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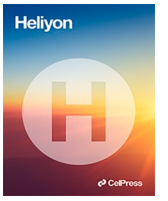

Research article

Comparative analysis of bacterial community structure and physicochemical quality in high-temperature Daqu of different colors in Qingzhou production area

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ABSTRACT

To compare the effects of differences in Daqu making technology and production regions on the bacterial composition and physicochemical properties of high-temperature Daqu (HTD), this study analyzed the bacterial community structure of three colors of HTD in the Qingzhou production area and measured their physicochemical quality. At the same time, a comparative analysis was conducted on the bacterial composition of Qingzhou and Xiangyang regions. The results revealed that the HTD in the Qingzhou area exhibited a diverse bacterial community dominated by *Lentibacillus*, *Scopulibacillus*, and *Staphylococcus*. The black HTD displayed the lowest bacterial richness ($P < 0.05$) and a relatively unique microbial structure. Significant variations were observed in the physicochemical qualities of the three colors of HTD. Notably, white HTD demonstrated higher moisture and ash content, saccharification and liquor-producing power. Yellow HTD exhibited higher amino nitrogen and protein content, and black HTD displayed higher water activity, acidity, and starch content. The variation in *Bacillus*, *Limosilactobacillus*, and *Weissella* distributions across different colors of HTD primarily contributed to these findings. From the HTD samples in the Qingzhou area, *Bacillus* (61.90 %) and lactic acid bacteria (17.46 %) being the predominant cultivable communities. Cluster analysis identified significant differences in bacterial communities among HTD samples from various production areas. It can enhance the understanding of HTD quality in the Qingzhou area and offer insights for optimizing HTD and Maotai-flavor Baijiu quality.

Abbreviations: HTD, High-temperature Daqu; OTUs, Operational Taxonomic Units; LDA, Linear Discriminant Analysis; LEfSe, Linear Discriminant Analysis Effect Size; PerMANOVA, Permutational Multivariate Analysis of Variance; MRS, deMan Rogosa and Sharpe; LB, Luria-Bertan; NA, Nutrient Agar.

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1. Introduction

"Daqu is the bone of liquor". High-temperature Daqu (HTD) serves as a saccharification starter for Maotai-flavor Baijiu, supplying essential enzymes and microorganisms crucial for its brewing, thereby influencing the quality of Baijiu [1]. HTD that is primarily composed of wheat undergoes crushing, water addition, and fermentation with mother Daqu. After formation, stacking fermentation, and maturation, finished Daqu was obtained (Fig. 1b) [2]. However, during high-temperature stacking fermentation in the Qu-room, the variation in environmental conditions, such as temperature, humidity, and oxygen concentration, due to the diverse spatial positions of the Daqu blocks, leads to differing degrees of browning in the Maillard reaction. Therefore, the HTD exhibits three colors: white, yellow, and black [3]. Among them, temperature is an important reason for the formation of three colors. Hou et al. [4] showed that the highest fermentation temperature of white HTD was 60–62 °C, that of yellow HTD was 62–64 °C, and that of black HTD was 64–65 °C. Recent studies have examined the microbial differences and quality attributes of HTD colors [5,6]. Y. Wang et al. [7] identified *Bacillus*, *Massilia*, and *Staphylococcus* as biomarkers corresponding to white, yellow, and black HTD in Xiangyang, Hubei Province, China. Pang et al. [8] suggested that in North China, white HTD demonstrated the highest liquefaction power and starch content, whereas black HTD exhibited the highest saccharification power and esterification power. Additionally, a significant correlation was observed between the formation of physical and chemical quality and microbial communities [8,9]. It can be seen that previous studies have shown that there are certain differences between the microbial composition and physical and chemical quality of HTD with different colors. Therefore, based on the analysis of microbial compositions and physical-chemical parameters, it is a prerequisite to understand the quality of HTD with three colors in different Maotai-flavor Baijiu brewing areas and how they play a role in Baijiu brewing.

Kweichow Moutai serves as the primary representative of Maotai-flavor Baijiu, known for its robust flavor and smooth body. Using Kweichow Moutai Town as the standard, China boasts numerous regions producing Maotai-flavored Baijiu. The Qingzhou production area is located in Weifang City, Shandong Province. This area is characterized by two main water systems, including the Mi River and the Xiaoqing River, and falls within a warm, temperate, semi-humid monsoon climate. Yunmen Wine Industry, affiliated with this area, stands as a prominent enterprise of Lupai Maotai-flavor Baijiu and contributes to the national standard for Maotai-flavor Baijiu (GB/T 26760). Xiangyang, Hubei, as a new Maotai-flavor Baijiu production area located approximately 832 km from Qingzhou, features distinct water systems and climatic conditions. Studies have indicated that geographical disparities, environmental temperatures, brewing raw materials, and production techniques can affect microbial succession in HTD, thereby affecting the flavor and quality of Maotai-flavor Baijiu across different regions [10]. Furthermore, the bacterial community, which is sensitive to environmental variation, can significantly affect the taste quality of Daqu [11]. Nevertheless, at present, there remains little research of the microbial and physicochemical aspects of HTD with different colors in Qingzhou, as well as there are also few studies on the comparative analysis of microbial resources in HTD with non-core production areas of Maotai-flavor Baijiu such as Xiangyang.

In recent years, Illumina MiSeq high-throughput sequencing technology has become prevalent for the analysis of brewing microbial communities [12,13]. It not only efficiently and accurately assesses microbial composition, but also reduces experimental costs [14]. This study focused on analyzing the bacterial communities of three types of HTD from the Qingzhou production area using high-throughput sequencing and pure culture techniques, along with measuring their physicochemical indicators. Simultaneously, to compare the microbial group differences in HTD across different production areas, sequencing data from HTD in Xiangyang, Hubei Province, were included in our previous study. This study then discussed the similarities and differences in the bacterial group structure between Qingzhou and Xiangyang through a combined analysis. This research can enhance the understanding of HTD quality and microbial communities in the Qingzhou production area, offering theoretical insights for optimizing subsequent Daqu-making processes.

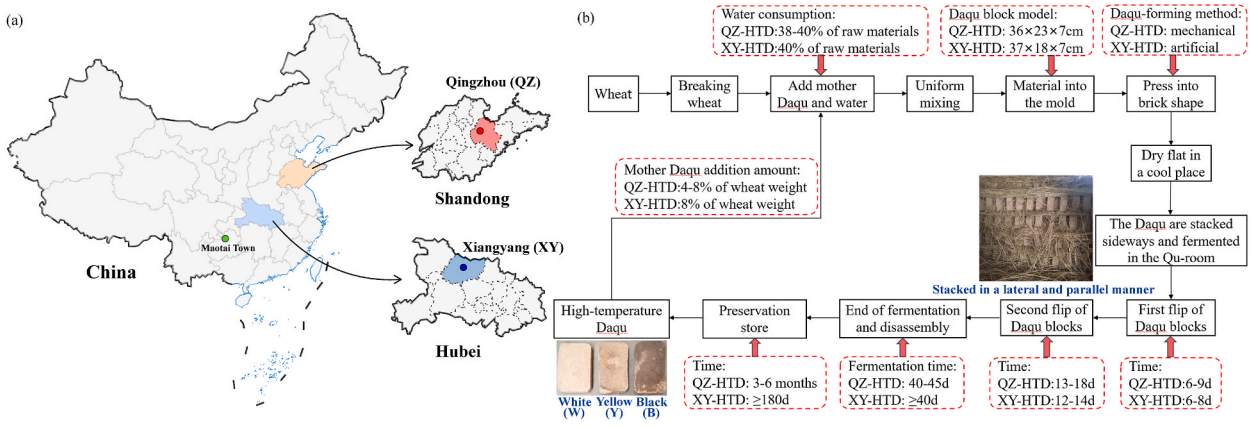

Fig. 1. Distribution map of sampling areas (a) and process flow diagram of Daqu making (b) for high-temperature Daqu (HTD).

