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Fermented foods: characterization of the autochthonous microbiota

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Fermented foods: characterization of the autochthonous microbiota

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Editorial: Fermented foods: characterization of the autochthonous microbiota

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autochthonous microbiota, artisanal fermented foods, fermentation dynamics, OMIC technologies, fermentation

Editorial on the Research Topic
Fermented foods: characterization of the autochthonous microbiota

Fermented foods have been essential in human diets for millennia, valued for their unique flavors, textures, and nutritional benefits. The microbial complexity underlying their production is particularly fascinating. In artisanal fermented foods, the absence of industrial starter cultures allows autochthonous microbiota to play a central role in driving the fermentation process. These microbes can originate from raw materials, the environment, equipment, and even human interaction during production. The result is a dynamic microbial ecosystem that contributes not only to the safety and quality of fermented foods, but also to their distinct organoleptic properties.

Historically, the characterization of these complex microbial communities was limited by traditional microbiological methods. Such approaches provided invaluable insights but often lacked the precision to fully capture the depth and diversity of fermentation microbiota. The advent of OMIC technologies revolutionized our ability to study microbial communities at a metabolic and functional level. These tools have shed new light on the interactions between microorganisms and their substrates, revealing how specific microbial populations influence flavor, aroma, texture, and even safety parameters.

Despite these advances, much of the research has concentrated on large-scale, commercially produced fermented foods, such as wine, beer, and bread. Artisanal products, which often rely solely on their natural microbiota for fermentation, remain

underexplored. This Research Topic seeked to address this gap by highlighting studies that focus on the characterization of autochthonous microbiota in artisanal fermented foods.

The papers published in this Research Topic cover a broad range of fermented products from different regions around the world:

"Bacterial composition and physicochemical characteristics of sorghum based on environmental factors in different regions of China" investigates the diverse microbial communities on sorghum used for Jiang-flavored baijiu fermentation (Xie et al.). This study reveals how environmental factors, including soil characteristics and climate, influence the bacterial composition and physicochemical properties of sorghum, ultimately impacting flavor development.

"Effects of sorghum varieties on microbial communities and volatile compounds in the fermentation of light-flavor Baijiu" is another research focusing on the fermentation process of light-flavor baijiu, which compares glutinous and non-glutinous sorghum varieties using PacBio single-molecule real-time (SMRT) sequencing and headspace solid-phase microextraction coupled with gas chromatography mass spectrometry (HS-SPME-GC-MS) (Tang et al.). This paper highlights how variations in microbial diversity correlate with the production of volatile compounds, offering insights into optimizing fermentation practices.

"Metagenomic analysis of core differential microbes between traditional starter and Round-Koji-mechanical starter of Chi-flavor *Baijiu*" is a metagenomic study comparing traditional and mechanical starters used in Chi-flavor baijiu production, and identifies key microbial taxa that contribute to the flavor and quality differences, providing guidance for improving fermentation processes (Liang et al.).

"Tradition unveiled: a comprehensive review of microbiological studies on Portuguese traditional cheeses, merging conventional and OMICs analyses" is a review gathering findings from conventional and OMIC studies on the microbiota of Portuguese traditional cheeses (Serrano et al.). This document explores the roles of various microbial species in cheese ripening and flavor development, emphasizing the importance of these communities for product safety and quality. Shedding light on the intricate interplay between microorganisms and cheese matrices contributes to unveiling the secrets behind the rich heritage and distinctiveness of these artisanal foods.

"Deciphering the microbial succession and color formation mechanism of "green-covering and red-heart" *Guanyin Tuqu*" correlates the temporal profiles of microbial community succession with the main environmental variables (temperature, moisture, and acidity) and spatial position (center and surface) in "Green-covering and red-heart" Guanyin Tuqu throughout fermentation, elucidating the physiological characteristics of core microorganisms responsible for the unique color formation in this special fermentation starter (Zhu et al.).

"Formic acid enhances whole-plant mulberry silage fermentation by boosting lactic acid production and inhibiting harmful bacteria" investigates the impact of four additives and combinations thereof on fermentation quality and bacterial communities associated with whole-plant mulberry silage, showing that formic acid significantly enhances lactic acid production while suppressing undesirable bacterial growth and improving overall silage quality (Hao et al.).

"Metagenomics profiling of the microbial community and functional differences in solid-state fermentation vinegar starter (seed *Pei*) from different Chinese regions", a research focusing on the seed Pei used in vinegar fermentation, employs metagenomic analysis to reveal the microbial composition and functional differences among starters from different regions, providing a new perspective on formulating vinegar fermentation starters and developing commercial fermentation agents for vinegar production (Han et al.).

"Rapid identification of lactic acid bacteria at species/subspecies level via ensemble learning of Ramanomes" introduces a novel approach for the rapid identification of lactic acid bacteria using single-cell Raman spectroscopy combined with machine learning techniques (Ren et al.). This method promises to enhance the efficiency and accuracy of lactic acid bacteria identification in fermented foods.

In "Isolation and *in vitro* screening of the probiotic potential of microorganisms from fermented food products" microorganisms from traditional fermented foods, of both animal and plant origin, are isolated and evaluated for their probiotic potential based on a set of *in vitro* assays, identifying promising candidates for future functional food applications (Ntiantiasi and Lianou).

Overall, the diversity of studies published in this Research Topic reflects the global importance of artisanal fermented foods and the ongoing scientific efforts to decode the intricate microbial processes behind them. These studies not only contribute to our scientific knowledge, but may also have practical implications for improving food quality, safety, and the preservation of artisanal products.

We are confident that this Research Topic will serve as a valuable resource for researchers, food technologists, and industry professionals interested in fermented foods. The papers herein provide a comprehensive overview of the role the autochthonous microbiota plays in shaping fermented foods, emphasizing the relevance of understanding these microbial communities to enhance food quality and safety.

Thanks to all the contributors and their excellent work, this Research Topic should inspire further research in this fascinating area of food science and technology.

Author contributions

MS: Writing – review & editing. ML: Writing – review & editing. MG: Writing – review & editing. CA-B: Writing – review & editing. TS-L: Writing – review & editing, Writing – original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Isolation and *in vitro* screening of the probiotic potential of microorganisms from fermented food products

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Introduction: Several human health benefits have been ascribed to probiotics, while traditional fermented foods have been acknowledged as rather important sources of these microorganisms. The objective of this study was the isolation of microorganisms from fermented food products of both animal and plant origin and the evaluation of their probiotic potential.

Methods: Microbial isolation was performed from milk kefir and table olives, while an olive mill waste sample also was analyzed given its anticipated association with the autochthonous microbiota of olive drupes. Among the 16 macroscopically distinct recovered microorganisms, 14 microbial isolates were identified as presumptive lactic acid bacteria (LAB), whereas two isolates corresponded to yeasts. The microbial isolates exhibiting a reproducibly robust growth profile in appropriate culture broth media (11 out of the 16 isolates) were assessed for their probiotic potential based on a set of *in vitro* assays: resistance to low pH; autoaggregation; biofilm formation; antioxidant activity; and safety assessment through evaluation of hemolytic activity.

Results and discussion: Based on the collective evaluation of the results of the abovementioned assays, five presumptive LAB as well as the two yeast isolates were identified as exhibiting desirable *in vitro* probiotic traits. Hence, these microbial isolates could be regarded as good candidates for inclusion in further studies aiming, ultimately, at their potential utilization in novel functional food products.

KEYWORDS

fermented foods, probiotic potential, milk kefir, table olives, lactic acid bacteria, yeasts

1 Introduction

Food fermentation is a long-lasting food processing technology which has traditionally allowed for enhanced preservation and considerable shelf-life extension of various perishable foods of both plant and animal origin. Fermented foods and beverages, defined as "foods made through desired microbial growth and enzymatic conversions of

food components" (Marco et al., 2021), have been consumed for thousands of years (Liu et al., 2019). Nonetheless, the claimed health benefits of their consumption have resulted in significant research interest and increasing popularity among consumers the last decades (Xiang et al., 2019). The functional character of fermented foods has been associated with the potential probiotic effect of their constituent microorganisms, the production (during fermentation) of bioactive compounds (e.g., short-chain fatty acids, peptides, and polyamines), as well as with the reduction of toxic compounds and anti-nutrients in the end products (Dimidi et al., 2019; Xiang et al., 2019; Annunziata et al., 2020). Moreover, assessment of the potential impact of fermented foods on human cognitive function has also attracted the interest of researchers recently (Casertano et al., 2022).

The functional attributes of fermented foods are regarded as being essentially delineated by their ample content in microorganisms with probiotic traits. According to the definition provided by the Food and Agriculture Organization (FAO) of the United Nations and the World Health Organization (WHO), probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2002; Hill et al., 2014). The contribution of probiotics to human health protection has been linked to various modes of action including: (i) modification of the gut microbiota (and its metabolic activity) contributing, among others, to the improvement of lactose fermentation (Ibrahim et al., 2021; Ma et al., 2023); (ii) reduction of the bowel's pH and prevention of its colonization by pathogenic organisms (Marco et al., 2006); (iii) prevention of gastric ulcer due to their activity against the bacterium *Helicobacter pylori* (Boltin, 2016); (iv) modulation of the host's immune responses (Yan and Polk, 2011); (v) protection against allergens (Lopez-Santamarina et al., 2021); and (vi) anti-carcinogenic activity (Legesse Bedada et al., 2020). Given their well-established benefits for human health, probiotics have been increasingly utilized as nutraceuticals in the production of food supplements and as ingredients of food products, with the latter being either fermented foods or non-fermented products enriched with probiotics. Numerous microbial strains have been identified as having probiotic traits, with most of them corresponding to species of the genus *Bifidobacterium*, lactic acid bacteria (LAB) species of the genera *Lactobacillus* (recently emended and reclassified into 25 genera), *Enterococcus*, *Streptococcus*, *Pediococcus* and *Leuconostoc*, as well as to the yeast species *Saccharomyces cerevisiae* (Fijan, 2014; Zheng et al., 2020; Soemarie et al., 2021; Staniszewski and Kordowska-Wiater, 2021).

