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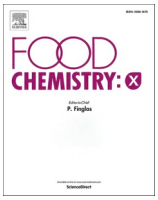

Precipitation and temperature drive microbial community changes affecting flavor quality of *Nongxiangxing Daqu*

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ABSTRACT

Nongxiangxing Baijiu is the most famous *Baijiu* flavor in China, and its characteristic style is closely related to *Nongxiangxing Daqu* used in fermentation. However, there are few reports about the difference of *Daqu* quality between seasonal variations. In this study, precipitation and temperature drove changes in microbial communities that resulted in differences in the flavor of *Daqu* produced in different seasons. For example, the average daily temperature in summer was as high as 27.29 ± 2.24 °C, which was significantly higher than other seasons ($p < 0.01$). *Bacillus* was abundant in the *Daqu* produced in this season, while tetramethylpyrazine flavor was more prominent, up to 1556.95 ± 153.92 μg/kg. Metabolomics studies identified major pathways associated with the weak flavor of spring_*Daqu*. In addition, LEFSe analysis revealed the marked microorganisms in different seasons. These results revealed the differences in seasonal *Daqu*, thus contributing to the scientific and rational use of *Daqu*.

1. Introduction

As one of the six most well-known distilled spirits in the world, Chinese *Baijiu* has a long history and plays a vital role in the Chinese food industry (Ma et al., 2022). Among all types of *Baijiu*, *Nongxiangxing Baijiu* is one of the most famous in China, which is distilled from sorghum and other grains through solid-state fermentation in a mud cellar, with a mellow taste and balanced aroma. This unique aroma is closely related to *Daqu*. *Daqu*, also known as *Jiuqu*, is made from wheat and other grains through open fermentation, and is the saccharifying and fermenting agent of *Baijiu*. During the fermentation process, microorganisms gradually enter the *Daqu* from the environment, causing the raw materials to form a complex and multifunctional microbial community and producing a variety of enzymes and compounds (Sakandar et al., 2020), which is the core indicator of the quality control of *Baijiu* brewing at present.

At present, the research on *Nongxiangxing Daqu* mainly focuses on microbial community succession (Huang et al., 2023; Zhu et al., 2022), screening of functional strains (Guan et al., 2023), and the composition of flavor compounds (Tan et al., 2019). In addition, many researchers have made a lot of explorations and attempts in accelerating and guiding the brewing of *Baijiu* by utilizing biofortification (Lv et al., 2023). It is worthwhile to recognize that these studies have improved our in-depth understanding of *Daqu* microbiology and quality. Previous researchers have done a lot of very valuable work on *Daqu* microorganisms and quality, but reports on the effects of climate on *Daqu* microorganisms and their quality are relatively rare, and people lack a systematic knowledge about them.

Climatic factors regulate the microbial structure and metabolism during spontaneous food fermentation (Kou et al., 2022). Changes in environmental conditions in different seasons are an important factor in microbial structural changes during the production of *Daqu*, such as the

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microbial community changes caused by the daily average temperature (Wang et al., 2020). Therefore, the microbial community structure of *Daqu* is closely related to the brewing microecological environment, and seasonal climate change are one of the main factors affecting the microecology of the brewing environment. Consequently, it is necessary to study the effect of seasonal climate change on the microbial community structure of *Nongxiangxing Daqu*.

This study elucidated the differences between *Daqu* produced in different seasons in terms of physicochemical indicators, flavor compounds, and microbial community structure. Biomarkers for seasonal *Daqu* were identified using LEfSe analysis, and correlation analysis revealed the relevance between climatic factors and microorganisms. To identify the key pathways leading to the observed quality differences, LC-MS metabolomics analysis of *Daqu* produced in different seasons was performed. This study contributes to the understanding of the differences in *Nongxiangxing Daqu* produced in different seasons, provides a certain degree of research on the controllability of *Daqu* and its scientific application in production.

2. Materials and methods

2.1. Sample collection and climate data acquisition

The sampling seasons were based on the 24 Chinese solar terms of "Spring Commences, Summer Commences, Autumn Commences, Winter Commences". The culture period for the spring_*Daqu* was from March 12, 2022 to April 11, 2022, summer_*Daqu* was from June 15, 2022 to July 15, 2022, autumn_*Daqu* was from September 12, 2022 to October 12, 2022, and winter_*Daqu* was from November 10, 2021 to December 10, 2021. Samples were collected at the end of the culture for the 30 days of fermentation of the *Daqu*, obtained from Chengdu Shuzhiyuan Liquor Co. Ltd. in Sichuan Province. The *Daqu* bricks, randomly selected in triplicate from the upper, middle, and lower stacked layers, were ground and mixed evenly. Then 100 g samples were collected using the quartering method (Wang et al., 2017), transferred to three aseptic bags, and stored at −20 °C until further analysis.

Climate data were collected at the National Meteorological Information Center-China Meteorological Data Network (https://data.cma.cn/). Meteorological data such as temperature, surface pressure, precipitation, relative humidity, wind speed, and sunlight hours during the fermentation period of the sampling site in 2021 and 2022 were collected (Table S1).

2.2. Analysis of physicochemical and enzymatic properties

The physicochemical and enzymological *Daqu* properties were measured in triplicate using national professional standard methods (QB/T 4257–2011, 2011). Next, 10 g of each sample was dried at 105 °C to determine the weight loss, which was used to measure the *Daqu* moisture level (Li et al., 2017). The pH was measured at a 1: 2.5 (wt/vol) ratio in double-distilled water (ddH_2O) using a pH meter-FE20 (Mettler Toledo, Shanghai, China). The starch level was determined using Fehling's titration method (Nozawa et al., 2005), while the acidity of the *Daqu* water extracts was evaluated via alkali titration (QB/T 4257–2011).

To assess the quality of the *Daqu* samples, the saccharification activity (U), liquefaction activity (U), esterification activity (U), and fermentation activity (U) were determined using national professional standard methods (QB/T 4257–2011, 2011). Saccharification was defined as 1 mg glucose hydrolyzed from soluble starch for an hour by 1.0 g starter at 40 °C pH 4.6. Liquefaction was defined as the mass (g) of soluble starch liquefied for an hour by 1.0 g starter at 60 °C pH 6. Esterification was defined as the mass (mg) of ethyl caproate produced by 1.0 g starter at 30–32 °C for 100 h. Fermentation activity was defined as the mass (g) of CO_2 produced from fermentable sugars for 72 h by 0.5 g starter at 30 °C.

2.3. Analysis of the volatile compounds

Previous research (Jin et al., 2019) used headspace solid-phase microextraction-gas chromatography–mass spectrometry (HS-SPME-GC–MS) to detect the volatile Daqu flavor compounds. An SPME fiber (50/30 μmDVB/CAR/PDMS; Supelco, Bellefonte, PA, USA) was used for volatile flavor compound sampling, while the GC–MS (Trace GC 1310 - ISQ mass spectrometer; Thermo Scientific, Austin, TX, USA) detected volatile flavor compounds. The volatile compounds were identified and quantified according to the internal standard 2-octanol response (Sigma-Aldrich, St. Louis, MO, United States). The obtained spectra were searched and analyzed using NSIT11.L and RTLPESRT, while peaks with a similarity of <80 % and siloxane-type impurities were screened and removed.