2. Material and methods

2.1. Sample collection

In April 2023, 30 HTD samples were collected from an HTD and Maotai-flavor Baijiu brewing enterprise located at coordinates 118°10′–118°46′ E and 36°24′–36°56′ N in Qingzhou, Weifang, Shandong Province. All the samples originated from the same batch and were stored in the same room. Ten white, yellow, and black HTD samples were randomly selected from various positions within the Qu-room and labeled as QZW1-10, QZY1-10, and QZB1-10, respectively. The collected samples were placed in sterile, self-sealing bags and rapidly transported to the laboratory for grinding. Each crushed sample was divided into three equal portions: one for high-throughput sequencing and bacterial strain isolation, another for determining physical and chemical indices, and the remaining portion stored at −40 °C for future analysis.

Our team previously analyzed the microbial communities of three HTD colors from Xiangyang City, Hubei Province [7]. Therefore, this study acquired bacterial sequence data from the MG-RAST database (https://www.mg-rast.org/mgmain.html?mgpage=metazen2&project=mgp96594) to compare and analyze the bacterial community structures across different HTD production areas. The key processes and parameters for HTD production in both areas are illustrated in Fig. 1b.

2.2. Macrogenomic DNA extraction, PCR amplification, and Illumina MiSeq high-throughput sequencing

Metagenomic DNA of HTD samples from Qingzhou was extracted using the QIAGEN DNeasy Mericon Food Kit (QIAamp DNA Microbiome Kit, QIAGEN Inc., Hilden, Germany). PCR amplification was conducted using the extracted DNA as a template following the method outlined by Cai et al. [15] PCR amplification was performed using forward and reverse primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-ACTCCTACGGGAGGCAGCAG-3′). The qualification of the PCR products was assessed via agarose gel electrophoresis, and PCR products displaying clear bands were submitted to the Illumina MiSeq platform (Majorbio Biotechnology Co., Ltd., Shanghai, China) for high-throughput sequencing under low-temperature conditions.

2.3. Bioinformatics and statistical analysis

Quality control and stitching of the offline sequence were conducted following the method described by Z. Zhang et al. [16] on the QIIME (v1.9.1) platform. After comparison and alignment using PyNAST (v1.2.2) software [17], the sequence set was partitioned into Operational Taxonomic Units (OTUs) using UCLUST at a 97 % similarity threshold [18]. The OTUs were eliminated using Chimera Slayer [19], followed by sequence alignment to elucidate the taxonomic status of HTD bacterial communities [20]. Additionally, α-diversity indices, including Chao 1, observed species, Shannon, and Simpson indices, were assessed using the QIIME (v1.9.1) platform. β-diversity was evaluated through principal coordinate analysis (PCoA) of weighted and unweighted UniFrac distances. Furthermore, Linear Discriminant Analysis (LDA) Effect Size (LEfSe) was employed to identify bacterial markers that distinguished HTD from different regions.

R (v4.1.3) software was applied to generate violin plots and PCoA diagrams for visualizing α and β diversity, and stack histograms and box pattern diagram were created to visualize bacterial community structure using R software. MATLAB (2016b) software was utilized for cluster analysis visualization based on Mahalanobis distance. LEfSe analysis was visualized through an online platform (http://huttenhower.sph.harvard.edu/galaxy/), and R software produced the upset and waterfall plots. The differences in HTD data across colors and areas were evaluated using Mann–Whitney, Kruskal–Wallis, and permutational multivariate analysis of variance (PerMANOVA).

2.4. Analysis of physical and chemical characteristics

The moisture content, acidity, ash content, amino nitrogen content, starch content, saccharification power, esterification power, liquor-producing power, fermenting power, and liquefaction power of HTD in Qingzhou were determined following the method outlined in the standard QB/T 4257-2011 (general methods of analysis for Daqu). The protein content was determined using the Kjeldahl nitrogen determination method specified in the GB 5009.5–2016 standard (the determination of protein in food). The water activity was measured using a water activity meter (Aqualab, Pullman, WA, USA). R (v4.1.3) software generated violin plots to visualize the physical and chemical indicator data, and the correlation analysis between physicochemical indicators and microorganisms was visualized using Cytoscape (v3.7.2) software.

2.5. Isolation and identification of bacterial strains

Daqu samples (10 g) were placed in a triangular flask containing 90 mL of physiological saline, with 6–8 glass beads. The mixture was shaken at 30 °C for 30 min, then diluted to a 10^{-5} gradient. The dilutions of 10^{-3}, 10^{-4}, and 10^{-5} were plated on deMan Rogosa and Sharpe (MRS) and Luria-Bertan (LB) agar plates and incubated inverted at 37 °C for 48 h. Single colonies of suspected lactic acid bacteria (MRS, anaerobic culture) and bacteria (LB, aerobic culture) were selected, purified for three generations, and preserved in 30 % glycerol at −80 °C for future applications. Concurrently, the triangular flask with a 10^{-1} gradient was heated in a water bath at 85 °C for 15 min, then diluted to a 10^{-5} gradient. The dilutions of 10^{-3}, 10^{-4}, and 10^{-5} were plated on Nutrient Agar (NA) plates and incubated inverted at 28 °C for 48 h. Single colonies of suspected *Bacillus* spp. were selected, purified for three generations, and stored

in 30 % glycerol at −80 °C for future applications.

DNA of the isolate was extracted and amplified using PCR. Amplified products were purified, linked, and transformed. The positive clones were then forwarded to Shanghai Sunny Biotechnology Co., Ltd. (Shanghai, China) for testing. BLAST analysis was conducted on the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) after sequencing, splicing, and primer removal. A phylogenetic tree was constructed using the species sequence most closely related to the isolate identified in the BLAST analysis, along with the 16S rRNA gene sequence of the isolate. Visualization was accomplished using MEGA5.0 (https://www.megasoftware.net/reltime) and R software.

3. Results

3.1. Comparative analysis of bacterial community structure of HTD with three different colors in Qingzhou area

The high-throughput sequencing process revealed 1,871,327 sequences in HTD samples of three colors from the Qingzhou area. After the exclusion of low-quality sequences, 1,859,806 valid sequences were observed. Partitioning at a 97 % similarity identified 55,993 OTUs. The α-diversity index was calculated at a sequencing depth of 31,010 sequences. Significant differences in the Chao 1 index and the observed species index among the three colored HTD were detected using the Kruskal-Wallis test ($P < 0.01$) (Fig. 2a). Conversely, no significant differences were observed in Shannon and Simpson indices ($P > 0.05$). The Mann-Whitney test indicated that the Chao 1 index and the observed species index were significantly lower in black HTD, whereas white HTD exhibited the opposite trend ($P < 0.05$). These findings suggested that white HTD harbored the highest bacterial richness, whereas black HTD exhibited the lowest, without significant diversity difference among the three colors. The PCoA based on weighted UniFrac distance (Fig. 2b) illustrated that white and yellow HTD samples predominantly clustered in the negative half axis of the Y-axis, while black HTD samples were distributed in the positive half axis. Moreover, the PCoA based on the unweighted UniFrac distance (Fig. 2b) demonstrated that white and yellow HTD samples were primarily distributed in the negative half axis of the X-axis, whereas black HTD samples

