the metabolism flux of glucose degradation in different moisture content systems, and it may serve as one of the contributing factors to the higher concentration of ethanol observed in A5. Remarkably, all differential pathways related to amino acid degradation, exhibited high abundance in A5, indicating that the nitrogen source was restricted under low moisture conditions. This finding provided an explanation for the intensified driving force of nitrogen sources on microbial succession as moisture content decreases (Fig. 4A).

The abundance of 21 pathways showed fluctuation throughout the fermentation stage, displaying higher abundances under the lowest moisture conditions during the early stages (5 – 10d) but exhibiting greater abundance under the highest moisture conditions at the later stages of fermentation (15 – 21d). These results revealed the underlying reasons for the acceleration of substrate consumption and product formation faster with the increased moisture content after the 10th day of fermentation (Fig. 2).

To sum up, moisture content exerted a significant impact on the metabolic flux of the microbial community. Consequently, distinct moisture content levels led to substantial differences in the content of flavor compounds.

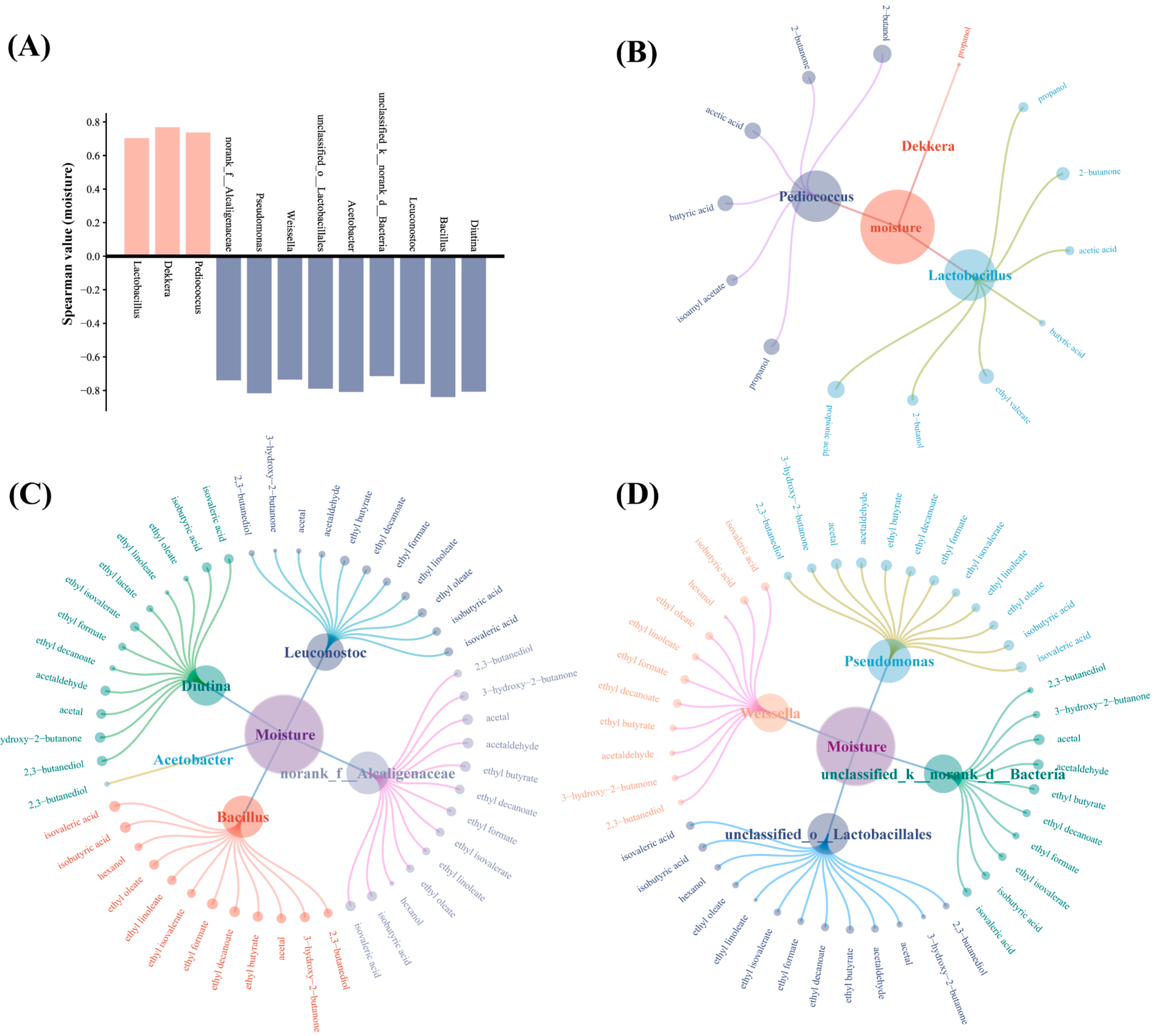

Fig. 6. Correlation among the moisture, microbial community and volatile flavor compound. (A). the correlation between moisture and microbiota ($P < 0.05$). (B) the correlation between positive genera and volatile flavor compounds ($P < 0.05$, $r > 0.6$), (C) and (D) the correlation between negative genera and volatile flavor compounds ($P < 0.05$, $r > 0.6$).