Assessment of the putative association of probiotics with the health benefits of traditional fermented foods is a research objective of enduring value. Such association is strongly supported by the profuse participation of the former microorganisms in these products' microbiota. Indeed, fermented foods constitute valuable natural reservoirs of microorganisms with probiotic traits (Rezac et al., 2018; Soemarie et al., 2021), with the latter being evaluated *in vitro*, *ex vivo* and/or in the context of clinical trials (Nuraida, 2015; Kim and Park, 2018). Fermented foods and beverages which have been evaluated as important sources of microorganisms with probiotic potential are various dairy products (e.g., yogurt, kefir, sour cream, ripened cheeses), meat products (e.g., salami, pepperoni), produce commodities (e.g., kimchi, sauerkraut, table olives, tempeh, tofu) and herbal extracts (e.g., kombucha) (Marco et al., 2017; Bonatsou et al., 2018; Kok and Hutkins, 2018; Marco et al., 2021; Soemarie et al., 2021). Isolation of microorganisms with probiotic potential from fermented foods is anticipated to provide a competitive advantage in their effective utilization as functional microbial cultures (starter or adjunct cultures) in the manufacture of the so-called "probiotic fermented foods" (Marco et al., 2021). Such microorganisms are highly likely to retain their probiotic traits *in situ* (i.e., in the food matrix) during the manufacturing process, and thus, to result in food products of enhanced functional quality throughout their shelf life. Furthermore, microorganisms with probiotic traits can be used for the functional supplementation of various products, even non-fermented ones, as well as for the development of nutritional supplements.

Given the above, the objective of the present study was the isolation of microorganisms from selected fermented food products of both animal and plant origin and the *in vitro* screening of their probiotic potential. The fermented foods used for microbial isolation were milk kefir and table olives, while an olive mill waste sample also was analyzed, given its anticipated association with the autochthonous microbiota of olive drupes.

2 Materials and methods

2.1 Samples

The food products used in the present study for microbial isolation purposes are compiled in Table 1 and included: (i) four kefir products (three industrially manufactured commercial products purchased from local retail stores and one homemade product), and (ii) natural black cv. Kalamata olives. Regarding kefir products, they were all made from cow's milk, while one of the industrial products (Kefir 3), was flavored and sweetened with steviol glycosides (Table 1). With reference to table olives, two distinct batches of fermented olives were provided by a local manufacturer, and microbial isolation was performed both from the olive drupes and the corresponding brines. Finally, an olive mill waste (OMW) sample provided by a local olive oil extraction plant also was analyzed, given its anticipated microbial association with the autochthonous microbiota of olive drupes.

2.2 Microbial isolation, phenotypic characterization and culture conditions

The aforementioned samples were subjected to microbiological analyses aiming at the isolation of microorganisms present at high concentration levels and as such, contributing appreciably to these products' microbiota. For this purpose, 1-mL aliquots of the kefir

TABLE 1 Samples analyzed in the present study, their origin, and characteristics.

Sample	Origin	Ingredients/Characteristics
Kefir 1	Industrial (Manufacturer 1)	Fresh semi-skimmed cow's milk, kefir grains; Fat: 1.0%
Kefir 2	Industrial (Manufacturer 2)	Fresh semi-skimmed cow's milk, lactic acid culture, kefir grains; Fat: 1.5%
Kefir 3	Industrial (Manufacturer 2)	Fresh skimmed milk, milk cream, sucrose, natural strawberry flavor, flavors (preservative: potassium sorbate), carrot concentrate, sweeteners: steviol glycosides, lactic acid culture, kefir grains; Fat: 1.3%
Kefir 4	Homemade	Fresh semi-skimmed cow's milk (1.5%), kefir grains
Table olives – Batch 1	Industrial*	Natural black cv. Kalamata (production year: 2019)
Table olives – Batch 2	Industrial*	Natural black cv. Kalamata (production year: 2020)
Olive mill waste	Olive oil extraction plant	Fresh olive mill wastewater

* The two batches of fermented table olives were provided by the same local manufacturer.

products were transferred aseptically to 9 mL of sterilized quarter strength Ringers solution (Neogen®, Lansing, MI, USA), and appropriate serial decimal dilutions in the same solution were pour/surface plated in/on selected agar media. The same procedure was followed for the microbiological analyses of the olive brines and the OMW. Regarding the isolation of microorganisms from olive drupes, three randomly selected drupes from each batch were rinsed with sterilized Ringers solution (10 mL per drupe), to remove brine and loosely attached microbial cells, and were depitted with sterile scalpel and forceps under aseptic conditions. The olive drupes' flesh was then transferred aseptically in 400-mL sterile stomacher bags (BagLight® Polysilk 400, Interscience, St Nom, France), was 1:10 diluted with Ringers solution and was homogenized in a Stomacher apparatus (BagMixer® 400, Interscience) for 120 s. Appropriate serial decimal dilutions of the homogenates were then pour/surface plated in/on selected agar media.

Overall, the microbiological analyses conducted and the agar media used in the present study were the following: (i) surface plating on Standard Methods Agar-PCA (Condalab, Madrid, Spain) for the total viable count (TVC) after incubation of plates at 28°C for 72 h; (ii) pour plating in de Man, Rogosa and Sharpe (MRS) agar (Condalab) along with medium's overlay for the determination of presumptive lactic acid bacteria (LAB) after incubation of plates at 28°C for 72–96 h; (iii) surface plating on potato dextrose agar (PDA, Condalab) supplemented with 0.05 g/L chloramphenicol (Sigma-Aldrich, Seelze, Germany) for the determination of yeasts and molds after incubation of plates at 28°C for 5 days; and (iv) pour plating in violet red bile glucose (VRBG) agar (Condalab) along with medium's overlay for the assessment of the presence (at detectable levels) of bacteria of the Enterobacteriaceae family after incubation of plates at 37°C for 24 h. Upon completion of the microbiological analyses, the pH values of the samples also were measured using a digital pH meter (Orion Model 420A, ATI Orion, Boston, MA, USA) with a glass electrode (HANNA® Instruments, Athens, Greece).

Morphologically distinct colonies growing in/on the different agar media (in the dilutions allowing for their accurate enumeration) were streaked on appropriate media aiming at the isolation of presumably different microorganisms. The isolated colonies were then used to inoculate different culture broth media, which were then incubated at different temperatures (depending on the medium) and for different time periods (24–48 h) in order to identify the incubation conditions better supporting their growth. Specifically, the microbial colonies recovered from PDA were grown in potato dextrose broth (PDB, Himedia, Mumbai, India) at 28°C, whereas the microbial colonies retrieved from MRS agar were grown in MRS broth (Condalab), M-17 broth (Himedia) and Columbia broth (Condalab) at 28, 37 and 45°C. The microbial isolates were phenotypically characterized based on microscopic observation using an optical microscope equipped with a digital camera (BioBlue BB.4267, Euromex Microscopen BV, Arnhem, The Netherlands), and in the case if bacterial isolates using Gram stain as well as the results of catalase and oxidase tests.

All microbial isolates were maintained as frozen (−80°C) stock cultures in MRS broth supplemented with 20% glycerol (presumptive LAB isolates) or PDB broth supplemented with 30% glycerol (yeast isolates). Working cultures were stored refrigerated (4°C) on slants of appropriate agar media (MRS agar and PDA for presumptive LAB and yeast isolates, respectively) and were renewed bimonthly. The probiotic potential of the microbial isolates was assessed based on a series of *in vitro* assays (described in detail in the following sections): resistance to low pH; autoaggregation capability; biofilm formation; antioxidant activity; and safety assessment through evaluation of hemolytic activity. The microbial cultures used in these assays were prepared by transferring a loopful from the working cultures (agar slants) in 10 mL of culture broth (MRS broth or PDB) and incubating at 28 or 37°C for 24 or 48 h, depending on the isolate. Beyond the isolated microorganisms, the *Saccharomyces cerevisiae* strain AXAZ-1, originally isolated from a Greek vineyard plantation (Argiriou

et al., 1992) and maintained at the cultures collection of the laboratory of Microbiology (Department of Biology, University of Patras), also was evaluated in the present study.

2.3 Resistance to low pH

The resistance of the microbial isolates to low pH was evaluated according to previously described procedures with some modifications (Argyri et al., 2013; Pavli et al., 2016). Specifically, cultures of the microbial isolates were centrifuged (Heraeus, Biofuge Stratos, Thermo Scientific, Osterode, Germany) at 10,000× g for 5 min at 4°C. The harvested cells were washed twice with phosphate buffered saline (PBS, pH 7.2) by centrifugation under the same conditions, and were finally re-suspended in 10 mL of PBS solution with pH adjusted to 2.5 using HCl (min. 37%; Sigma-Aldrich). The pH adjustment of the PBS solution was performed using a digital pH meter with a glass electrode, and its pH value was also measured (and confirmed not to be considerably different) after autoclaving. The microbial suspensions were incubated at 37°C with constant stirring at medium amplitude in a water bath (Memmert WB22, Schwabach, Germany) for a total period of 3 h. At 0 and 3 h of the applied acid challenge, samples were taken from each microbial suspension to determine the initial and surviving population, respectively. The populations of presumptive LAB isolates were enumerated by pour-plating of appropriate serial decimal dilutions (in Ringers solution) in MRS agar, while the populations of the yeast isolates by surface plating on PDA. Microbial colonies were counted after incubation of plates at 28°C for a total of 120 h (colonies were counted at 72 h and the counts were confirmed at 96 and 120 h). The acid resistance of each isolate was assessed in three independent experiments (n=3).