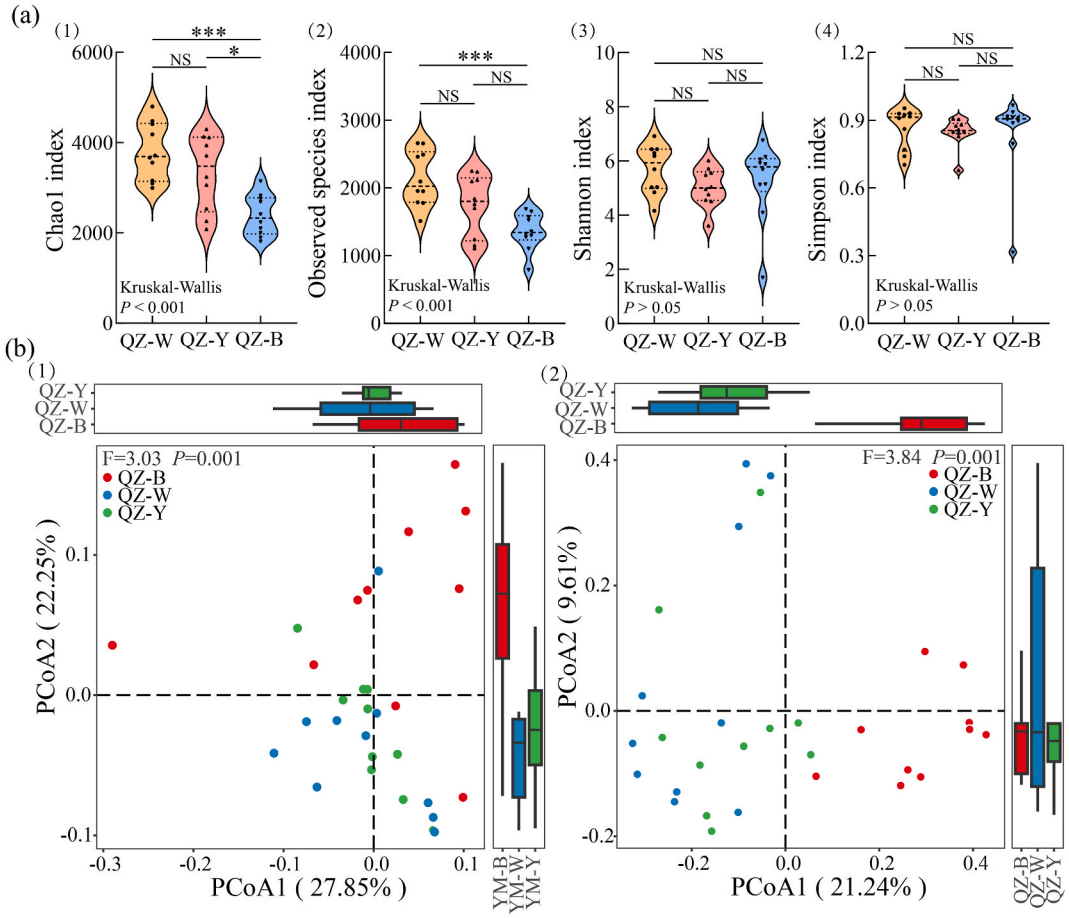

Fig. 2. HTD with different colors in the Qingzhou area: *α* diversity analysis (a) and *β* diversity analysis (b). a: Chao1 index (1); observed species index (2); Shannon Index (3); Simpson index (4). b: PCoA based on weighted UniFrac distance (1) and unweighted UniFrac distance (2). * indicates $P < 0.05$; ** indicates $P < 0.01$; *** indicates $P < 0.001$; NS indicates $P > 0.05$, the same below.

predominantly clustered in the positive half axis. According to the PERMANOVA analysis, significant disparities were observed in the spatial distribution of the HTD samples with varying colors ($P = 0.001$). Additionally, β-diversity analysis confirmed significant differences in the bacterial community structure among the three colored HTD samples in the Qingzhou area. Compared to other two types, the bacterial community structure in the black HTD was relatively unique.

3.2. Analysis of bacterial community structure of three colors of HTD in the Qingzhou area

After comparing all high-quality sequences with the database and annotating their taxonomic positions, a total of 22 phyla, 42 classes, 85 orders, 173 families, and 408 genera were identified from the HTD samples of three colors in the Qingzhou area. Bacterial phyla and genera with an average relative content exceeding 1.0 % were defined as being dominant. As shown in Fig. 3a, the dominant bacterial phyla in the HTD samples from the Qingzhou area primarily consisted of Firmicutes (88.49 %), Actinobacteria (6.46 %), and Proteobacteria (4.82 %). Additionally, Fig. 3b demonstrates that the dominant bacterial genera were primarily *Lentibacterius* (16.98 %), *Scopulibacillus* (16.13 %), *Staphylococcus* (12.88 %), *Thermoactinomyces* (11.76 %), *Bacillus* (10.56 %), *Kropenstedtia* (6.60 %), *Saccharopolyspora* (4.60 %), *Limosilicobacillus* (2.18 %), *Oceanobacillus* (1.33 %), and *Weissella* (1.30 %). Notably, the Kruskal-Wallis test revealed differences in the distribution of Firmicutes and Proteobacteria among the dominant bacterial phyla in the three colored HTD samples. ($P < 0.01$) Among these, the Firmicutes content in the black HTD samples was significantly lower ($P < 0.05$), while the Proteobacteria content was significantly higher ($P < 0.01$) (Fig. 3c). Furthermore, the Kruskal-Wallis test identified differences in the content distribution of *Scopulibacillus*, *Staphylococcus*, *Bacillus*, *Limosillactobacillus*, and *Weissella* among the dominant bacterial genera in the three colored HTD samples ($P < 0.01$). The Mann-Whitney test revealed that the content of *Scopulibacillus*, *Bacillus*, *Limosillactobacillus*, and *Weissella* in black HTD samples with different colors were significantly lower, whereas the *Staphylococcus* content was significantly higher ($P < 0.05$). Notably, apart from *Scopulibacillus*, which exhibited a significantly higher content in yellow HTD than in white HTD ($P < 0.05$), no significant difference was observed in the content of other dominant bacterial genera between white and yellow HTD ($P > 0.05$). These findings indicated a diverse bacterial community in the HTD samples from the Qingzhou area, with significant variations in bacterial composition among the three colored HTD samples, particularly in the uniquely black HTD. According to Mann Whitney's test, *Scopulibacillus*, *Bacillus*, *Limosilicobacillus*, and *Weissella* have significantly lower content in black HTD samples, while *Staphylococcus* has significantly higher content ($P < 0.05$).