2.4. Total DNA extraction and PCR amplification

The total DNA was extracted using a commercial E.Z. N.A™ Mag-Bind Soil DNA Kit (Omega Bio-Tek, Norcross, GA, USA). The quality of the extracted DNA was assessed via electrophoresis in 1 % agarose gel and spectrophotometry at an optical density ratio at 260 nm/280 nm. For the bacteria, the 338F (5′-ACTCCTACGGGAGGCAGCAG-3′)/806R (5′-GGACTACHVGGGTWTCTAAT-3′) universal primer was used to amplify the V3-V4 hypervariable region of the 16S rRNA gene (Soergel et al., 2012). For the fungi, the ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′)/ITS2R (5′-GCTGCGTTCTTCATCGATGC-3′) primers were used to amplify the internal transcribed spacer (ITS) region (Usyk et al., 2017). A method described by Ma et al. (2022) was used for PCR mixture and amplification, after which the products were separated using 2 % agarose gel. The purified amplicons were analyzed via paired-end sequencing using an Illumina NovaSeq PE250 platform (Illumina, San Diego, CA, USA) according to the standard protocols described by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China).

The Toolkit software was combined with FLASH software for sequence quality control and filtration (Patel & Jain, 2012). The QIIME (V 1.9.1) software was employed for the quality control, demultiplexing, and subsequent analysis of the amplicon sequencing data. The high-quality pattern data were divided into operational taxonomic units (OTUs), while a 97 % consistency was considered the cut-off value after subsampling to the same sequencing depth. The taxonomic information of each OTU was added to the BIOM file via the RDP Classifier Ribosomal Database Project, which provides data and tools for high throughput rRNA analysis.

2.5. Analysis of the metabolites

Here, 50 mg *Daqu* samples were accurately weighed, after which the metabolites were extracted using a 400 μL methanol:water (4: 1, *v*/v) solution with 0.02 mg/mL L-2-chlorophenylalanin as an internal standard. The mixture was allowed to settle at −10 °C and treated in a high throughput tissue crusher Wonbio-96c (Shanghai Wanbo Biotechnology Co. Ltd.) at 50 Hz for 6 min, followed by ultrasonic exposure at 40 kHz for 30 min at 5 °C. The proteins in the samples were precipitated at −20 °C for 30 min and centrifuged at 13000*g* at 4 °C for 15 min, after which the supernatant was carefully transferred to sample vials for LC-MS/MS analysis.

LC-MS analysis was performed using a UHPLC-Q Exactive system (Thermo Fisher Scientific, Waltham, MA, USA). The chromatographic conditions involved using an HSS T3 column (100 mm × 2.1 mm i.d., 1.8 μm) to separate 2 μL of the sample for mass spectrometry detection. The mobile phases consisted of 0.1 % formic acid in water:acetonitrile (95: 5, *v*/v) (solvent A) and 0.1 % formic acid in acetonitrile:isopropanol: water (47.5: 47.5: 5, v/v) (solvent B). The solvent gradient conditions included 0 % B to 5 % B from 0 to 0.1 min, 5 % B to 25 % B from 0.1 to 2 min, 25 % B to 100 % B from 2 to 9 min, 100 % B to 100 % B from 9 to 13 min, 100 % B to 0 % B from 13 to 13.1 min, and 0 % B to 0

% B from 13.1 to 16 min for system equilibration. The analysis occurred at a sample injection volume of 2 μL, a flow rate of 0.4 mL/min, and a constant column temperature of 40 °C, while all samples were stored at 4 °C. The MS conditions involved collecting the mass spectrometric data using a Thermo UHPLC-Q Exactive Mass Spectrometer equipped with an electrospray ionization (ESI) source operating in either positive or negative ion mode. The optimal conditions included a heater temperature of 400 °C, a capillary temperature of 320 °C, a sheath gas flow rate of 40 arb, an Aux gas flow rate of 10 arb, an ion-spray voltage floating (ISVF) value of −2800 V in negative mode and 3500 V in positive mode, respectively, a normalized rolling collision energy of 20–40-60 V for MS/MS, a full MS resolution of 70,000, and an MS/MS resolution of 17,500. The data were acquired in data-dependent acquisition (DDA) mode, while detection occurred in a mass range of 70–1050 *m/z*.

2.6. Statistical analysis

The bar chart was obtained using GraphPad Prism (version 9.0.3), while the microbial composition determination and LEfSe were performed using the online tool Majorbio Cloud Platform (https://cloud.majorbio.com/page/tools/) (Segata et al., 2011). The redundancy analysis (RDA) based on the pearson correlation coefficients were performed using R (version 3.2), while the R igraph package was employed to plot the related graphs (Huhe Chen et al., 2017). The statistical significance of the differential metabolites in the raw liquor was examined via one-way analysis of variance (ANOVA), while the correlation analysis was performed using R ropls (Version 1.6.2).

3. Results and discussion

3.1. The physicochemical properties and enzymatic activity at different seasonal scales

The *Daqu* starter is characterized by various enzymes, multiple microbial species, and diverse flavor compounds (Wang et al., 2017), significantly influencing the quality and flavor of the final fermented products. The physicochemical indicators and enzymatic properties of the *Daqu* varied among the four seasons. As shown in Table S2, the moisture content of the fermented spring_*Daqu* and winter_*Daqu* was relatively high with no obvious differences ($p > 0.1$), while the autumn_*Daqu* displayed the lowest moisture level at 10.50 ± 0.24 %. The moisture content differences may be caused by the external environmental temperature and precipitation, as well as the influence of filamentous microorganisms. A lower moisture content was not conducive to microbial growth and metabolic activity. For instance, the moisture content, liquefaction, and fermentation activity were low in the autumn_*Daqu*. The liquefaction activity in the *Daqu* results from enzymes secreted by mycorrhizal fungi, which grow at temperatures between 20 and 30 °C (Han et al., 2016). Although the average daily temperature in summer reached 27.29 ± 2.24 °C, it did not promote mold growth, consequently reducing the liquefaction activity in the *Daqu*. The pH differed significantly between the four seasons ($p < 0.01$). Overall, the fermented *Daqu* appeared weakly acidic regardless of the season, with a pH level as low as 6.11 ± 0.01 in autumn, which increased the acidity (1.24 ± 0.03 mmol/10 g). Since *Daqu* is prepared using starchy raw grains, such as wheat, grain, and peas, it has a high starch content that is saccharified and fermented into wine during the B*aijiu* brewing process. As for enzymatic activity, it was evident that the fermentation and saccharification activity of the spring_*Daqu* was significantly higher than during the other seasons, reaching 0.57 ± 0.04 U and 927.00 ± 11.45 U, respectively. The saccharification activity was substantially affected by temperature, possibly causing significant ($p < 0.01$) related seasonal variation between the *Daqu* samples. However, too high saccharification activity will make the temperature rise too fast in the subsequent fermentation, and the yeast would be inhibited, resulting in the loss of liquor flavor.