2.4 Autoaggregation capability

The autoaggregation assay was performed based on previously described protocols (Ogunremi et al., 2015; Bonatsou et al., 2018). In brief, cultures of the microbial isolates were centrifuged as described above, washed once with PBS (pH 7.2) and resuspended in 10 mL of the same solution. The microbial suspensions were vortexed for 10 s and incubated at 37°C for 24 h. At 0, 2, 4 and 24 h of incubation, 1-mL aliquots of the supernatant of each isolate were carefully transferred in plastic cuvettes and their optical density (OD) was measured at 600 nm using a UV spectrophotometer (UV-1800, Shimadzu Europa GmbH, Duisburg, Germany). The autoaggregation capability was quantified based on the following equation:

$$Autoaggregation\ (\%) = [1 - \frac{A_t}{A_0}] \times 100$$

where A_t and A_0 is the OD measurement at time t and zero, respectively.

The autoaggregation assay was performed twice (independent assays), with the OD at 2 and 4 h, however, being recorded once.

2.5 Biofilm formation

The biofilm-forming ability of the microbial isolates was evaluated *in vitro* using a colorimetric microtiter plate method, frequently used for the indirect quantification of biofilm formation. The biofilm formation assay applied herein is based on the measurement of the OD of the biofilm mass in microtiter plate wells after crystal violet staining and was performed according to previously described procedures (Lianou and Koutsoumanis, 2012; Pachla et al., 2021) with some modifications. More specifically, 20-μL aliquots of each microbial isolate's culture were added to 180 μL of appropriate culture broth, namely MRS broth for the presumptive LAB isolates and PDB for the yeast isolates, dispensed in 96-well polystyrene microtiter plates (CytoOne®, STARLAB International GmbH, Hamburg, Germany). Each microbial isolate was tested in eight replicate wells, while negative control wells (containing only 200 μL of culture broth) also were included. The microplates were incubated statically at 37°C for 72 h. The content of the microplates was then discarded, and the wells were rinsed with 200 μL of Ringers solution, aiming at the removal of the planktonic or reversibly attached cells. During the applied rinsing step, the microplates were agitated on a rocking platform (Stuart STR9 3D Rocking Platform, Stuart Scientific, Staffordshire, UK) at 40 rpm for 5 min. Next, the adherent bacterial cells were fixed with 200 μL of methanol (min. 99.8%; Fisher Scientific, Loughborough, UK) per well for 15 min, the wells were emptied by inversion of the microplates and the latter were air dried for 20 min. Afterward, the biofilm mass was stained with crystal violet (Gram's crystal violet solution, Sigma-Aldrich) for 20 min, after which the excess stain was rinsed off (at least three times) through filling the microplates' wells with deionized water and emptying them by inversion. Then, the microplates were vigorously tapped on absorbent paper and air dried for 1 h. The bound to the formed biofilm stain was solubilized in 200 μL of ethanol (min. 99.8%; Fisher Scientific) per well and the OD of each well was measured at 595 nm using a microplate reader (MRX Microplate Absorbance Reader, Dynex Technologies, Chantilly, VA, USA). The average value of the OD measurements of the control wells was subtracted from the OD of each test well, and this difference, referred to as $\Delta OD_{595\ nm}$, was used as an index of the biofilm-forming ability of the microbial isolates. Two independent biofilm formation assays were performed for each microbial isolate, with eight replicate wells per treatment (isolates and negative control) being included in each assay (n=16).

2.6 Antioxidant activity

The antioxidant activity was assessed as the percentage of reduction of 2,2-diphenyl-1-picrylhydrazyl (DPPH) according to previously described protocols (Gil-Rodríguez et al., 2015; Cho et al., 2018) with some modifications. One-milliliter aliquots of each microbial culture were centrifuged (LabNet Hermle Z 233 MK-2 microcentrifuge, Hudson, MA, USA) at 12,000 rpm for 5 min

at 4°C, and the harvested cells were washed twice with Ringers solution and finally resuspended in 1 mL of the same solution. One-milliliter aliquots of freshly prepared DPPH (Sigma-Aldrich) solution (0.2 mM in methanol) were then added to the cell suspensions, and the mix was vortexed and incubated at 37°C in darkness for 30 min. Afterward, the reaction tubes were centrifuged under the same conditions, 200 μL of the mixtures' supernatants were transferred into 96-well polystyrene microtiter plates, and absorbance measurements were recorded at 490 nm using a microplate reader (MRX Microplate Absorbance Reader). The scavenged DPPH was calculated using the following equation:

$$Scavenging\ \ activity\ \ (\%) = [1 - \frac{A_{sample}}{A_{blank}}] \times 100$$

where A_{sample} is the absorbance of the DPPH solution with the sample and A_{blank} is the absorbance of the DPPH solution and Ringers solution. The absorbance values of pure DPPH solution as well as of ascorbic acid (Sigma-Aldrich) solution (0.2 mM in methanol), serving as positive control of the applied assay, were also recorded.

The antioxidant activity of each microbial isolate (and the positive control) was evaluated in four replicate microplate wells (n=4). Given the anticipated directly proportional association of the scavenging activity of each microbial isolate with the cell concentration of the tested microbial suspension, the cell concentration of the used microbial cultures was determined by pour/surface plating (as described in Section 2.3), and the attained antioxidant activity results were standardized for a microbial concentration of 10^7 CFU/mL.

2.7 Hemolytic activity

For assessing the safety of the isolated microorganisms, fresh microbial cultures were streaked on duplicate plates of Columbia agar containing 5% (v/v) sheep blood (Bioprepare® Microbiology, Keratea, Attica, Greece). The plates were incubated at 28°C for 48–72 h and were examined for signs of hemolytic activity. Specifically, green-hued zones around colonies are regarded as sign of α-hemolysis, clear zones around colonies as sign of β-hemolysis, whereas absence of zones around the colonies is indicative of no hemolytic activity also referred to as γ-hemolysis.

2.8 Statistical analysis

The experimental data derived from the acid resistance, autoaggregation capability, biofilm formation and antioxidant activity assays were evaluated by analysis of variance using the general linear model procedure of the software IBM® SPSS® Statistics 27 (IBM Corp., Armonk, NY, USA). Means were separated using the Tukey HSD *post-hoc* test at a significance level of α=0.05.

3 Results

The total mesophilic populations and presumptive LAB constituting the autochthonous microbiota of the fermented food samples and the OMW sample tested herein, along with the samples' pH values are compiled in Table 2. Bacteria belonging to the Enterobacteriaceae family were not recovered at detectable levels from none of the tested samples. Aiming at the recovery of microorganisms present at high concentration levels in the tested samples and as such, contributing appreciably to their microbiota, microbial isolation was conducted at the dilutions of the tested samples allowing for their enumeration. The microorganisms isolated from the selected fermented food products and the OMW are presented in Table 3, while the results of the *in vitro* trials aiming at the evaluation of their probiotic traits are illustrated in Figures 1–5. The characterization of the probiotic potential of the isolated microorganisms was based on the collective evaluation of the compiled data, allowing for the selection of isolates that would be of interest for further investigation and utilization in industrial applications.

In total, 16 microbial isolates with distinct macroscopic characteristics were recovered from the tested food/OMW samples. The macroscopic distinctiveness (i.e., evidently different colony morphology) of the isolated microorganisms was aimed to maximize the likelihood of isolating different microbial strains from each tested sample. Based on their phenotypic characteristics, the majority of

TABLE 2 Microbial populations and pH values of the tested samples.

Sample	PCA [1]	MRS agar [1]	pH
Kefir 1	7.60 ± 0.22	6.54 ± 0.33	4.22 ± 0.01
Kefir 2	8.14 ± 0.17	6.62 ± 0.25	4.58 ± 0.04
Kefir 3	8.69 ± 0.19	7.06 ± 0.21	4.52 ± 0.06
Kefir 4	6.44 ± 0.25	6.52 ± 0.30	4.00 ± 0.06
Table olives – Batch 1	4.15 ± 0.56	3.00 ± 0.39	4.03 ± 0.18
Brine – Batch 1	5.76 ± 0.32	5.54 ± 0.41	3.92 ± 0.16
Table olives – Batch 2	3.87 ± 0.46	2.98 ± 0.40	4.09 ± 0.15
Brine – Batch 2	3.48 ± 0.34	5.90 ± 0.36	3.98 ± 0.06
Olive mill waste	4.05 ± 0.29	3.74 ± 0.27	4.75 ± 0.04

[1] Microbial populations in log CFU/ml (olive brine, kefir, olive mill waste) or log CFU/g (table olives), as enumerated on Standard Methods Agar-PCA (total viable count) and in MRS agar (presumptive lactic acid bacteria). Values are means ± standard deviations (n=2).

TABLE 3 The characteristics of the isolated microorganisms.