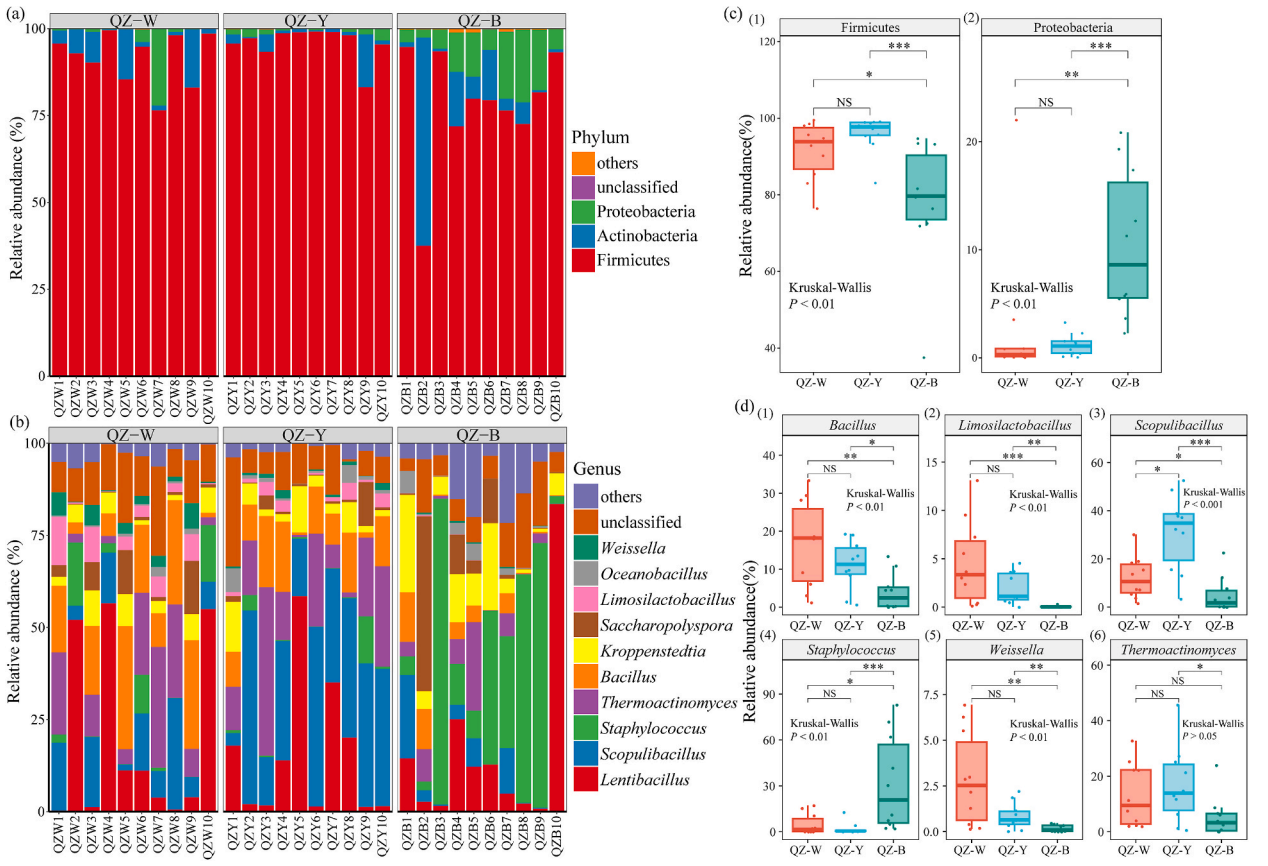

Fig. 3. Stack histogram of dominant bacterial phyla (a) and genera (b) in HTD in Qingzhou area. Comparative analysis of differentially dominant bacterial phyla (c) and genera (d) with different colors of HTD.

3.3. Physical and chemical quality of three colors of HTD in the Qingzhou area and its correlation with bacterial community

Determining physical and chemical indices can be crucial for assessing the fermentation status and quality of Daqu. The analysis revealed that moisture content of the HTD ranged from 8.32 % to 10.62 %, water activity from 0.50 to 0.61 aw, acidity from 3.36 to 19.85 mmol/10g, the ash content from 1.82 to 2.15 g/100g, the protein content from 14.70 % to 18.40 %, the amino nitrogen from 2.71 to 4.35 g/kg, and the starch content from 56.96 to 209.23 g/100g. Significant differences were observed in physicochemical indices among the three colored HTD samples ($P < 0.05$), according to the Kruskal-Wallis test. The moisture content and ash content were notably higher in white HTD, averaging 9.83 % and 1.98 g/100 g, respectively. Yellow HTD exhibited relatively higher protein and amino nitrogen contents, averaging 16.19 % and 3.75 g/kg, respectively. However, black HTD had relatively high water activity, acidity, and starch content, averaging 0.57 aw, 16.70 mmol/10g, and 179.98 g/100 g, respectively (Fig. 4a). The correlation analysis with dominant bacterial genera revealed significant negative correlations between water activity and *Scopulibacillus* and *Limosilactobacillus* ($P < 0.05$), and significant positive correlations with *Staphylococcus* and *Kropenstedtia* ($P < 0.05$). Additionally, protein content demonstrated a significant positive correlation with *Scopulibacillus* ($P < 0.05$), and starch content exhibited a significant negative correlation with *Scopulibacillus* ($P < 0.01$) and a significant positive correlation with *Staphylococcus* ($P < 0.05$). In addition, ash content displayed a significant negative correlation with *Staphylococcus* ($P < 0.05$) and a significant positive correlation with *Bacillus* ($P < 0.01$), while acidity showed a significant negative correlation with *Bacillus* ($P < 0.05$), *Weissella* ($P < 0.01$), and *Limosilolactobacillus* ($P < 0.01$) and a significant positive correlation with *Oceanobacillus* ($P < 0.05$) (Fig. 4c).

Determining enzyme activity in Daqu is essential for assessing biochemical properties, such as saccharification, ethyl acetate, alcohol, and carbon dioxide production abilities, as well as alcohol yield. Fig. 4b illustrates that saccharification power of HTD samples in the Qingzhou area ranged from 0.4 to 364.4 mg/g·h, esterification power ranged from 1.70 to 421.10 mg/50 g·7d, liquor-producing power ranged from 0.00 to 9.98 % vol, fermenting power ranged from 0.01 to 0.24 g/0.5 g·72h, and liquefaction power ranged from 0.00 to 0.60 g/g·h. The Kruskal-Wallis test revealed significant differences in saccharification, esterification, and liquefaction power among the three colored HTD samples ($P < 0.05$). Among them, white HTD exhibited significantly higher saccharification ability and