3.2. The difference of volatile flavor compounds in Daqu at different seasonal scales

80 volatile flavor compounds were identified in *Daqu* samples from different seasons using HS-SPME-GC–MS, including alcohols, aldehydes, esters, acids, hydrocarbons, ketones, ethers, aromatics, and pyrazines (Table S3). There were less esters in the spring_*Daqu*, while more esters were found in the winter_*Daqu*. For example, ethyl valerate (145.88 ± 6.08 μg/kg) and ethyl butyrate (399.90 ± 32.51 μg/kg) might have made the winter_*Daqu* more expressive of fruity aroma. Furfuryl alcohol (8.53 ± 0.62 μg/kg) detected in summer_*Daqu* showed coffee aroma, malt aroma and roasted aroma. 3-Methyl-1-butanol, 2,3-butanediol, 2,6-dimethylpyrazine, benzaldehyde, 2-octanone, phenylacetaldehyde, nonanal, and tetradecane were the flavor compounds common to the four seasons of *Daqu* production, which may be the basic substances produced during the production of dacquoise to bring the grain aging flavor to the *Daqu*.

A total of 28 differential volatile flavor compounds displayed values of VIP > 1 with different flavor profiles, possibly playing a vital role in the aroma characteristics of the *Daqu* (Fig. 1). Esters often present fruity, flowery aromas and are considered the main contributors to the aromatic character of *Daqu* (Zhao et al., 2018). In this study, esters presented the highest content and broader diversity types than the other flavor compounds in the winter_*Daqu* samples, where ethyl heptanoate, ethyl butyrate, ethyl valerate, and ethyl caprylate were detected. Ethyl hexanoate, the main flavor substance in *Nongxiangxing Baijiu*, was detected at levels of 154.41 ± 15.21 μg/kg and 2181.46 ± 146.49 μg/kg in the autumn_*Daqu* and winter_*Daqu*, respectively, while it was not present in the other seasons. More alcohols were contained in the autumn_*Daqu*, such as 2-pentanol (1450.39 ± 103.28 μg/kg), 9,12-Octadecadien-1-ol (438.80 ± 37.91 μg/kg), and 3-penten-2-ol (709.03 ± 70.26 μg/kg). The presence of phenylethyl alcohol presented a flowery aroma and was only detected in the summer_*Daqu* and winter_*Daqu*. Pyrazine, considered beneficial to health, provides *Daqu* with a roasted and coffee aroma and is indispensable for its flavor and liquor (Shi et al., 2022). Pyrazines were present and varied in the fermented *Daqu* of all four seasons, which may be due to the differences in microbial communities in the macros of the seasons. 2,3-Dimethylpyrazine was more predominant in summer and 2,3,5-trimethylpyrazine was present up to 424.536 ± 32.98 μg/kg in autumn. 2,3,5-Trimethylpyrazine has a strong roasted peanut aroma and has been a common premium spice in recent years, bringing a roasted aroma to autumn_*Daqu*. Besides, The key flavor compounds in the autumn_*Daqu* mainly included aldehydes and alcohols, typically produced by microorganisms during the *Daqu* fermentation process or via microbial starch degradation in the raw materials during glycolysis (Xiao et al., 2021). Benzyl alcohol, produced by benzoate degradation, presents jasmine oil and hyacinth aromas (Wang, 2022). Overall, the flavor compound levels were lower in spring, exhibiting only small amounts of 2,6-dimethylpyrazine and 2,3,5,6-tetramethylpyrazine. 2,3,5,6-Tetramethylpyrazine was more abundant in summer_*Daqu*, with a higher nutty aroma flavor profile than in the other three seasons, and moderated bitterness and acidity. The esters in winter_*Daqu* were the most diverse of the four seasons of *Daqu*, whereas aldehydes, ketones, alcohols, phenols in autumn_*Daqu* were the most diverse.

3.3. The microbial compositions at different seasonal scales

The amplicon sequencing data were used to show the microorganism succession during the *Daqu* fermentation in the four seasons. The OTUs in the samples were defined with a ≥ 97 % sequence identity cut-off, and all samples displayed high coverage (1.00). The rarefaction curves of the bacterial and fungal communities approached the saturation plateau, indicating that the microbial communities were well represented at the sequencing depth (Fig. S1).The ace index and other information are shown in Supplementary Table S4, displaying obvious diversity in the

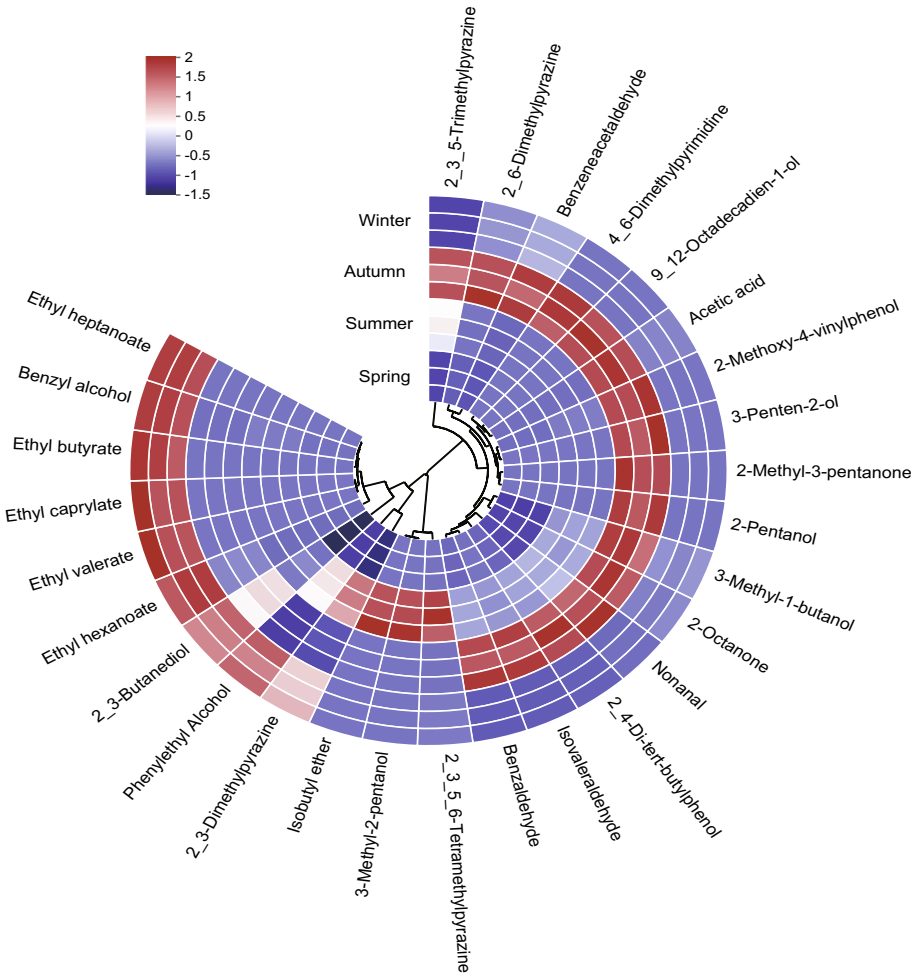

Fig. 1. The heatmap visualization of the volatile flavor compounds in the *Daqu* during different seasons.

four seasons.