Isolate No.	Source	Phenotypic characteristics	Culture conditions
1*	Kefir 1	Bacterium (coccobacilli)	MRS broth, 37°C, 24 h
2*	Kefir 1	Bacterium (bacilli)	MRS broth, 37°C, 24 h
3	Kefir 2	Bacterium (coccobacilli)	**
4*	Kefir 2	Bacterium (cocci)	MRS broth, 28°C, 24 h
5	Kefir 3	Bacterium (coccobacilli)	**
6	Kefir 3	Bacterium (cocci)	**
7*	Kefir 4	Bacterium (bacilli)	MRS broth, 37°C, 24 h
8*	Kefir 4	Yeast	Potato dextrose broth, 28°C, 48 h
9*	Table olives – Batch 1 (drupes)	Bacterium (bacilli)	MRS broth, 28°C, 48 h
10*	Table olives – Batch 1 (drupes)	Bacterium (bacilli)	MRS broth, 28°C, 48 h
11*	Table olives – Batch 1 (brine)	Bacterium (bacilli)	MRS broth, 37°C, 24 h
12	Table olives – Batch 2 (drupes)	Bacterium (coccobacilli)	**
13*	Table olives – Batch 2 (brine)	Bacterium (bacilli)	MRS broth, 37°C, 24 h
14	Table olives – Batch 2 (brine)	Bacterium (bacilli)	**
15*	OMW	Bacterium (coccobacilli)	MRS broth, 37°C, 24 h
16*	OMW	Yeast	Potato dextrose broth, 28°C, 24 h

* Microbial isolates which were evaluated for their probiotic potential.
** Inability of reproducibly robust growth under the culture conditions evaluated in the study.

microbial isolates (14/16) were recovered from MRS agar and were identified as presumptive LAB (Gram-positive, catalase- and oxidase-negative bacteria), while two isolates were recovered from PDA with chloramphenicol and were identified as yeasts. From the isolated microorganisms, 11 exhibited reproducibly robust growth in culture broth under the conditions of this study and were therefore, selected to be further investigated for their probiotic potential. The phenotypic and culture characteristics of the isolated microorganisms, as well as the isolates selected for further study are presented in Table 3. Microscopy images of the 11 selected microbial isolates (nine bacteria and two yeasts) are illustrated in Supplementary Figures 1, 2. The *S. cerevisiae* strain AXAZ-1 (maintained at the laboratory's cultures collection) also was included in all conducted trials.

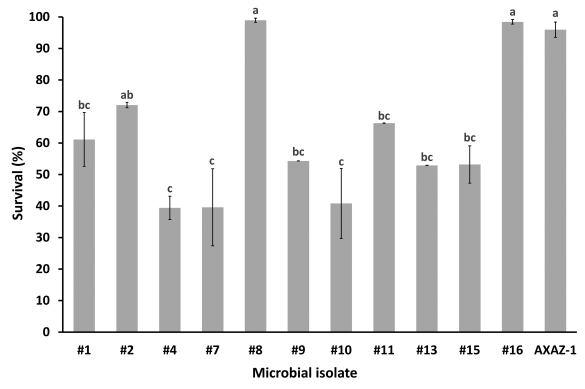

FIGURE 1
Survival percentage of microbial populations after 3 h of exposure to pH 2.5 in phosphate buffered saline. Values are means ± standard errors (n=3). Means lacking a common letter are significantly different (P<0.05).

When the microbial isolates were assessed regarding their resistance to low pH, the microorganisms which were noted for their acid tolerance were the isolates #1, #2, #8, #11, #16 as well as the strain AXAZ-1, with the estimated survival (%) after 3 h of acid challenge (pH 2.5) being 61.1, 72.0, 98.9, 66.3, 98.4 and 96.0, respectively. Nonetheless, sufficient acid resistance was also recorded for the rest of the microbial isolates, for which the survival percentages varied from 39.4 to 54.3% (Figure 1).

The abovementioned isolates also exhibited considerable autoaggregation capability. Particularly, the isolates #2, #8, #11 and #16, along with the yeast strain AXAZ-1, were capable of autoaggregating at levels exceeding 80% after 24 h (Figure 2). Appreciable autoaggregation was also demonstrated by the microbial isolates #1, #4, #7, #13 and #15, with the estimated percentages after 24 h varying from 64.0 to 78.7%. The yeasts investigated in the context of this study, namely the isolates #8 and #16 and the strain AXAZ-1, were evidently prompter to form cell aggregates than the bacterial isolates, since high autoaggregation percentages were noted even within the first 2 h of incubation (Figure 2). On the other hand, most of the bacterial isolates exhibited either a similarly low autoaggregation capability at 2 and 4 h followed by a considerably higher capability at 24 h of incubation (e.g., isolates #1 and #2), or a gradually increasing autoaggregation as a function of time (e.g., isolates #9 and #15). The microbial isolate exhibiting the lowest (P<0.05) autoaggregation capability was the bacterial isolate #10 (Figure 2).

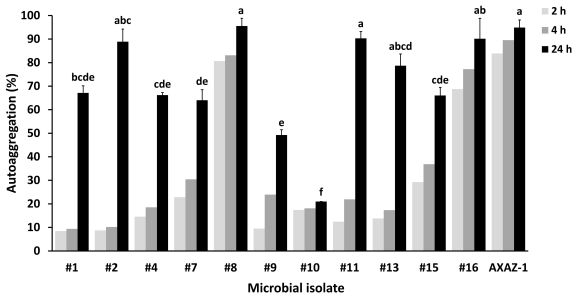

FIGURE 2
Autoaggregation capability (as percentage) of the microbial isolates after incubation in phosphate buffered saline for 2, 4 and 24 h and optical density measurements at 600 nm. The values corresponding to 24 h are means ± standard errors (n=2). Means lacking a common letter are significantly different (P<0.05).

With reference to the biofilm-forming ability of the microbial isolates, which was quantified *in vitro* via a colorimetric microtiter plate method, the collected ΔOD_{595nm} data are illustrated in Figure 3. According to these data, which are indicative of the total biofilm mass produced under the conditions of this study by each one of the tested microorganisms, the bacterial isolates #2, #10, #11, #13 and #15, as well as the yeast isolate #16, could be characterized as strong biofilm formers. Significantly (P<0.05) poorer biofilm formation was recorded for the rest of the tested microorganisms, including the yeast AXAZ-1, with the bacterial isolate #4 being evaluated as the weakest biofilm former (Figure 3). Since the autoaggregation capability of microorganisms constitutes a putatively important parameter for their initial adhesion on surfaces, it is worth and relevant for someone to comparatively evaluate this capability with the corresponding biofilm formation of the tested isolates. Indeed, important capability of both autoaggregation and biofilm formation was recorded for all the aforementioned isolates, except for the bacterial isolate #10, which, albeit demonstrating considerable biofilm formation, did not exhibit remarkable autoaggregation (Figures 2, 3).

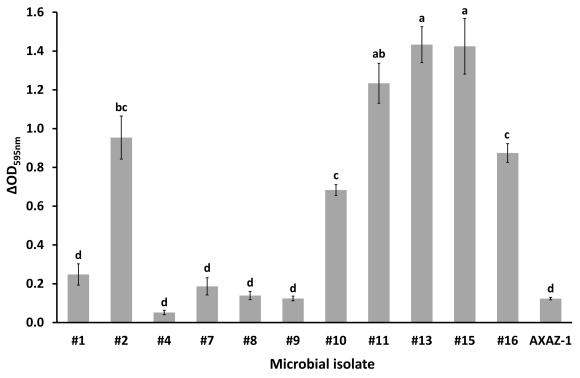

FIGURE 3
Biofilm formation in polystyrene microtiter plates by the microbial isolates, based on crystal violet staining and the difference between the optical density measurements of the test and negative control samples (ΔOD_{595nm}). Values are means ± standard errors (n=16). Means lacking a common letter are significantly different (P<0.05).

The antioxidant activity of the microbial isolates, expressed as the percent reduction of DPPH, was evaluated as rather variable (Figure 4). The highest (P<0.05) antioxidant activity was exhibited by the yeast isolate #16, with the estimated scavenging activity being 38.6%. The minimum value of the estimated scavenging activity was 3.3% (isolate #11), while the yeast strain AXAZ-1 demonstrated negligible antioxidant activity. The suitability of the wavelength of 490 nm for the determination of the microbial isolates' scavenging activity in the context of the applied methodology (section 2.6) was assessed and confirmed in preliminary experimental trials; according to the results of these trials, the absorbance of DPPH at 490 nm was maximized to 1.35 ± 0.08 (mean ± standard deviation, n=4) compared to the significantly lower absorbance values recorded at the wavelengths of 450 nm, 550 nm, 595 nm and 630 nm at which the microplate reader could operate.

Finally, the vast majority of the microbial isolates investigated in the present study were evaluated as safe in terms of hemolysis, namely as γ-hemolytic. The sole exception to this was the isolate #1 (bacterial isolate from kefir), which exhibited α-hemolysis (partial hemolysis) with green-hued zones being formed around the colonies of the isolated microorganism on Columbia agar containing sheep blood (Figure 5).

4 Discussion

The presence at high populations of microorganisms, being characterized as presumptive LAB and/or yeasts in fermented food products was anticipated, justifying the selection of such products in the search of potential probiotics in the first place. Indeed, the fermented milk beverage of kefir, originating from the Caucasus region, is manufactured through the addition to milk of a symbiotic starter culture referred to as "kefir grains" (Lopitz-Otsoa et al., 2003). This symbiotic culture typically consists of lactic and acetic acid-producing bacteria and yeasts (lactose-fermenting and/or non-lactose fermenting), being housed and interacting within kefiran, a polysaccharide and protein matrix (Lopitz-Otsoa et al., 2003; Dimidi et al., 2019). Among the eight macroscopically different microorganisms isolated from the kefir products tested in this study, seven were identified as presumptive LAB and one as yeast, with five of them being ultimately selected to be further investigated. Although a wide range of microbial species have been identified in kefir grains, the microorganisms most recovered from the final product are LAB species belonging to the genera *Lactobacillus* and *Lactococcus*, acetic acid bacteria of the genus *Acetobacter*, and the yeast species *Kluyveromyces lactis* and *Saccharomyces cerevisiae* (Dimidi et al., 2019). Furthermore, it has been shown that even though the abundance of yeasts in kefir grains may be relatively low compared to that of bacteria, their contribution to the fermentation process and the characteristics of the final product (e.g., acid production and aroma) can be significant (Chen et al., 2021). In the case of the kefir products investigated herein, the sole yeast isolate (isolate #8) originated from the homemade product, while only bacterial isolates were recovered from the industrial products, probably reflecting corresponding differences in the microbial consortia used as starter cultures.