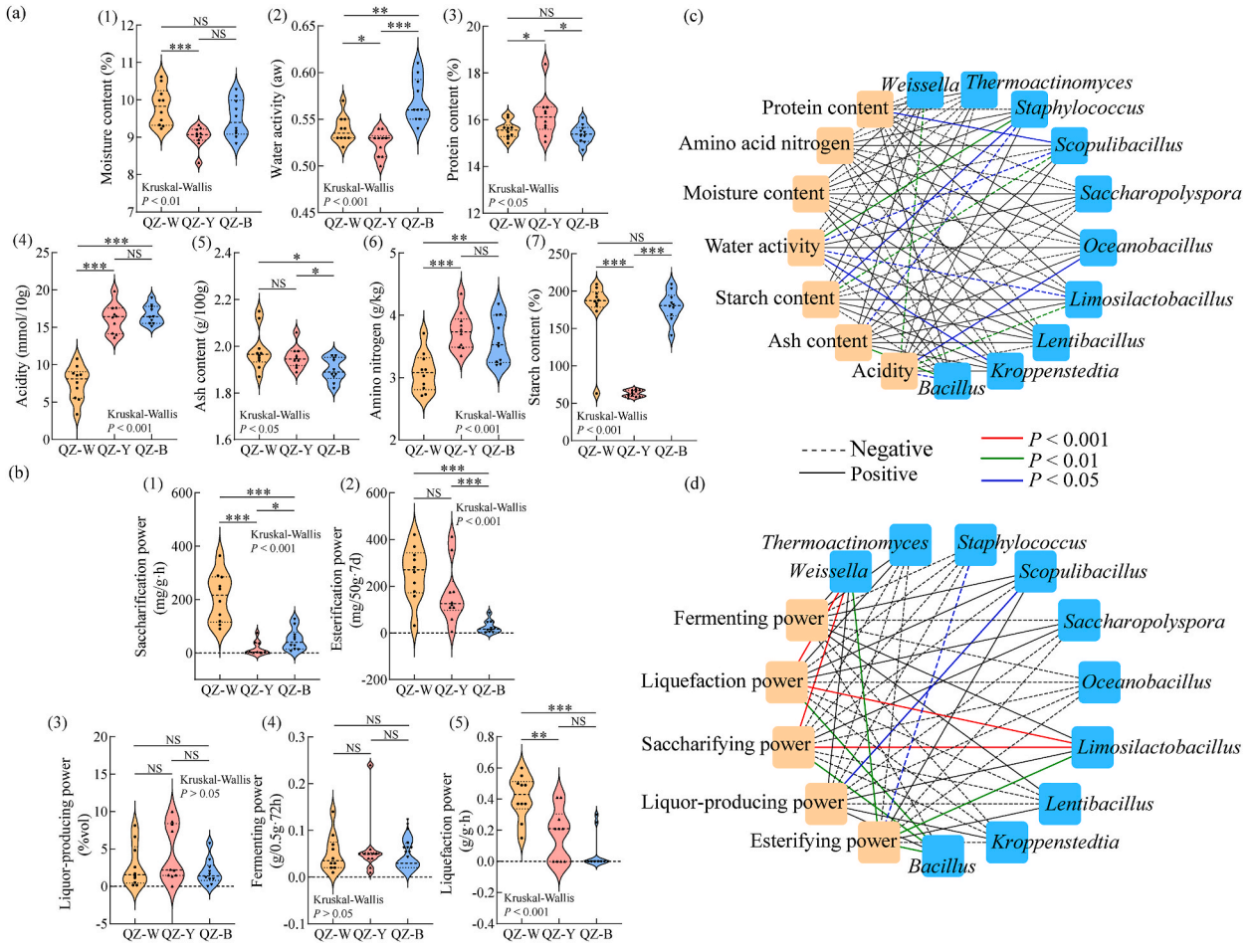

Fig. 4. Violin plot of physicochemical indices (a) and enzyme activity (b) of HTD in Qingzhou area. Correlation network diagram between physicochemical indices and dominant bacterial genera (c), as well as enzyme activity and dominant bacterial genera (d).

alcohol yield ($P < 0.05$), with saccharification and liquefaction power of 207.98 mg/g·h and 0.41 g/g·h, respectively. In contrast, black HTD demonstrated a significantly lower ethyl acetate production ability ($P < 0.05$), with an esterification power of only 25.88 mg/50 g·7d. However, there were no notable differences in alcohol and carbon dioxide production abilities among the three HTD colors ($P > 0.05$). It is worth noting that in this study, different white HTD samples showed great differences in saccharification power, which may be related to the differences in microbial communities contained in different samples. Studies have shown that the saccharification power in Daqu is positively correlated with the content of *Staphylococcus* [6], so it is of positive significance to further study the microbial composition of Daqu. The correlation analysis with the dominant bacterial genera revealed a significant positive correlation between a liquor-producing power and *Scopulibacillus* ($P < 0.05$). Moreover, esterification ability presented a significant negative correlation with *Staphylococcus* ($P < 0.05$), and a significant positive correlation with *Bacillus*, *Limosillactobacillus*, and *Weissella* ($P < 0.01$). Furthermore, the saccharification ability and liquefaction power were significantly positively correlated with *Bacillus* ($P < 0.01$), *Limosillactobacillus* ($P < 0.001$), and *Weissella* ($P < 0.001$) (Fig. 4d). These findings indicated significant differences in physical and chemical indices and certain enzyme activities among the three colored HTD samples in the Qingzhou area, demonstrating a correlation between the formation of physical and chemical characteristics and the composition of their bacterial communities.

3.4. Isolation and identification of cultivable bacteria of three colors of HTD in Qingzhou area

Based on uncovering a specific correlation between the physical and chemical properties of Daqu and its bacterial community structure, we isolated, purified, and identified bacterial communities from three differently colored samples of HTD through pure cultivation. As shown in Fig. 5, 63 bacterial strains were isolated and identified from 30 HTD samples from the Qingzhou area, representing 19 species across 10 genera. Among them, 25 strains, including HBUAS69425, were identified as *Bacillus*

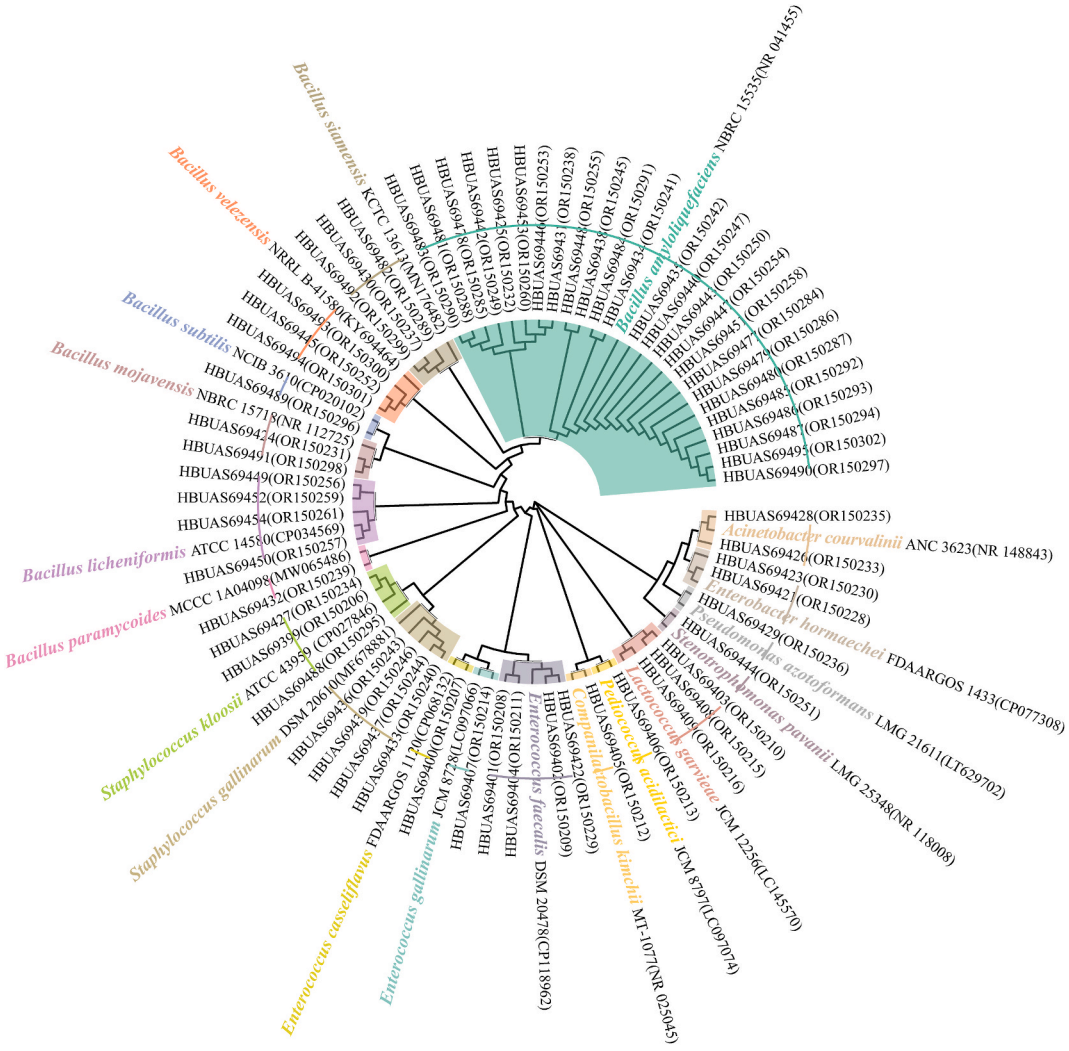

Fig. 5. Phylogenetic tree of cultivable bacteria in HTD in Qingzhou area.