In this work, the bacterial and fungal communities of *Daqu* responded differently at various seasonal scales, especially the change of bacterial communities was more sensitive. The microbial distribution at the genus level is shown in Figs. 2A-B, and 14 bacterial genera with an average relative abundance above 1 % had been identified. Colder climates favor the growth of cold-loving microorganisms and vice versa. Previous studies (Cho et al., 2006) indicated that *Weissella* represented the main species in pickled cabbage produced at −1 °C. Therefore, the temperature may be instrumental in its superiority in the spring *Daqu* (42.08 %) and winter *Daqu* (28.92 %). The spring *Daqu* also contained *Lactobacillus* (19.22 %), *Pediococcus* (14.14 %), and *Thermoactinomyces* (11.29 %). *Bacillus* (75.81 %) dominated in the summer *Daqu*, while *Pantoea* (51.89 %) was most abundant in the autumn *Daqu*. *Thermoascus*, *Aspergillus*, and *Saccharomycopsis* represented the main fungal genera. *Aspergillus* dominated during all four seasons with a high relative abundance since it displayed strong environmental adaptability to acid and ethanol (Zhuansun et al., 2022). The higher abundance of thermophilic fungi, such as *Thermomyces* (4.11 %), during the summer *Daqu* fermentation might be attributed to more significant biological heat accumulation in the *Daqu* during summer production due to higher temperatures, which promoted heat-resistant microbial growth. These results indicated that the climate changes during different seasons disrupted species interactions, forcing them to adapt, migrate and be replaced by other species or become extinct. Interestingly, *Qingxiangxing Daqu* produced in summer was found to have high abundance of *Lactobacillus*, while *Qingxiangxing Daqu* produced in fall was found to have high abundance of *Kroppenstedtia* and *Bacillus* in the study by Fu et al. (2021). This is inconsistent with our results, possibly due to differences in preparation methods and culture environments of *Daqu* types, but further research is needed to confirm these results in larger, more varied sample groups.

Figs. 2C-D shows the high-dimensional biomarkers of the fermented *Daqu* as revealed by LEfSe analysis (LDA > 3, $p < 0.05$). Bacterial genera significantly enriched in spring *Daqu* are mainly lactic acid bacteria, including *Weissella* and Lactobacillus. They can survive high acidity and low moisture conditions. Additionally, they convert glucose or starch into lactic acid. This increases the acidity of the production environment (Zhu et al., 2022). Lactic acid is the precursor of ethyl lactate and can also be used as a substrate for the synthesis of flavor substances such as butyric acid and hexanoic acid by Clostridia to enhance the flavor and quality of *Baijiu* (Chwialkowska et al., 2019). In addition, lactic acid bacteria produce bacteriocins that adversely affect the growth of other microorganisms, which in turn affects the enzymes and flavor, which may have affected the flavor of spring *Daqu*.

The main bacteria enriched in the summer *Daqu* are *Bacillus*, *Acinetobacter*, *Kroppenstedtia*, *Sphingobacterium* and *Massilia*. *Bacillus* and *Kroppenstedtia* can produce hydrolase and α -amylase respectively, which can convert macromolecules (including starch and protein) into glucose and amino acids, and produce various flavor precursors and substances (Zeng et al., 2022). At the same time, the free amino acids produced by *Bacillus* through Maillard reaction during fermentation increased the unique baking aroma and contributed to the flavor of *Baijiu*. *Massilia* is rare in *Daqu*, which may be brought in from the environment during *Daqu* processing. *Massilia* can not only synthesize a variety of secondary metabolites and enzymes, but also ferment with