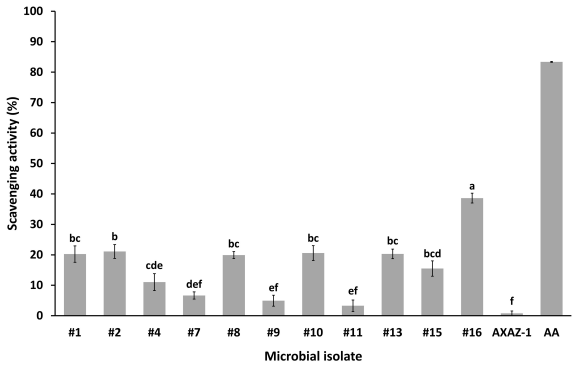

FIGURE 4
Antioxidant effects of intact cells of the microbial isolates on DPPH (1,1-diphenyl-2-picrylhydrazyl) radical-scavenging activity. Values are means ± standard errors (n=4). Means lacking a common letter are significantly different (P<0.05). AA: ascorbic acid (positive control).

Table olives are regarded as one of the oldest fermented vegetables in the Mediterranean area, constituting an important element of the economy of several countries, with the main producers being Spain, Greece, Italy, and Portugal (IOC, 2022). Olive fermentation, which still adheres to traditional processes, is based on the enzymatic activity of various members of the autochthonous microbiota of olive drupes. This indigenous microbiota is mainly composed of LAB and yeasts; the most identified bacteria are *Lactobacillus* spp., while yeast species of the genera *Candida*, *Pichia* and *Saccharomyces* have also been isolated (Argyri et al., 2020; Torres et al., 2020). As is the case for kefir, fermented table olives also exhibit a favorable health profile which, beyond their high content in bioactive compounds (e.g., unsaturated fatty acids and phenolic compounds), expands to their putative role as food vehicles for probiotics' delivery (Bonatsou et al., 2017; Perpetuini et al., 2020). The specific microbial species that will shape both the technological characteristics (e.g., texture, flavor) and functional traits of the final product seem to depend largely on the applied fermentation process. Specifically, in natural black olives which were one of the microbial isolation sources in this study, the fermentation is principally driven by lactobacilli, with *Lactobacillus pentosus* and *Lb. plantarum* being identified as the main LAB species involved, while *Leuconostoc mesenteroides* has also been recovered from the final product (Doulgeraki et al., 2013; Bonatsou et al., 2017; Perpetuini et al., 2020). The contribution of yeasts is also undeniably important, with this microbial group playing a decisive role in the development of the organoleptic characteristics of the final product, while at the same time enhancing LAB growth through the release of nutritive compounds (Nisiotou et al., 2010; Perpetuini et al., 2020). However, certain yeast species can cause significant product deterioration due to spoilage (Nisiotou et al., 2010), justifying to some extent why the microbiota of successfully fermented table olives is predominantly populated by LAB species. The outgrowth and prevalence of LAB over yeasts during olive fermentation results in the acidification of brine (due to the production of lactic acid) which, in turn, ensures the microbiological stability of the final product (Perpetuini et al., 2020). Consistent with the anticipated dominance of LAB, six macroscopically distinct bacterial isolates were recovered from table olives in the present investigation (three isolates from drupes and three isolates from brine samples), while no yeast was isolated, not at least at the sample dilution levels required for TVC determination. On the other hand, the isolation of yeast #16 from OMW demonstrates that even if not present in the final product, yeasts are likely to participate in the indigenous microbiota of olive drupes, contributing mainly to the first stages of the fermentation process. From the table olives' isolates, four (two from drupes and two from brines) were selected for further investigation. In quest of probiotics originating from table olives, the ones recovered from the surface of drupes may have a competitive advantage over microorganisms recovered from the brine. Such advantage is mainly associated with the indigenous ability of these microorganisms to adhere, an ability which is highly likely to allow for enhanced maintenance of microbial cell integrity and stability during transit in the gastrointestinal tract and ultimately, to

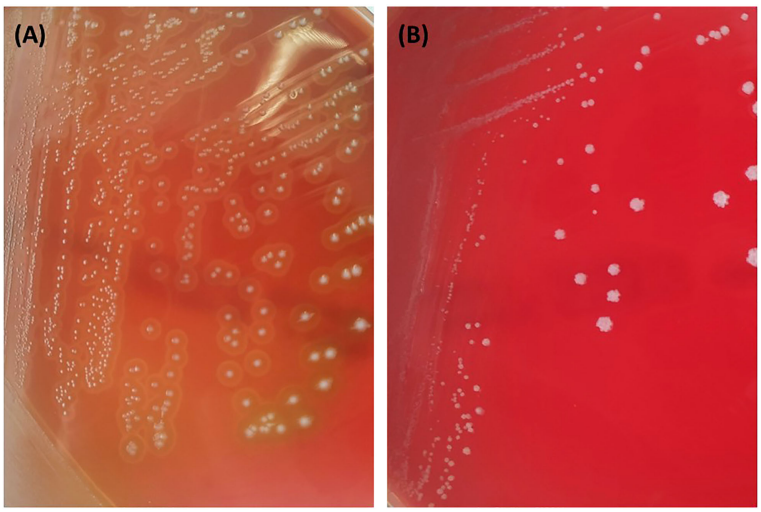

FIGURE 5
Observation of α-hemolysis (partial hemolysis, **(A)**) and γ-hemolysis (absence of hemolysis, **(B)**) for the microbial isolate #1 and #8, respectively, on Columbia agar containing 5% (v/v) sheep blood.

favorably contribute to microbial adhesion to the intestinal epithelium (Larsen et al., 2009; Peres et al., 2015).

Based on the results of this study, the microbial isolates exhibiting the highest acid resistance were the bacteria #1 and #2 originating from kefir, the bacterial isolate #11 recovered from olive brine, and both isolated yeasts, namely the isolates #8 (from kefir) and #16 (from OMW). High survival rates under low pH conditions have been commonly recorded among LAB and yeasts isolated from fermented foods in studies assessing the probiotic potential of these organisms (Argyri et al., 2013; Pavli et al., 2016; Oliveira et al., 2017; Bonatsou et al., 2018). The resistance of microorganisms to low pH is an important prerequisite for their putative probiotic label, since in the absence of such inherent acid resistance they will not be able to survive the transit through gastric fluids and thus, successfully colonize the intestinal epithelium. Although most of the tested isolates (8/11) exhibited survival exceeding 50% after 3-h exposure to the rather low pH of 2.5, the acid survival recorded for the two yeast isolates was well above 90%, and a similarly high survival was also noted for the yeast strain AXAZ-1. The remarkable acid resistance of yeasts has been well identified and documented in the scientific literature, constituting essentially one of their most important traits for their value as putative probiotics (Oliveira et al., 2017; Bonatsou et al., 2018). The collected in this study resistance data would be useful to be further substantiated in future research through additional experimental trials involving either exposure of the microbial isolates to simulated gastric fluid or utilization of gastrointestinal model systems.

Most of the tested in this study microbial isolates exhibited considerable autoaggregation capability. Indeed, 10 out of the 12 tested microorganisms demonstrated autoaggregation equal to or higher than 60% after 24 h, with five of them (i.e., isolates #2, #8, #11, #16 and the yeast strain AXAZ-1) demonstrating a rather high autoaggregation capability (>80%). The remarkable autoaggregation demonstrated by the yeasts (isolates #8 and #16 as well as AXAZ-1), even within the first 2 h of incubation, is most likely associated with the larger size of yeast cells compared to that of bacterial cells, facilitating the precipitation of the formed cell aggregates. The autoaggregation capability of microorganisms is regarded as indicative, to some extent, of their ability to adhere on biotic and/or abiotic surfaces, including the intestinal epithelium, an apparently desirable trait for microorganisms evaluated for their putative probiotic traits (Javanshir et al., 2021). Nonetheless, additional parameters may play an important role, such as the hydrophobicity and co-aggregation of microbial cells, while autoaggregation is commonly evaluated in conjunction with the biofilm-forming ability of microorganisms (Ogunremi et al., 2015; Bonatsou et al., 2018). As demonstrated by the biofilm formation data generated in the present study, the bacterial isolates #2, #10, #11, #13 and #15, as well as the yeast isolate #16, could be regarded as strong biofilm formers, and all these isolates, excluding #10, also demonstrated a high autoaggregation capability. It is also noteworthy that when the isolation source is considered, it seems that table olives may be a more propitious reservoir of microorganisms with high biofilm-forming ability than kefir. Only one of the kefir isolates (out of the five that were tested) was a strong biofilm former (isolate #2), while the rest of the isolates evaluated as such originated from fermented table olives (#10, #11, #13) and OMW (#15, #16). The high biofilm-forming ability of both LAB and yeast isolates originating from table olives has been also reported by other researchers (Grounta et al., 2016; León-Romero et al., 2016; Bonatsou et al., 2018; Vergara Alvarez et al., 2023), while findings relevant to the important probiotic properties of microorganisms isolated from table olive biofilms are certainly very interesting (Benítez-Cabello et al., 2020). Extracellular polymeric substances (EPS), being composed primarily of polysaccharides, are a vital structural and functional component of biofilms (Flemming, 2016). As supported by research findings, EPS production enhances the attachment of probiotics to the intestinal epithelium, inhibiting in this manner its colonization by foodborne pathogenic bacteria (Tatsaporn and Kornkanok, 2020). The biofilm-forming ability of the microbial isolates tested in this study was assessed via an indirect quantification methodology which refers to the total biofilm mass, not allowing the distinction among viable cells, dead cells, and EPS matrix components. Thus, a further research step could be the compositional characterization of the biofilms formed by the isolates identified herein as strong biofilm formers. Moreover, the attained *in vitro* results should be also ascertained *in situ* and ultimately *ex vivo*/*in vivo*, since biofilm formation on abiotic surfaces is not necessarily associated with microbial adhesivity on biotic surfaces (Peres et al., 2015).