amyloliquefaciens, 4 strains, including HBUAS69450, belonged to *B. licheniformis*, 3 strains, including HBUAS69445, belonged to *B. velezensis*, 3 strains, including HBUAS69430, belonged to *B. siamensis*, 2 strains, including HBUAS69424, were identified as *B. mojavensis*, HBUAS69432 as *B. paramycoides*, HBUAS69489 as *B. subtilis*, HBUAS69405 as *Companilactobacillus kimchii*, 4 strains, including HBUAS69401, were identified as *Enterococcus faecalis*, HBUAS69400 as *Enterococcus casselifalavus*, HBUAS69407 as *Enterococcus gallinarum*, 2 strains, including HBUAS69421, belonged to *Enterobacter hormaechei*, HBUAS69403 as *Lactococcus garvieae*, and HBUAS69406 as *Pediococcus acidilactici*, HBUAS69429 as *Pseudomonas azotoformans*, 4 strains, including HBUAS69436, belonged to *Staphylococcus gallinarum*, 3 strains, including HBUAS69399, were identified as *Staphylococcus kloosii*, HBUAS69444 as *Stenotropiomonas pavanii*, and 2 strains, including HBUAS69428, were identified as *Acinetobacter courvalinii*. This analysis revealed that *Bacillus* and lactic acid bacteria constituted 61.90 % and 17.46 % of the main cultivable bacterial communities in HTD in the Qingzhou area, respectively.

3.5. Difference analysis of bacterial communities in HTD from Qingzhou and Xiangyang areas

Bacterial sequences were retrieved from the MG-RAST database (accession number: mgp96594) for 10 white, 10 yellow, and 10 black HTD samples from the Xiangyang area. Further investigation focused on discerning differences in the bacterial community structure across various regions and the processing methods involved in HTD production. As depicted in Fig. 6a, the Chao 1, observed species, Shannon, and Simpson indices of HTD samples from the Qingzhou area significantly surpassed those from the Xiangyang area ($P < 0.001$), indicating notably higher richness and diversity in bacterial communities within HTD from Qingzhou ($P < 0.001$). In Fig. 6b, PCOA analysis based on the unweighted UniFrac distance revealed dispersed distributions of HTD samples from both regions in two-dimensional space. Specifically, the Qingzhou HTD samples predominantly occupied the negative half axis of the X-axis, whereas the Xiangyang HTD samples were mainly situated in the positive half axis of the X-axis. Additionally, PERMANOVA analysis highlighted a substantial disparity in the spatial distribution of HTD samples between the two regions ($P = 0.001$), indicating that β diversity analysis confirmed significant differences in the bacterial community structure of HTD between the Qingzhou and Xiangyang areas. Furthermore, clustering analysis based on Mahalanobis distance was performed on white, yellow, and black HTD samples from both areas. As depicted in Fig. 6c, the HTD samples of the three colors from Qingzhou formed a distinct cluster under a major branch, indicating a significant disparity between the HTD samples from the two regions ($P < 0.001$). In summary, considerable differences were observed among HTD samples produced in various regions and under different processes. Moreover, a substantial number of unique bacterial communities were identified in HTD samples from Qingzhou and Xiangyang.

To analyze the distinct variances in the bacterial community structure of HTD between the Qingzhou and Xiangyang areas, we

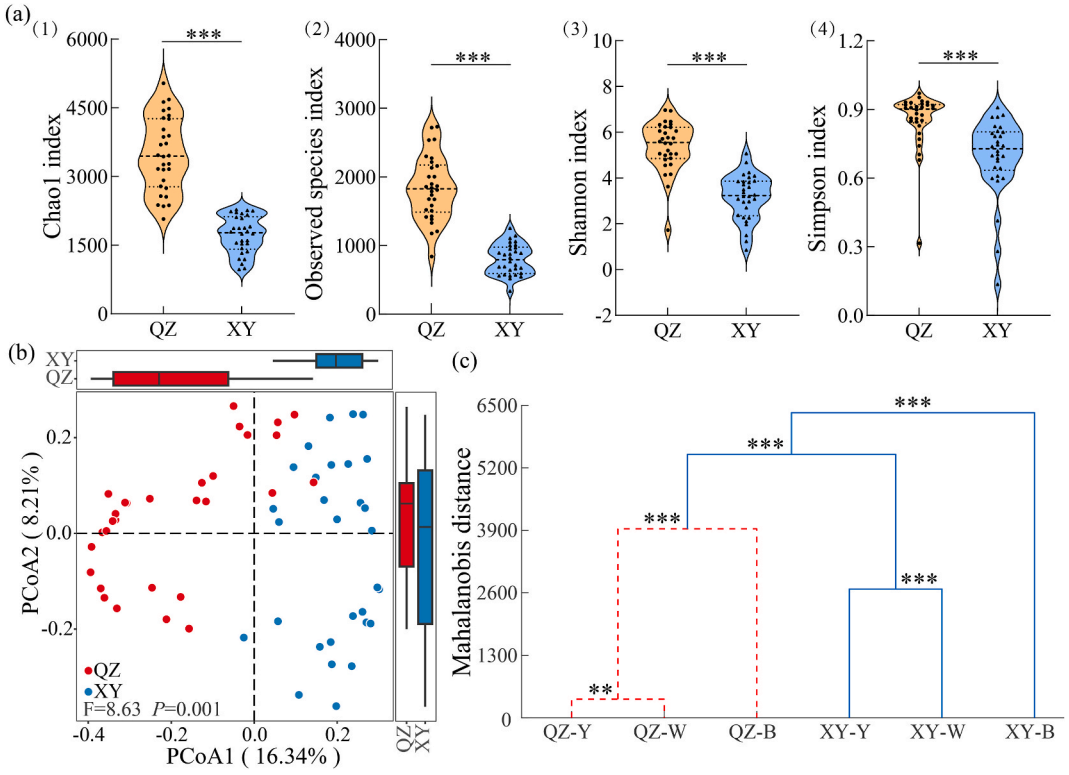

Fig. 6. Bacterial communities in HTD in Qingzhou and Xiangyang regions: *α* diversity analysis (a); PCoA analysis based on unweighted UniFrac distance (b); cluster analysis based on Mahalanobis distance (c).

utilized Lefse analysis to identify biomarkers specific to each region. Fig. 7a illustrates that at the class level, prominent bacterial taxa in the Qingzhou HTD primarily belonged to Alphaproteobacteria and Gammaproteobacteria branches, while no significant bacterial community was observed in the Xiangyang HTD samples at this level. Fig. 7b reveals that when the LDA score exceeded 3.0, 30 bacterial taxa exhibited significant differences between HTD samples from the two regions ($P < 0.05$). Notably, key bacterial categories were markedly more abundant in the Qingzhou HTD samples than in the Xiangyang HTD samples. At the genus level, *Pantoea*, *Lactobacillus*, *Enterococcus*, *Companilactobacillus*, *Lactococcus*, *Weissella*, *Oceanobacillus*, *Limosillactobacillus*, *Bacillus*, and *Scopulibacillus* were abundant in HTD samples from Qingzhou ($P < 0.05$), whereas *Kroppenstedtia*, *Rhodococcus*, and *Ralstonia* were significantly enriched in HTD samples from Xiangyang ($P < 0.05$). This indicated that the bacterial community of HTD in Qingzhou exhibits a greater diversity and abundance of *Bacillus* and lactic acid bacteria than that in Xiangyang, consistent with the results of the α diversity index analysis mentioned earlier.