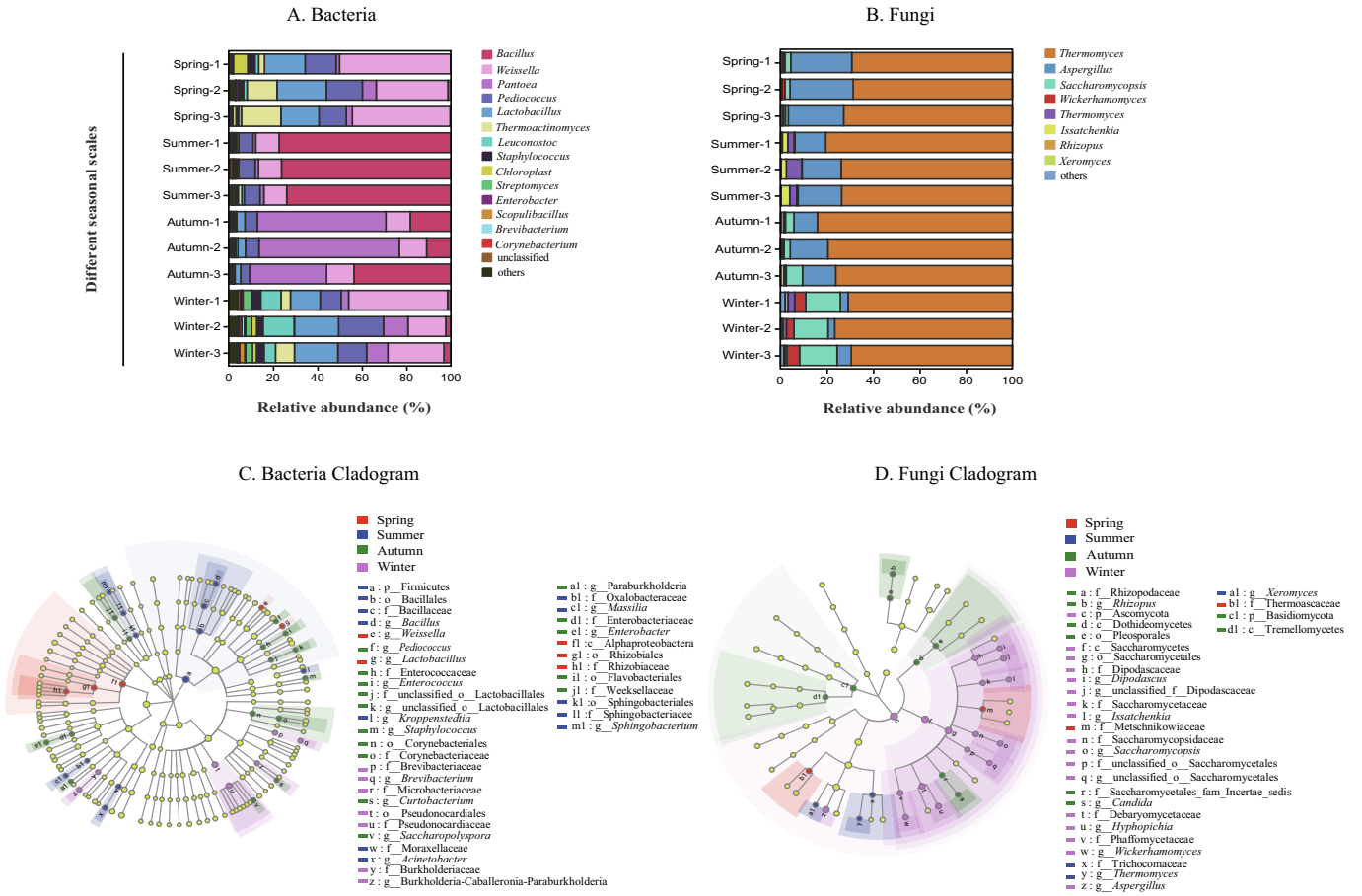

Fig. 2. The bacterial (A) and fungal (B) community structures in the *Daqu* at the genus level. Species with relative abundance levels below 1 % were classified as others. The LEfSe identification of the discriminant bacterial (C) and fungal (D) taxa in the Daqu produced during different seasons. The non-discriminant taxon nodes are marked in yellow and branch areas are shaded according to the highest-ranked variety for that taxon. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

starch, maltotriose or maltose as the only carbon source (Han et al., 2014). Its role and application in fermented foods need to be further clarified.

The marker microorganisms significantly enriched in autumn_*Daqu* were *Pediococcus*, *Enterococcus*, unclassified_o_Lactobacillales, *Staphylococcus*, *Curtobacterium*, and *Saccharopolyspora*, *Achromobacter*, *Enterobacter*, *Candida*, and *Rhizopus*. *Staphylococcu* produce hydrolytic enzymes and synthesize aldehydes (Wang et al., 2021), and *Pediococcus* produce amino acids, alcohols and esters (Liu et al., 2019). *Rhizopus* has a high amylase production capacity and degrades starch from raw materials into reducing sugars that can be utilized by yeast (Fan et al., 2020). Thus, they stimulate yeast growth as well as facilitate the metabolic flow of carbon from raw materials to alcohol (the end product of fermentation) and are commonly used as saccharifying microorganisms in the brewing industry. In addition, *Rhizopus* produce fumaric, lactic, succinic and other organic acids, as well as aromatic esters, and are also involved in the synthesis of some low-boiling substances.

Significantly enriched bacterial genera in winter_*Daqu* are *Burkholderia-Caballeronia-Paraburkholderia* and *Brevibacterium*. It has been shown that *Burkholderia-Caballeronia-Paraburkholderia* may produce lipolytic lipases and are positively associated with many volatile ester compounds (Wu et al., 2022). The marker fungal microorganisms of winter_*Daqu* are mainly enriched in Ascomycota. Among them, yeast genera such as *Issatchenkia*, *Saccharomycopsis*, and *Wickerhamomyces* produce pleasant volatile compounds such as ethyl acetate and ethyl butyrate, which are the main aroma-producing fungi in *Baijiu* (Su et al., 2020). The enrichment of these microorganisms is also corroborated with the results of ester enrichment in winter_*Daqu*.

The biomarkers of seasonal *Daqu* have different functions. The marker microorganisms in spring_*Daqu* are conducive to lactic acid production. The marker microorganisms in summer_*Daqu* are conducive to the degradation of raw materials and the production of baking aroma. The biomarkers in autumn_*Daqu* can metabolize organic acids, which is conducive to the production of alcohols, aldehydes, and acids, while the biomarkers in winter_*Daqu* mainly consist of microorganisms with aroma production function, which is conducive to the fermentation and aroma production of *Daqu*.

3.4. The relationship between the volatile flavor compounds and microorganisms

As shown in Fig. 3, Spearman's correlation analysis was used to evaluate the potential relationship between the key volatile compounds and microorganisms (microbial genera) in the different *Daqu* samples (Pang et al., 2023).

Weissella and *Lactobacillus*, the marker microorganisms in spring_*Daqu*, were negatively correlated with 19 flavor substances, probably due to the inhibitory effect of lactobacilli on other microorganisms, which affects flavor production. Signature microorganisms *Bacillus* in summer_*Daqu* were significantly and positively correlated with pyrazine compounds (2,3,5,6-tetramethylpyrazine, 2,3-dimethylpyrazine, and 2,3,5-trimethylpyrazine), which is in agreement with the results of a number of studies. For example, He et al. (2019) showed a significant increase in the content of volatile compounds such as esters, pyrazines