The antioxidant activity of microbial metabolites constitutes an important field of study in Microbiology and Microbial Biochemistry, since the formation of reactive oxygen species (ROS) and the resulting oxidative stress is an important etiologic factor for many human diseases including diabetes, rheumatoid arthritis, and cardiovascular diseases (Forman and Zhang, 2021). Probiotics and their fermented metabolites, referred to as "postbiotics" have been shown to exhibit antioxidative activities and as such, are rather promising for the regulation of oxidative stress and eventually the protection of cells from oxidative damage (Hoffmann et al., 2021; Lin et al., 2022). Regarding the microorganisms investigated herein, the greatest antioxidant activity was exhibited by the isolate #16, corresponding to yeast isolated from OMW. The rest of the microbial isolates, being in their vast majority presumptive LAB, were evaluated as having variable antioxidant activity, with the estimated scavenging activity (expressed as percent reduction of DPPH) varying from 0.8 to 21.1%. These observations are generally in good agreement with the findings of previous studies assessing, among other probiotic traits, the antioxidant activity of LAB (Chen et al., 2014) and yeasts (Gil-Rodríguez et al., 2015). Although the mechanisms of their antioxidant action have not been fully elucidated, it has been proposed that probiotics may modulate the redox status of the host via their metal ion chelating ability, antioxidant enzyme systems (e.g., superoxide dismutase and catalase), antioxidant metabolites (e.g., glutathione, butyrate, and folate), as well as through regulation of signaling pathways, enzymes producing ROS and the intestinal microbiota (Wang et al., 2017).

For microorganisms with beneficial activity to be utilized by the food, pharmaceutical and/or cosmetic industries, their safety for humans must be explicitly demonstrated. The hemolytic activity of microbial strains is among the safety criteria that are commonly assessed since its absence is indicative of their safe use in consumer

products' applications (FAO/WHO, 2002). Hemolytic activity is regarded as an indication of potential pathogenicity, and thus, microbial isolates exhibiting β-hemolysis cannot be considered as potential probiotics. Excluding potentially isolate #1, which exhibited α-hemolysis (partial hemolysis), the rest of the microbial isolates investigated in the present study were evaluated as safe in terms of hemolysis. Alpha hemolysis is mainly caused by the microbial production of hydrogen peroxide, oxidizing oxy-hemoglobin to met-hemoglobin (McDevitt et al., 2020). In any case, however, observations based on *in vitro* trials should be confirmed through virulence assays in cell lines and subsequently, in the context of *in vivo* investigations.

Based on the collective evaluation of the results of the *in vitro* assays carried out in this study, five presumptive LAB (#2, #10, #11, #13, #15) as well as two yeast isolates (#8, #16) were identified as exhibiting desirable *in vitro* probiotic traits. Hence, these microbial isolates could be regarded as good candidates for inclusion in further studies aiming, ultimately, at their potential utilization in novel functional food products. The *S. cerevisiae* strain AXAZ-1 is an alcohol-resistant and psychrotolerant strain previously isolated from the agricultural area of North Achaia, Greece (Argiriou et al., 1992). Given its biotechnological interest, related mainly to the valorization of agro-industrial residues for the production of high-added value products such as ethanol (Kallis et al., 2019; Dourou et al., 2021), and the fact that *S. cerevisiae* strains have been commonly identified as having probiotic potential (Staniszewski and Kordowska-Wiater, 2021), it was thought as worthy and interesting to also evaluate the probiotic traits of this strain. Nevertheless, a probiotic potential was not plainly conveyed for AXAZ-1 under the conditions of this study; despite its high acid resistance and autoaggregation capability, this yeast strain was evaluated as a poor biofilm former with negligible antioxidant activity.

Microbial strains with functional properties, such as probiotic potential, may be used in various food product development applications. Such applications involve utilization of such strains as starter cultures (provided of course that they also possess the required technological properties) to produce probiotic fermented foods, or as adjunct cultures in various novel multi-functional food products of both enhanced health benefits and microbiological safety (Blana et al., 2014; Botta et al., 2015; Peres et al., 2015). The seven microbial isolates that were identified herein as exhibiting the most desirable *in vitro* probiotic traits, namely isolates #2, #8, #10, #11, #13, #15 and #16, could be regarded as good candidates for inclusion in further studies. Such studies should include: (i) molecular characterization of the identified isolates; (ii) assessment of additional probiotic properties (e.g., antimicrobial activity) and safety criteria (e.g., antibiotic resistance); (iii) *in situ* assessment of their performance as starter/adjunct cultures in selected food products; and (iv) *ex vivo* evaluation of both their safety and potential health benefits. The data collected in the present preliminary study provide the information needed for the targeted genetic characterization of the microbial isolates exhibiting probiotic potential, a characterization which is of even greater importance in the light of the recently emended taxonomy of LAB (Zheng et al., 2020). The genetic characterization of the identified microbial isolates, and the assessment of their behavior and performance *in situ* constitute our imminent research goals.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

NN: Formal Analysis, Investigation, Writing – original draft. AL: Conceptualization, Methodology, Resources, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author Alexandra Lianou declared that she was an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/finmi.2023.1257483/full#supplementary-material

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Rapid identification of lactic acid bacteria at species/subspecies level via ensemble learning of Ramanomes

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Rapid and accurate identification of lactic acid bacteria (LAB) species would greatly improve the screening rate for functional LAB. Although many conventional and molecular methods have proven efficient and reliable, LAB identification using these methods has generally been slow and tedious. Single-cell Raman spectroscopy (SCRS) provides the phenotypic profile of a single cell and can be performed by Raman spectroscopy (which directly detects vibrations of chemical bonds through inelastic scattering by a laser light) using an individual live cell. Recently, owing to its affordability, non-invasiveness, and label-free features, the Ramanome has emerged as a potential technique for fast bacterial detection. Here, we established a reference Ramanome database consisting of SCRS data from 1,650 cells from nine LAB species/subspecies and conducted further analysis using machine learning approaches, which have high efficiency and accuracy. We chose the ensemble meta-classifier (EMC), which is suitable for solving multi-classification problems, to perform in-depth mining and analysis of the Ramanome data. To optimize the accuracy and efficiency of the machine learning algorithm, we compared nine classifiers: LDA, SVM, RF, XGBoost, KNN, PLS-DA, CNN, LSTM, and EMC. EMC achieved the highest average prediction accuracy of 97.3% for recognizing LAB at the species/subspecies level. In summary, Ramanomes, with the integration of EMC, have promising potential for fast LAB species/subspecies identification in laboratories and may thus be further developed and sharpened for the direct identification and prediction of LAB species from fermented food.

KEYWORDS

Ramanome, rapid classification, deep learning, LAB species/subspecies, fermented food

1 Introduction

Lactic acid bacteria (LAB) are important members of the probiotic family and are often used as starter cultures of dairy products with important economic and nutritional value. "Identification of starter culture organism(s)" is a primary prerequisite for documenting the microbiological safety of LAB (Zhang et al., 2023). Several approaches have been developed to identify LAB strains. Classical phenotypic identification methods for LAB are based on a

combination of their morphological and physiological characteristics (Jarocki et al., 2020). Genotype-based identification techniques include those based on 16S ribosomal RNA (16S rRNA) gene sequence, housekeeping genes (e.g., phenylalanyl-tRNA synthase alpha subunit, RNA polymerase alpha subunit, β-tubulin, and calmodulin), genome-wide single-nucleotide polymorphisms (SNPs), multilocus sequence typing (either core genome or whole-genome), or amplified fragment length polymorphism (Keith and Jolley, 2014; Mareike et al., 2014; Morovic et al., 2016; Lugli et al., 2019). Additionally, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), Fourier Transform Infrared (FT-IR) spectroscopy, and Tandem Mass Spectrometry (MS/MS) are frequently used for identification (Wenning et al., 2014). Although these methods have been well developed and proven to be reliable, they have several disadvantages. First, these methods usually require a culture-based strain isolation step that can take up to seven days (even longer for slow-growing cells), thus greatly delaying the time to report. Second, most of these methods are invasive and living cells must be broken down to extract DNA or proteins for identification. Third, these methods cannot be used to identify LAB in real-world settings. Accordingly, a fast, culture-independent, non-invasive and low-cost identification approach is highly desirable.

Single-cell Raman spectroscopy (SCRS) can provide the "molecular fingerprint" of a cell, and cells with different phylogenetic backgrounds can potentially be distinguished despite their varied metabolic states (He et al., 2019). SCRS is culture-independent and can promote "phenotype detection before culture." In a previous study (Teng et al., 2016), we introduced the concept of Ramanome, the collection of SCRS data sampled from a cell population or consortium, as a type of single-cell-resolution metabolic phenome, and demonstrated the ability of SCRS to rapidly classify microbial species in a culture-free and label-free manner. Since its proposal in 2016, Ramanome has been applied in many microbial fields, including species classification (usually via pure cultures) and metabolic feature identification (Lu et al., 2020; Heidari et al., 2021; Liu et al., 2022). However, most previous studies have focused on microalgae or pathogens, but not LAB species or subspecies. Furthermore, Ramanomes are sensitive to not only "phylogeny" but also the "state" of a cell, yet existing experimental designs have generally failed to distinguish them. Consequently, a broadly applicable and reliable approach for the identification of LAB remains unexplored. The typical Ramanome of a single microbial cell contains thousands of variables based on wavenumbers. Thus, the greatest challenge in reliable microbial classification using Ramanome analysis is to retrieve characteristic information for each species that is normally not evident. Therefore, accurate species classification requires the application of advanced statistical algorithms to recognize differences in the Ramanomes of different species.