3.6. Similarity analysis of bacterial communities in HTD in Qingzhou and Xiangyang areas

After the analysis of significant differences in the bacterial community composition between HTD samples from both areas, this study revealed common bacterial communities at the OTU level in samples from Qingzhou and Xiangyang. The OTU present in all HTD samples were defined as the core OTU. As illustrated in Fig. 8a, 7578 OTUs were exclusively found in HTD samples from Qingzhou, 2140 OTUs were unique to HTD samples from Xiangyang, and 6576 OTUs were presented in HTD samples from both regions. Fig. 8b illustrates five core OTUs with an average relative content exceeding 1.0 % in HTD samples from both Qingzhou and Xiangyang. These core dominant OTUs included OTU51717 (*Kropentedtia*, 15.96 %), OTU41869 (*Lentibacterillus*, 14.84 %), OTU46054 (*Staphylococcus*, 6.16 %), OTU74937 (*Saccharopolyspora*, 4.11 %), and OTU352 (*Kropentedtia*, 3.03 %), collectively constituting 44.09 % of the total bacterial population. The Mann-Whitney test revealed that among the five-core dominant OTUs, only OTU51717 exhibited significant differences in HTD samples from the Qingzhou and Xiangyang areas ($P < 0.05$), with average relative contents of 7.31 % and 24.61 %, respectively. This indicated that *Kropentedtia*, *Lentibacillus*, *Staphylococcus*, and *Saccharopolyspora* were common core bacterial communities in both regions, with only *Kropentedtia* significantly more abundant in Qingzhou HTD.

4. Discussion

In this study, it was found that black HTD exhibited the lowest richness, whereas the bacterial community structures of white and yellow HTD were relatively similar. Similar to our findings, Y. Wang et al. [7] also found that the bacterial community structure of black HTD in Xiangyang, Hubei Province is more specific, whereas the bacterial community structures in white and yellow HTD were more similar. The reason for this phenomenon may be related to the difference of temperature and humidity in the process of HTD accumulation and fermentation. Temperature variations can significantly influence the physicochemical characteristics of HTD with varying colors. Q. Zhu et al. [3] observed that temperature discrepancies induced by stacked fermentation not only led to varying degrees of Maillard reaction in HTD, but also significantly affected enriched microbial groups. Specifically, *Bacillus* dominated Kweichow Moutai white HTD, *Virgibacillus* and *Staphylococcus* prevailed in Kweichow Moutai yellow HTD, and *Kroppenstedtia* thrived

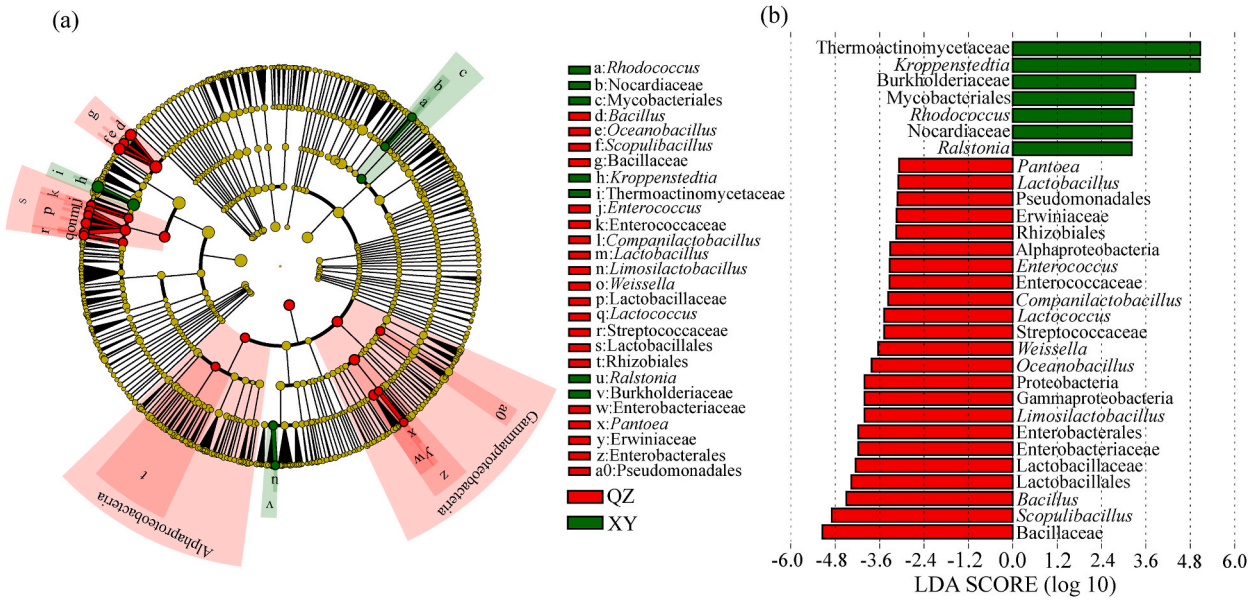

Fig. 7. Linear Discriminant Analysis (LDA) Effect Size (LEfSe) analysis of significantly enriched microorganisms in HTD in Qingzhou and Xiangyang. a: Evolutionary branching diagram; b: LDA distribution histogram.

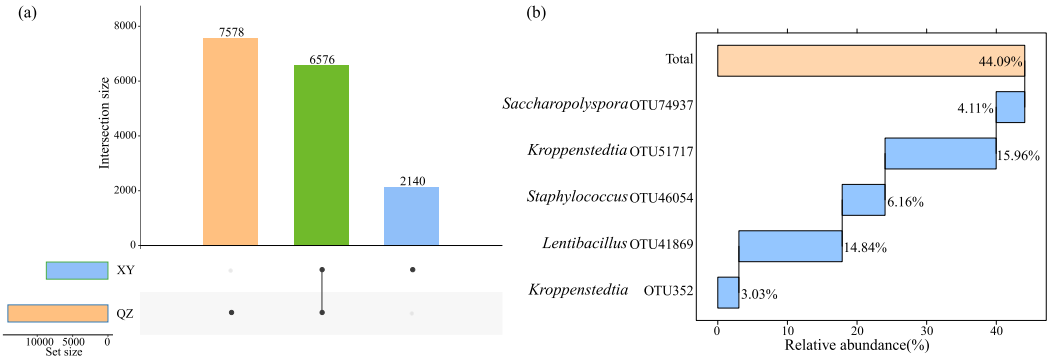

Fig. 8. Analysis of common microorganisms in HTD in Qingzhou and Xiangyang. a: Upset plot of Operational Taxonomic Unit (OTU) content distribution in HTD from the two areas. b: Waterfall diagram of core advantage OTUs in the two areas.