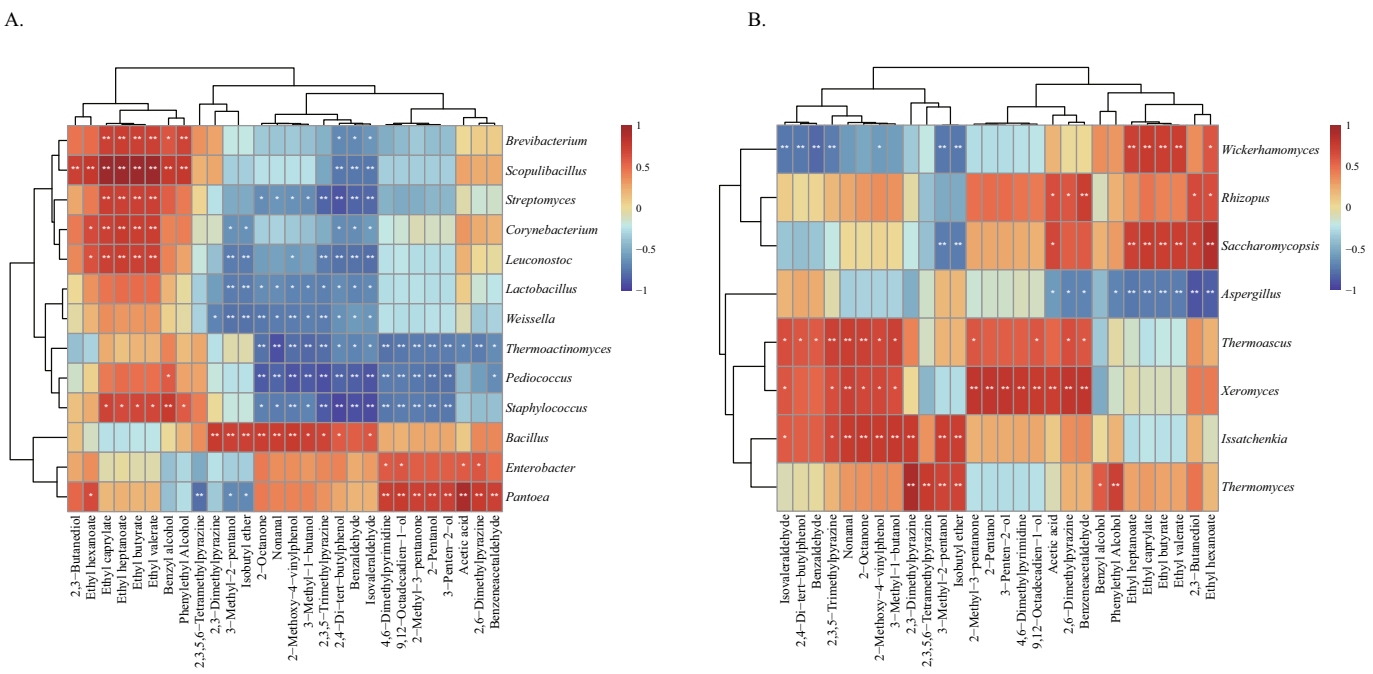

Fig. 3. The correlation heatmap between the microorganisms and the 28 main volatile flavor compounds. The *, ** and *** indicate statistical significance at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

and alcohols, especially tetramethylpyrazine, after inoculation of *Bacillus velezensis* and *Bacillus subtilis* to *Daqu*. The results proved that *Bacillus velezensis* is an important functional bacterium for the production of pyrazine flavor compounds, but the current research on tetramethylpyrazine in *Daqu* is mostly focused on *Jiang-flavor* and *Sesame-flavor Daqu*. In this study, tetramethylpyrazine was found to be abundant in summer-produced *Nongxiangxing Daqu*, with a content of 1556.95 ± 153.92 μg/kg. there is little information about the relationship between *Bacillus* and tetramethylpyrazine and the application of tetramethylpyrazine in the *Nongxiangxing Daqu*. Therefore, the potential for exploring high-yielding tetramethylpyrazine strains from summer-produced *Daqu* can be investigated further. Besides, *Thermomyces* was more abundant in summer, and its secretion of heat-stable hydrolases not only improves the rate of substrate hydrolysis under high-temperature environment, but also facilitates the rate of pyrazine synthesis through non-enzymatic reactions such as Merad (McClendon et al., 2012). *Pantoea* enriched in the autumn_*Daqu* showed significant positive correlation with 4,6-dimethylpyrimidine, linolenol, 2-methyl-3-pentanone, 2-pentanol, 3-penten-2-ol, acetic acid, 2,6-dimethylpyrazine, and phenylglyoxal, and significant negative correlation with tetramethylpyrazine. However, Zhang et al. (2021) found that *Pantoea* was negatively correlated with several flavor substances, such as phenylacetaldehyde, in high-temperature *Daqu*. This may be due to the differences in flavor caused by the different types of *Daqu* production processes, which in turn affects the interrelationship between microorganisms and flavor. In addition, it was found that *Pantoea* was positively correlated with fructose content, which is a precursor substance for the formation of 2,3-butanediol and phenylethanol, and contributed to the enhancement of the floral and fruity aroma of autumn_*Daqu*. *Rhizopus*, the hallmark microorganism of autumn_*Daqu*, was also significantly and positively correlated with phenylacetaldehyde. The marker microorganisms *Brevibacterium*, *Saccharomycopsis* and *Wickerhamomycess* were positively correlated with five esters (ethyl hexanoate, ethyl caprylate, ethyl heptanoate, ethyl butyrate and ethyl pentanoate) and two alcohols (benzyl alcohol and phenylethanol), which favored esterification reactions in winter_*Daqu*.

3.5. The key climatic factors and physicochemical indicators for the microbial determination

Studies showed that some environmental variables mainly drove the microbial community dynamics during the *Daqu* fermentation process. RDA was performed on the dominant genera (relative abundance >1 %) and physicochemical variables to investigate the effect of physicochemical factors on the microbial community distribution during *Daqu* fermentation (Ma et al., 2022). As illustrated in Fig. 4A, RDA1 explained 64.07 % of the bacterial species distribution, while RDA2 clarified 30.88 %, indicating that the physicochemical parameters had an important influence on microbial composition. *Bacillus* was positively correlated with acidity, temperature, and precipitation. The spring_*Daqu* displayed lower acidity, which was possibly related to a decrease in *Bacillus*. It acts as a key thermophilic protease-producing bacterium during the *Daqu* manufacturing process due to a certain temperature tolerance while metabolizing proteins to produce a variety of free amino acids, increasing the acidity of the fermentation environment (Huang et al., 2017). Consistent with previous studies (Yang et al., 2017), *Weissella* was significantly correlated with glucoamylase activity and liquefaction enzyme activity. *Issatchenkia*, an aromatic yeast genus, was positively associated with the impact of esterification in the fermentation system. Additionally, *Aspergillus* and *Candida* can metabolize and accumulate citric acid (Zhang et al., 2021). The considerable number of organic acids produced by these dominant bacteria during the late fermentation period inhibited the growth of acid-intolerant microorganisms and increased the *Daqu* acidity. Therefore, *Lactobacillus* and *Aspergillus* were positively correlated with the *Daqu* acidity. These results indicated that the discrepancies in the physicochemical properties of the *Daqu* produced during different seasons resulted in microbial community variation. Yeast is closely related to fermentation and alcohol production ability. The winter_*Daqu* displayed *Saccharomycopsis* and *Wickerhamomyces* abundance, possibly increasing the fermentation capacity.

Six climatic factors, including temperature, surface pressure, precipitation, relative humidity, wind speed, and sunlight hours, were monitored during *Daqu* fermentation (Table S1). As shown in Figs. 4C-D, precipitation was significantly correlated with 15 bacterial and 14 fungal genera, with the greatest effect on *Wickerhamomyces*,