In recent years, researchers have begun to focus on the interpretability of machine learning models, which can significantly improve prediction accuracy and are more credible (Guo et al., 2021; Barton et al., 2022). To further improve the classification accuracy and stability, we adopted a model integration strategy to build an ensemble meta-classifier (EMC). The EMC is a blend of base classifiers (linear discriminant analysis (LDA), linear support vector machine (SVM), random forest (RF), extreme gradient boosting (XGBoost), k-nearest neighbors (KNN), partial least squares discriminant analysis (PLS-DA), convolutional neural network (CNN), and long short-term memory network (LSTM)) for each training performance compared with base classifiers, which has proven the application value of model integration in the Raman spectrum (Heidari et al., 2021). Although much research has employed Raman spectroscopy to identify bacteria, less research has been conducted on LAB. In this study, we established a method that combined Ramanome and EMC to discriminate the following nine closely related LAB species/subspecies: *Lacticaseibacillus paracasei* subsp. *paracasei*, *Lacticaseibacillus paracasei*, *Lacticaseibacillus rhamnosus*, *Lactiplantibacillus plantarum*, *Lactiplantibacillus plantarum* subsp. *plantarum*, *Lactiplantibacillus argentoratensis*, *Lactiplantibacillus pentosus*, *Lactobacillus gallinarum* and *Pediococcus pentosaceus*. We then compared the classification of nine machine-learning algorithms, and the results showed that the EMC model was the best classifier, with an average prediction accuracy of 97.3%, which was 3.66% higher than the maximum accuracy of the CNN of the single deep-learning model. This EMC utilizes the Ramanome approach for rapidly identifying single LAB cells, greatly accelerating the mining of LAB from fermented food.

2 Materials and methods

2.1 Chemical and biological materials

Nine LAB strains belonging to nine different LAB species/subspecies were collected from fermented dairy samples (Table 1). A total of 1,650 Raman spectrum fingerprints (600–1800 cm^{-1}) were obtained for all of the bacterial species/subspecies. All LAB strains were stored in the School of Life Science and Technology, Inner Mongolia University of Science and Technology, Baotou, China, after isolation. These strains were identified using biochemical methods and 16S rRNA and stored in a ThermoFisher freezer at −80°C.

2.2 SCRS acquisition and parameter setting

After culturing each strain on MRS agar plates overnight, single colonies were inoculated in 5 mL MRS broth. Then, 1% bacterial culture was inoculated into a new MRS broth culture and incubated at 37°C for 16 h at 200 rpm. Thereafter, 1 mL sample from each strain was centrifuged at 8000 rpm for 2 min and washed thrice with sterile water. Sterile water samples with a moderate-weighted drop hanging on a calcium fluoride (CaF_2) slide were air-dried prior to Raman analysis.

TABLE 1 LAB samples.

No.	Strain number	LAB species/subspecies
001	IMUST00001	*Lacticaseibacillus paracasei subsp. paracasei*
016	IMUST00016	*Lacticaseibacillus paracasei*
036	IMUST00036	*Lactobacillus gallinarum*
063	IMUST00063	*Lactiplantibacillus plantarum*
067	IMUST00067	*Lactiplantibacillus plantarum subsp. plantarum*
114	IMUST00114	*Lactiplantibacillus argentoratensis*
138	IMUST00138	*Pediococcus pentosaceus*
143	IMUST00143	*Lacticaseibacillus rhamnosus*
146	IMUST00146	*Lactiplantibacillus pentosus*

All SCRS data were acquired using a RACS instrument (Qingdao Single-Cell Biotechnology, Qingdao, China). The system is equipped with a microscope with a 100 × dry objective (NA = 0.80) and a 532 nm Nd: YAG laser with a maximum power of 100 mW. Each cell was exposed to a laser for 1 s, and the spectra were recorded using a diffraction grating with 300 grooves/mm. A total of 180 cells were analyzed using SCRS for each biological replicate of each LAB strain.

2.3 Data preprocessing and machine learning

To process and analyze the Ramanome data effectively, we adopted the following comprehensive processing flow to ensure consistency, comparability, and reliability of the data (Guo et al., 2021). Our processing pipeline primarily included baseline correction, spectral smoothening, and normalization, which played key roles in extracting spectral features and reducing noise (Senger and Scherr, 2020). Firstly, we use the polynomial fitting method to perform baseline correction on the spectrum. By estimating the background baseline of the spectrum and subtracting it from the original spectrum, we could reduce the background interference and highlight the characteristics of the spectral peak. Then, to further eliminate the noise in the data, we applied the Savitzky–Golay smoothing method to smooth the spectral data by fitting polynomials, so as to retain important information while reducing unnecessary fluctuations. In the final stage of spectrum pre-processing, we employed subsequent analysis, a step that involves dividing each data point of the spectral data by the maximum value of that spectrum, mapping the data to between 0 and 1, thus achieving a uniform amplitude scale. The pre-processing diagram is shown in Supplementary Figure S1. After preprocessing the SCRS data, the average SCRS data for each bacterial species/subspecies were generated by calculating the intensities at each Raman shift, and standard deviations (shaded error bands) were also calculated. The averaged SCRS data were imported into the LabSpec software (HORIBA Scientific, Japan).

2.4 Comparative evaluation of machine learning algorithms

A comprehensive list of machine-learning techniques, including six machine-learning models (LDA, SVM, RF, XGBoost, KNN, and PLS-DA) and two deep-learning models (CNN and LSTM), was employed to generate the base machine-learning classifiers. Metrics (Accuracy, Mean Sensitivity, Mean Specificity, Kappa) are essential for evaluating the effects of machine-learning algorithms during data analysis. In this study, different evaluation metrics were used to measure the performance indicators in spectrum-signal recognition.

3 Results

3.1 Average SCRS spectra and characteristic peaks

In this study, we calculated the average Raman intensity at each Raman shift to generate the average SCRS data for each of the nine LAB species/subspecies. Hence, we built a reference Ramanome database that spans a wide range of nine LAB species/subspecies. Notably, nine LAB species/subspecies from each of the genera *Lacticaseibacillus*, *Lactiplantibacillus*, *Lactobacillus*, and *Pediococcus* were included to determine the feasibility of the species/subspecies-level classification (Table 1). Each species was cultured in triplicates under optimal growth conditions. From each biological replicate culture, 60 cells were randomly selected for SCRS spectrum acquisition as Ramanomes; thus, 180 cells were sampled per species. Different LAB species show differences in their Raman intensities and distributions of their characteristic peaks, which can be used to discriminate them. In addition, the standard error band was visualized in the averaged Raman spectrum of each bacterium to determine whether the spectral data exhibited good repeatability during SCRS spectrum generation. The narrower the error band, the higher the repeatability of the Raman spectrum. According to the results shown in Figure 1, the reproducibility of the Raman spectra for each LAB species/subspecies was good.

We found that the spectra of the nine samples were different at the wave intensities of 805, 851, 1,000, 1,095, 1,240, 1,328, 1,450, 1,570, and 1,653 cm^{-1} (Figure 2). The specific characteristic peaks and their corresponding biological meanings are listed in detail in Table 2. Distributions of the characteristic peaks of all nine LAB species and subspecies are shown in Supplementary Figure S2A. The difference in the significance of each characteristic peak is shown in Supplementary Figure S2B, where the value represents the degree of significance (0, $p > 0.05$; 1, $0.01 < p \leq 0.05$; 2, $0.001 < p \leq 0.01$; 3, $0.0001 < p \leq 0.001$; 4, $p \leq 0.0001$). All bacteria share basic structures, such as cell walls and cell membranes, but the composition and types of proteins, lipids, and nucleic acids vary depending on the species or subspecies (Rodriguez et al., 2017). Proteins make up 40–50% of bacterial cells. The characteristic Raman peaks at 851 and 1,000 cm^{-1} were associated with proline, tyrosine, and phenylalanine. The amide I band of the proteins (1,653 cm^{-1}) contributed substantially to the accurate discrimination of the nine LAB species/subspecies. Lipids make up 10–15% of bacterial cells. The lipid of the CH_2 bending was associated with 1,450 cm^{-1}. Bacterial cells contain 2–4% DNA and 5–15% RNA. The PO_2^- symmetric stretching and PO_2^- asymmetric phosphate were associated with 1,095 cm^{-1} and 1,240 cm^{-1}, while the characteristic Raman peaks at 805 and 1,328 cm^{-1} were due to backbone geometry, phosphate ion interactions, and CH_3CH_2. Furthermore, the Raman peak at 1570 cm^{-1} was attributed to guanine or adenine (Wang et al., 2016; Sun et al., 2023).

We used t-distributed Stochastic Neighbor Embedding (t-SNE) to cluster the nine species/subspecies (Figure 3). T-SNE cluster analysis results showed that strains 001 (*Lacticaseibacillus paracasei* subsp. *paracasei*), 036 (*Lactobacillus gallinarum*), and 016 (*Lacticaseibacillus paracasei*), (*Pediococcus pentosaceus*) and 143 (*Lacticaseibacillus rhamnosus*) had many overlapping regions that could not be distinguished but could be distinguished from other strains; strains 063 (*Lactiplantibacillus plantarum*) and 067 (*Lactiplantibacillus plantarum* subsp. *plantarum*) could not be distinguished but could be distinguished from the other strains; and strains 114 (*Lacticaseibacillus argentoratensis*) and 146 (*Lacticaseibacillus pentosus*) could be distinguished from the other strains. Therefore, accurate differentiation was not possible when comparing the Raman spectra of the nine LAB species/subspecies. To overcome this difficulty, six machine-learning and two deep-learning models were built to identify the nine LAB species/subspecies.