3.4. Construction of a model between moisture, microbes, and volatile flavor compounds

The Spearman correlation analysis revealed significant relationships among moisture content, microbial community, and volatile flavor compounds. Specifically (Fig. 6A), *Lactobacillus, Dekkera, Pediococcus* were significantly positive with moisture, while *Acetobacter*, *Bacillus*, *Pseudomonas*, *Leuconostoc, Weissella*, unclassified_o_Lactobacillales, unclassified_k_norank_d_Bacteria, *norank_f_Alcaligenaceae,* and *Diutina* for significant negative correlation ($P < 0.05$, $r > 0.6$). Of these, *Lactobacillus* was a facultative anaerobe or anaerobe (Goldstein et al., 2015), and thus increasing the moisture content favors its proliferation. *Dekkera* was robust to large changes in pH (Blomqvist et al., 2010). *Pediococcus* a type of lactic acid bacteria (LAB), was characterized by its ability to withstand acidic conditions (Li et al., 2021). Therefore, the species with high tolerance or adapting to high moisture content might have gained competitive advantages and replaced those species with low tolerance, making them become the dominant species with the increase of moisture content, and causing the successional dynamics of the microbial community.

Then, the relationship between microbe and volatiles was shown in Fig. 6B-D. *Lactobacillus* exhibited positive correlations with 2-butanone, propanol, ethyl valerate, acetic acid, propionic acid, and butyric acid. It has been reported that *Lactobacillus* was an important microbial source of propanol production in baijiu fermentation (Lu et al., 2019). Besides, *Lactobacillus* has been reported to produce lactic acid, butyric acid, and acetic acid (Angelis et al., 2016). *Dekkera* demonstrated a positive correlation with propanol and previous studies have shown that it has excellent alcohol production capacity (Blomqvist et al., 2010). *Pediococcus* displayed positive correlations with 2-butanone, 2-butanol, propanol, isoamyl acetate, acetic acid, and butyric acid. *Pediococcus* was regarded as the main functional genera in the fermentation process of baijiu and contributed to many flavor substances (Li et al., 2013).

On the other hand, nine genera that exhibited negative correlations with moisture demonstrated positive correlations with various volatiles, including acetaldehyde, acetal, 3-hydroxy-2-butanone, 2,3-butanediol, hexanol, ethyl formate, ethyl butyrate, ethyl isovalerate, ethyl hexadecanoate, ethyl linoleate, and ethyl oleate. Many microorganisms (*Acetobacter*, *Bacillus*, and *Leuconostoc*) could convert sugars to acetoin (Chai et al., 2020; Gumustop & Ortakci, 2022; Thanh et al., 2010). Besides, 2,3-butanediol is a by-product of metabolizing acetoin (Thanh et al., 2010). Consequently, microorganisms related to acetoin metabolism were also associated with 2,3-butanediol production. Furthermore, ethyl lactate was found to be related exclusively to the presence of *Diutina*. *Diutina* was a genus separated from *Candida* and can produce various lipases (Antarctica lipase A, rugosa lipases, glucose ester synthesis lipase, etc.) during fermentation (Wang et al., 2019). In a word, with the increasing moisture content, the microorganisms exhibiting positive correlations with moisture contributed to the characteristic flavor compounds (such as 2-butanone, propanol, 2-butanol, acetic acid, etc.). This finding indicated that the disparity in flavor compounds under different moisture content was primarily attributed to variations in microbial community structure.

4. Conclusion

In summary, our results highlighted the importance of moisture content in flavor compound formation, microbial community succession, microbial interaction, and metabolic function. Among them, rising moisture content increased the total volatile contents, the proportion of acids and alcohols within the total volatiles, and the ratio of ethyl acetate to ethyl lactate. Meanwhile, increasing moisture content promoted the microbial community to rapidly form a fermentation system microbiota dominated by *Lactobacillus*. Besides, increasing moisture content enhanced the complexity of ecological networks, as well as changed the metabolic flux of the microbial community. *Lactobacillus, Dekkera,* and *Pediococcus* were positively correlated with moisture, promoting the production of propanol, acetic acid, butyric acid, and 2-butanol. Hence, controlling the initial moisture content can serve as a strategy for managing and optimizing the fermentation process, ultimately improving the flavor characteristics of Xiaoqu baijiu and other fermented foods.

CRediT authorship contribution statement

Huan Wang: Methodology, Investigation, Formal analysis, Visualization, Writing – original draft. **Chunhong Sun:** Investigation, Data curation, Formal analysis. **Shengzhi Yang:** Investigation, Resources. **Yulei Ruan:** Data curation, Formal analysis. **Linjie Lyu:** Resources. **Xuewu Guo:** Supervision, Writing – review & editing. **Xiaole Wu:** Supervision, Writing – review & editing. **Yefu Chen:** Conceptualization, Supervision, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.fochx.2023.100981.

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