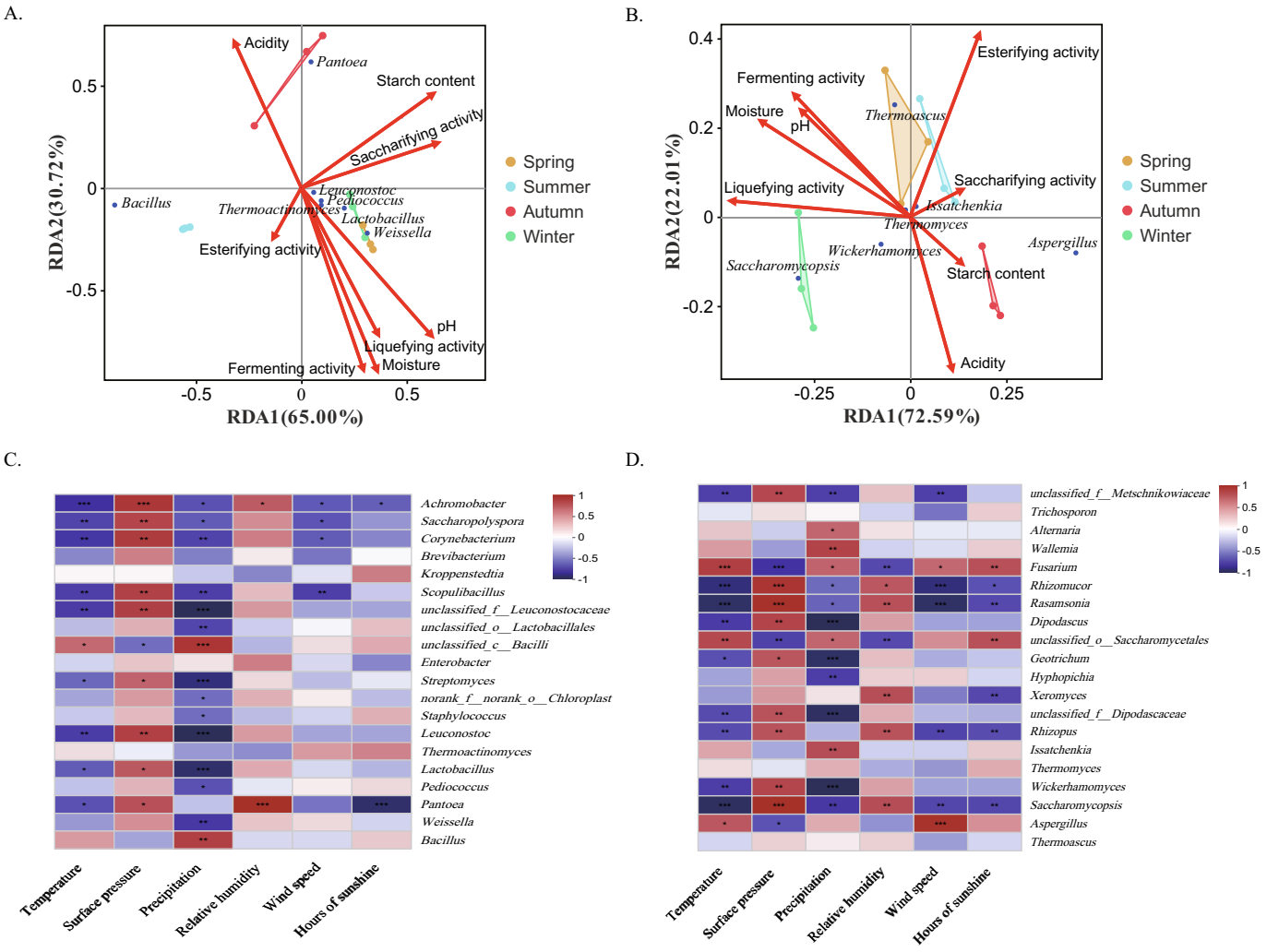

Fig. 4. The RDA of the dominant genera, physicochemical parameters, and enzymatic properties: bacteria (A) and fungi (B). The heatmap visualization of the impact of climatic factors on bacteria (C) and fungi (D). The *, ** and *** indicate statistical significance at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

Issatchenkia, *Lactobacillus*, *Bacillus*, *Lactobacillus*, *Leuconostoc* and *Streptomyces*. *Wickerhamomyces*, *Issatchenkia* as the dominant yeast genera in winter showed significant negative correlation with precipitation.Apart from that temperature was positively correlated with 12 bacterial and 17 fungal genera. The Mantel test based on the Bray-Curtis measure of variance also verified the significant correlation between precipitation and temperature changes and microbial communities (precipitation: rM = 0.56, $p = 0.004$; temperature: rM = 0.51, $p = 0.004$). The differences in microbial abundance reflected by seasonal variations are consistent with previous studies (Hutchins et al., 2019), indicating that anthropogenically controlled fermentation systems can be greatly influenced by their surroundings, and these fluctuations determine microbial community structure and function. It has been shown that the average daily air temperature is an important factor in changing the microbial population dynamics (Wang et al., 2020), probably because the production process of *Daqu* is in direct contact with the atmosphere, and the form of energy interaction between the internal system and the external natural environment is mainly the heat transfer, which is determined by the temperature of the internal system of the production of *Daqu* and the external natural environment, and in turn affects the internal system of the production of *Daqu*. This heat transfer is determined by the temperature of the internal system of *Daqu* production and the external natural environment, and thus affects the temperature of the internal system of *Daqu* production. The response of microbial communities to precipitation changes was complex. It has been shown that precipitation changes can affect soil microbial communities by altering water availability leading to different microbial responses in terrestrial ecosystems (Nielsen & Ball, 2015). In addition, increased precipitation leads to higher potential extracellular enzyme activity enzyme activity, greater microbial activity, and enhanced nutrient cycling (Szejgis et al., 2023). Similarly, precipitation has been shown to affect *Daqu* fermentation system microbes and metabolites in previous study (Wang et al., 2020). This may be due to the fact that precipitation is usually accompanied by changes in other climatic factors such as temperature, which in turn affected the structure of microbial communities and the flavor of *Daqu*, but the specific mechanism of the effect should be further explored.

3.6. *Analysis of metabolite pathway causing quality difference of spring_Daqu*

In order to explore the reasons for the poor flavor in spring_*Daqu*, the metabolites of *Daqu* produced in different seasons were analyzed by LC-MS. A total of 926 and 587 metabolite ion features were obtained in the ESI+ and ESI− modes, respectively. PCA of the extracted peaks showed QC sample clustering in both modes (Figs. 5A-B), indicating the stability of the instrument and the reliability of the data. On the PC1 axis, the summer_*Daqu* and spring_*Daqu* were noticeably separated in both modes, while the PC2 axis distinguished between the autumn_*Daqu* and winter_*Daqu*. Therefore, a supervised PLS-DA model was constructed to better differentiate between the four types of *Daqu* (Figs. 5C-D). A