in Kweichow Moutai black HTD. Moreover, studies have indicated that bacterial communities in HTD are more susceptible to environmental factors and species interactions than fungal communities in HTD [5]. However, contrary to our results, Deng et al. [6] observed greater microbial species richness and diversity in black HTD from Luzhou, Sichuan Province. It can be seen that temperature is an important factor affecting the composition of bacterial communities in Daqu, but it is not the only one. Additionally, disparities in HTD quality may arise from variations in geographical location, environmental conditions, and production methods. Previous studies have indicated that pH, temperature, moisture, and acidity interactions within the Qu-room, along with microbial community variations across habitats, contribute to divergent microbial structures in HTD from the same batch stacked at different locations [21]. Appropriate temperatures and moisture levels are fundamental for forming microbial communities and Maillard reactions in HTD [22]. M. Zhu et al. [23] showed that environmental humidity was a key driving force for microbial succession in Daqu, and it was positively correlated with the content of bacterial communities such as *Pantoea* and *Staphylococcus*. All HTD samples in this study had moisture contents below 13 %, meeting the requirements for moisture standards for finished Daqu [24]. Notably, white HTD exhibited a relatively higher moisture content, potentially related to its shorter exposure to high-temperatures during the fermentation, reducing its browning intensity. On the contrary, the water activity in the inner layer of black HTD is relatively high but the water content is relatively low. This may be due to the fact that water may gradually transfer from the inner to the outer layers of Daqu [25], driven by spontaneous processes or active microorganisms. Upon optimal temperature and moisture conditions, the Maillard and Caramelization reactions during HTD production led to a yellow appearance and a rich, robust aroma reminiscent of soy sauce [26].

In addition to temperature and humidity, various factors such as regional disparities, environmental conditions, and production methodologies contribute partially to the bacterial community structure and physicochemical quality of Daqu, consistent with previous research results [27–29]. Therefore, further investigation into microorganisms within HTD across diverse production regions is crucial to comprehensively understand both the similarities and distinctions in microbial resources among different Daqu areas, thereby elucidating the fermentation mechanisms of HTD and Maotai-flavor Baijiu. The cluster analysis further revealed close proximity among bacterial communities in HTD samples of different colors from the same production area. This indicated that compared to variations in Daqu production temperatures, the composition of bacterial communities differed among the three colored HTD samples, with regional disparities, raw materials, and production techniques exerting a more pronounced impact on the microbial community structure of Daqu. Utilizing single-molecule real-time sequencing technology, Xiang et al. [29] observed discrepancies in the composition and content of lactic acid bacteria and thermophilic bacterial communities in low-temperature Daqu from the Taiyuan and Suizhou regions due to differing Daqu production processes. Similarly, Ma et al. [28] noted substantial variations in microbial composition among Nongxiangxing Daqu from different production areas, resulting in diverse volatile metabolites and potential functionalities, thereby contributing to differences in flavor quality across various production regions of Nongxiangxing Baijiu. LEfSe analysis identified *Scopulibacillus* and *Kroppenstedtia* as bacterial genera with the highest LDA values in HTD samples from the Qingzhou and Xiangyang areas, respectively, with relatively richer types and contents of *Bacillus* and lactic acid bacteria biomarkers in the Qingzhou HTD samples. Similar to the results of this study, Y Zhang et al. [30] found that *Scopulibacillus* was more abundant in northern HTD than in southern HTD, with an average relative content of 19.40 % and 6.69 %, respectively. Although *Scopulibacillus* is one of the main bacterial communities in HTD, further research is needed to determine its specific contribution in HTD [31]. *Kropentedtia* was a significant source of glucose in the Daqu [30], which can significantly increase the content of volatile components such as esters, alcohols, acids, and pyrazines in Daqu, and promote the synthesis of esters [32]. At the OTU level, *Kroppenstedtia* (18.99 %), *Lentibacillus* (14.84 %), *Staphylococcus* (6.16 %), and *Saccharopolyspora* (4.11 %) were identified as the common core bacterial communities in all HTD samples from both regions. *Lentibacillus* exhibited active amino acid metabolism and secreted various proteases beneficial for flavor formation in Baijiu [33], whereas *Saccharopolyspora* facilitated aroma substance formation and accumulation in Daqu [33]. Despite *Staphylococcus aureus* being mostly pathogenic [7], Y. Yang et al. [34] highlighted *Staphylococcus gallinarum* as the primary species potentially producing carboxylesterase in Daqu, suggesting the utility of *Staphylococcus* strains as starter cultures in food fermentation. These core bacterial groups including *Kroppenstedtia*, *Lentibacillus*, *Staphylococcus* and *Saccharopolyspora*, enriched in HTD from both regions, notably contributed to Daqu and Baijiu flavor quality formation.

Maotai-flavor Baijiu, renowned as the most intricate type of Baijiu in China, is epitomized by Kweichow Moutai, which is located in

the upper reaches of the Yangtze River. Numerous other regions across China also exhibit significant HTD and Maotai-flavor Baijiu brewing areas, differences in Daqu production techniques and environmental factors exert a substantial influence on HTD quality, consequently influencing the taste and aroma variations in Maotai-flavor Baijiu across different production areas [35]. In addition, bacteria, as pivotal microbial agents in Baijiu fermentation, actively contribute to the enzymatic and aromatic profile development in HTD and Maotai-flavor Baijiu. This study employed high-throughput sequencing technology to analyze the bacterial flora in three varieties of white, yellow, and black HTD from the Qingzhou production area, situated in the lower reaches of the Yellow River, and isolated brewing microbial resources via pure culture. Simultaneously, the physical and chemical qualities of the three HTD colors were assessed. The findings of this study can offer valuable insights for enhancing the quality of HTD and Maotai-flavor Baijiu originating from the Qingzhou production area. In the future, we can analyze the microbial structure across more HTD-producing areas to explore high-quality brewing microbial resources, and further conduct in-depth research on the metabolites of common dominant microorganisms. This exploration forms the basis for understanding the complex flavor quality of Maotai-flavor Baijiu, with significant implications for the safety and quality enhancement of both HTD and Maotai-flavor Baijiu.

5. Conclusion

This study investigated the bacterial community structure and physicochemical quality of HTD in Qingzhou production area. The results revealed diverse bacterial groups in the Qingzhou HTD, with black HTD exhibiting the most unique bacterial composition, while white and yellow HTD displayed relatively similar bacterial community structures. *Bacillus* and lactic acid bacteria were the primary cultivable bacterial groups in the Qingzhou HTD. Significant differences in physical and chemical indicators, along with partial enzyme activity, were observed among the three HTD colors, with a correlation between the physicochemical characteristics and bacterial community composition. The comparative analysis of the Xiangyang production area demonstrated significantly higher richness and diversity of the bacterial communities in the Qingzhou HTD. Notably, *Scopulibacillus*, *Bacillus*, and *Limosillactobacillus* were significantly enriched in the Qingzhou HTD, whereas *Kroppenstedtia*, *Rhodococcus*, and *Ralstonia* were enriched in the Xiangyang HTD. Furthermore, common core bacterial communities, such as *Kroppenstedtia*, *Lentibacillus*, *Staphylococcus*, and *Saccharopolyspora*, were identified in HTD from both areas.

CRediT authorship contribution statement

Dongying Ge: Writing – review & editing, Visualization. **Wenchao Cai:** Software, Methodology, Formal analysis. **Zhuang Guo:** Visualization, Funding acquisition. **Bangkun Wang:** Resources. **Minwan Liu:** Resources. **Chunhui Shan:** Visualization, Supervision, Data curation. **Yurong Wang:** Methodology, Funding acquisition, Conceptualization.

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.

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Data availability statement

Raw sequence data are publicly available online under the National Center for Biotechnology Information Sequence Read Archive database (BioProject ID: PRJNA1070552).

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