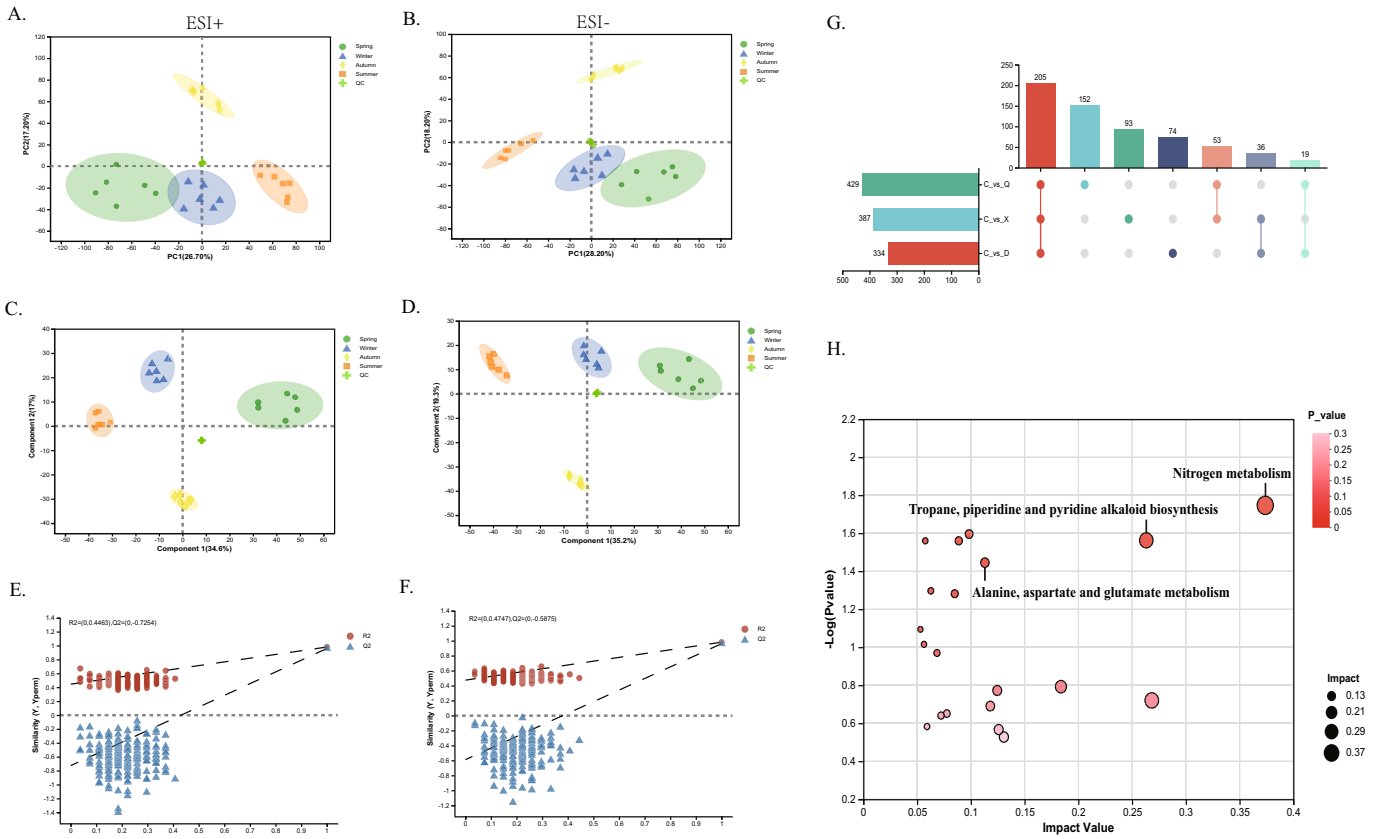

Fig. 5. The PCA (A-B), PLS-DA (C—D), and 200 permutation tests (*E*-F) in positive and negative ion modes based on reliable metabolites. The number of different metabolites between spring_*Daqu* and other seasons(G). C, X, Q, D represent *Daqu* produced in spring, summer, autumn, and winter, respectively. The histogram in the lower left corner of the figure is the statistics of the number of elements in each metabolic set. The histogram on the right is the statistical result of the number of elements after intersection of different metabolic sets. The single point below represents the unique elements of a metabolic set, and the connecting line between points represents the unique intersection of metabolic sets. Enriched bubble diagram of important significantly different metabolites pathways (bubble size is proportional to the degree of influence of each pathway; bubble color indicates the significant degree of influence) (H).

decline in the replacement retention decreased the R^2 and Q^2, while the regression line increased, indicating that the replacement test was passed, and no over-fitting was evident in the model (Figs. 5E-F).

Fig. 5G showed the number of different metabolites between spring and other seasons. Although there is no significant difference in daily average temperature and surface pressure between spring_*Daqu* and autumn_*Daqu*, their metabolites and flavors were quite different in the end. MetaboAnalyst 5.0 was used to analyze the metabolic pathway enrichment of the above 632 different metabolites (Fig. 5). Bubble diagram pathway analysis showed that these metabolites involved 59 metabolic pathways. Based on Pathway impact >0.1 and $p < 0.05$, "nitrogenmetabolism", "tropane, piperidine and pyridine alkaloid biosynthesis" and "alanine, aspartate and glutamate metabolism" were identified as the most important metabolic pathways.

4. Conclusions

This study showed that precipitation and temperature influenced microbial community structure, which in turn caused differences in seasonal *Daqu*. Firstly, there were significant differences in the physicochemical indexes of *Daqu* produced in different seasons. The moisture content of spring_*Daqu* and winter_*Daqu* was significantly higher than that of the other two seasons, and summer_*Daqu* had a higher utilization of starch. For the enzyme activity, the fermentation and saccharification power of spring_*Daqu* was the strongest, and the esterification and liquefaction power of winter_*Daqu* was the strongest. Secondly, the composition of microbial communities of the *Daqu* produced in different seasons was also different, with *Bacillus* being the dominant microorganism in summer_*Daqu*, *Weissella* being dominant in spring_*Daqu* and winter_*Daqu*, and *Pantoea* being dominant in autumn_*Daqu*. This may be closely related to the average daily temperature because *Bacillus* is thermophilic and *Weissella* is cryophilic, and they congregate in the *Daqu* produced under different climatic conditions. Since the quality of *Daqu* can not be judged from physical and chemical properties alone, the flavor compounds of *Daqu* were then analyzed using HS-SPME-GC–MS. Among the 80 flavor compounds detected, only 8 flavor substances were common to the *Daqu* produced in the four seasons. Overall, it appeared that spring_ *Daqu* had fewer types and lower contents of flavor compounds, and winter_ *Daqu* was rich in esters. The seasons influenced the average daily temperature, an important environmental factor, which led to the accumulation of *Wickerhamomyces* and *Saccharomycopsis* in winter, and finally led to an increase in the content and types of esters in winter_*Daqu*. From the metabolic point of view, "nitrogenmetabolism", "tropane, piperidine and pyridine alkaloid biosynthesis " and "alanine, aspartate and glutamate metabolism" were found to be the differential metabolic pathways that differed from other seasons in spring. In the actual production process, it is possible to improve the quality by mixing the spring_ *Daqu* with the *Daqu* from other seasons, and these results can help to guide the future production process of *Baijiu*.

CRediT authorship contribution statement

Tongwei Guan: Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Xinyue Wei:** Writing – original draft. **Xianping Qiu:** Validation, Resources. **Ying Liu:** Resources.

Jianshen Yu: Project administration, Methodology, Funding acquisition. **Rui Hou:** Software, Formal analysis, Data curation. **Maoke Liu:** Visualization, Validation, Software. **Yichen Mao:** Supervision, Project administration. **Qingru Liu:** Writing – review & editing, Visualization, Validation, Software, Methodology. **Lei Tian:** Validation, Supervision, Investigation, Formal analysis, Data curation. **Zongjun He:** Resources, Investigation. **Shuangquan Xiang:** Software, Resources, Project administration.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.102063.

Data availability

Data will be made available on request.

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