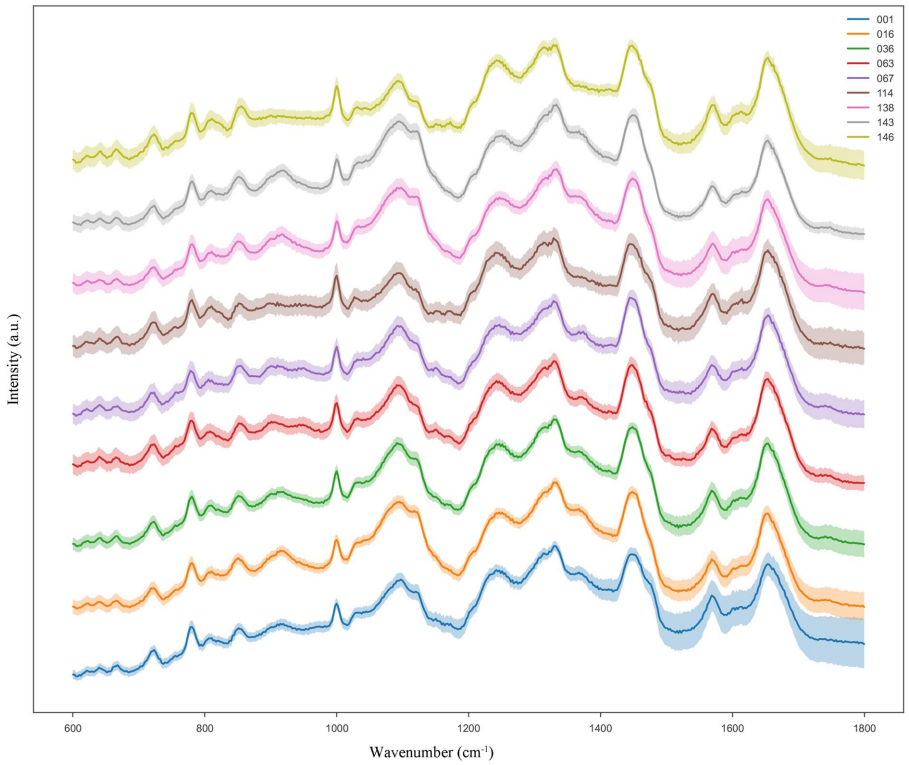

FIGURE 1
Typical Ramanome of the nine LAB species/subspecies. The mean Raman spectra are indicated by the solid line (colored), and the standard deviations are represented by the shadow. For each species/subspecies, the Ramanomes of more than 160 single cells were acquired via SCRS analysis.

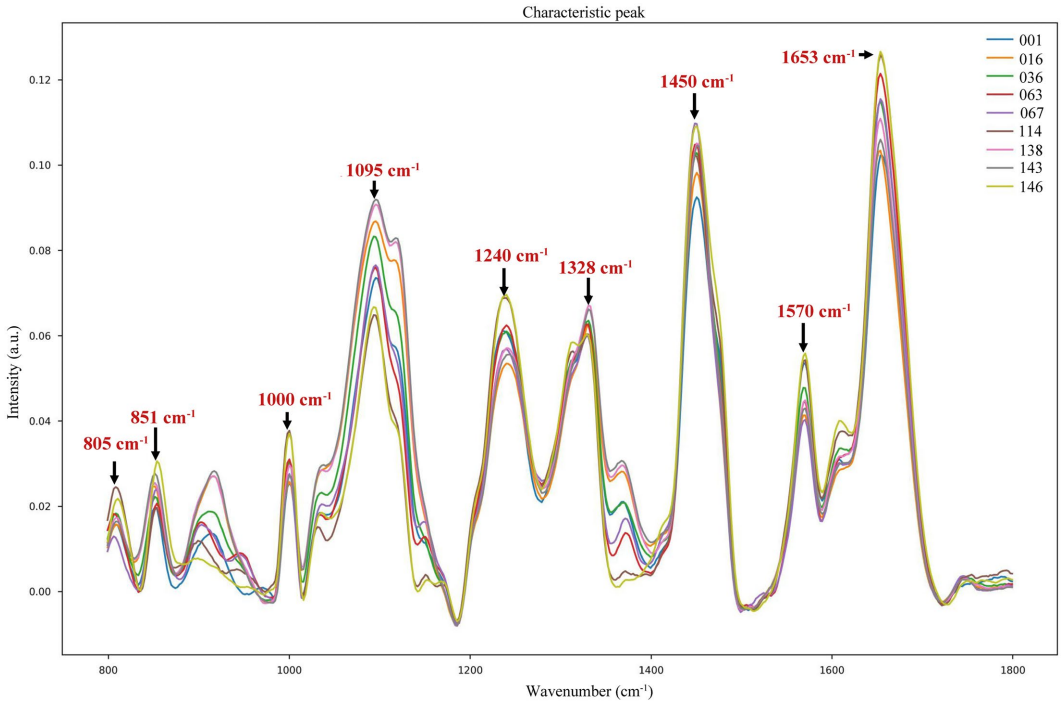

FIGURE 2
Characteristic peaks of different LAB species/subspecies in the fingerprint region.

3.2 Machine learning analysis and ensemble meta-classifier

In this study, six machine learning models (LDA, PLS-DA, XGBoost, KNN, RF, and SVM) and two deep learning models (LSTM and CNN), with eight independent classification models, were employed for the classification of LAB Ramanome data. Each model exhibited a specific classification performance during separate training and validation (70% for the training set and 30% for the test set). Four evaluation metrics, accuracy (ACC), mean sensitivity, mean specificity, and kappa, were applied to measure the performance of all machine learning models (Figure 4). Ten-fold cross-validation was used to determine whether the models were overfitted during training. The results are shown in Table 3. The accuracy of the eight classifiers was between 70.41 and 93.64%. LSTM and CNN achieved comparatively good classification results with accuracies of 92.07 and 93.64%, respectively. Meanwhile, the specificity of the eight classifiers was between 69.07 and 93.14%. LSTM and CNN also achieved comparatively good classification results with specificities of 91.77 and 93.14%, respectively. A schematic illustration of the CNN and LSTM analyses of the Ramanome data is shown in Supplementary Figures S3A,B. Excluding the LDA, PLS-DA, and XGBoost algorithms, the KNN and RF achieved an accuracy and a specificity of more than 80%.

TABLE 2 Biological meaning of the characteristic peaks for the nine LAB species as per the literature.

Peak (cm^{-1})	Assignment	References
805	Backbone geometry and phosphate ion interactions	Chan et al. (2006)
851	Proline, hydroxyproline, and tyrosine	Laska and Widlarz (2005)
1,000	Phenylalanine	Wang et al. (2016) and Stone et al. (2004)
1,095	Lipid	Loan et al. (2004)
1,240	Asymmetric phosphate [PO^{2-}(asym.)] stretching modes	Chan et al. (2006)
1,328	CH_3CH_2 wagging mode in purine bases of nucleic acids	Wang et al. (2016)
1,450	CH_2 bending	Jyothi Lakshmi et al. (2002)
1,570	Guanine, adenine, and TRP (protein)	Stone et al. (2004)
1,653	amide I	Farquharson et al. (2005)

The sensitivity and specificity of each model were similar to the accuracy results. To further validate the performance of different machine learning models for different bacterial species/subspecies, we used kappa values to measure the specificity and sensitivity of each model. The larger the kappa value, the better the performance of the model. By comparing the accuracy (93.64%), sensitivity (93.32%), specificity (93.14%), and kappa (0.9416), we concluded that the CNN model had the best performance for Raman data analysis at the bacterial species/subspecies level. The model with a similar predictive performance was LSTM. The first five models with the best performances were CNN, LSTM, SVM, RF, and KNN. Conversely, the LDA, PLS-DA, and XGBoost models failed to correctly distinguish among the nine LAB species/subspecies.

To further improve the accuracy and stability of the classification, we adopted the EMC strategy (Table 3). First, we evaluated eight models on the test set and their accuracies in the classification task. Each algorithm exhibited a certain classification performance during individual training and verification. By comparing the performance of the models, we chose the highest accuracy of the first five types of models (CNN, LSTM, SVM, RF, and KNN) as the components of the integration. During the integration process, we used a voting strategy to combine the predictions of various models (Figure 5). First, we (1) evaluated the performance of each classification model and used the

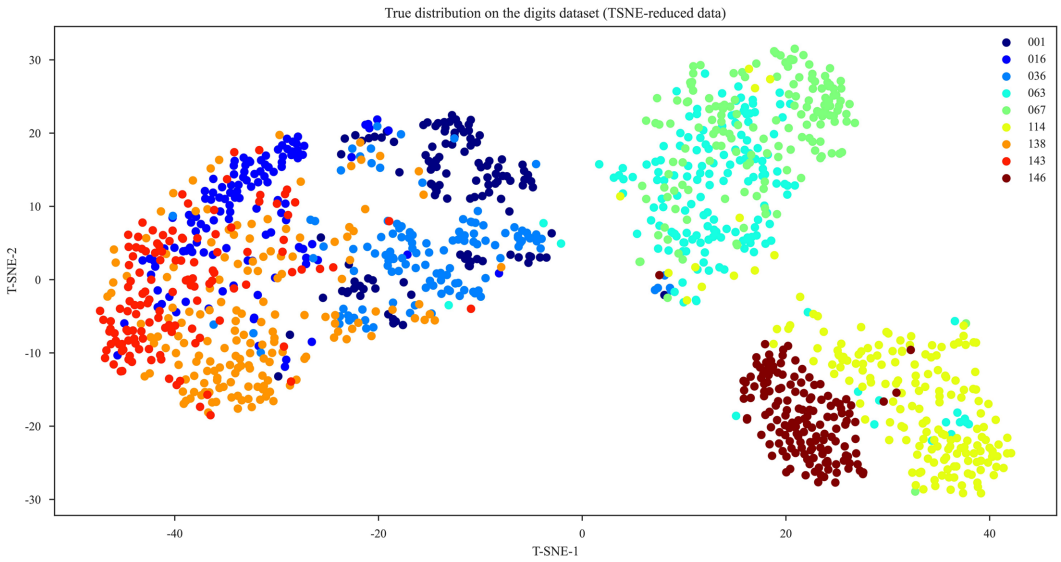

FIGURE 3
T-SNE cluster analysis of the nine LAB species/subspecies.

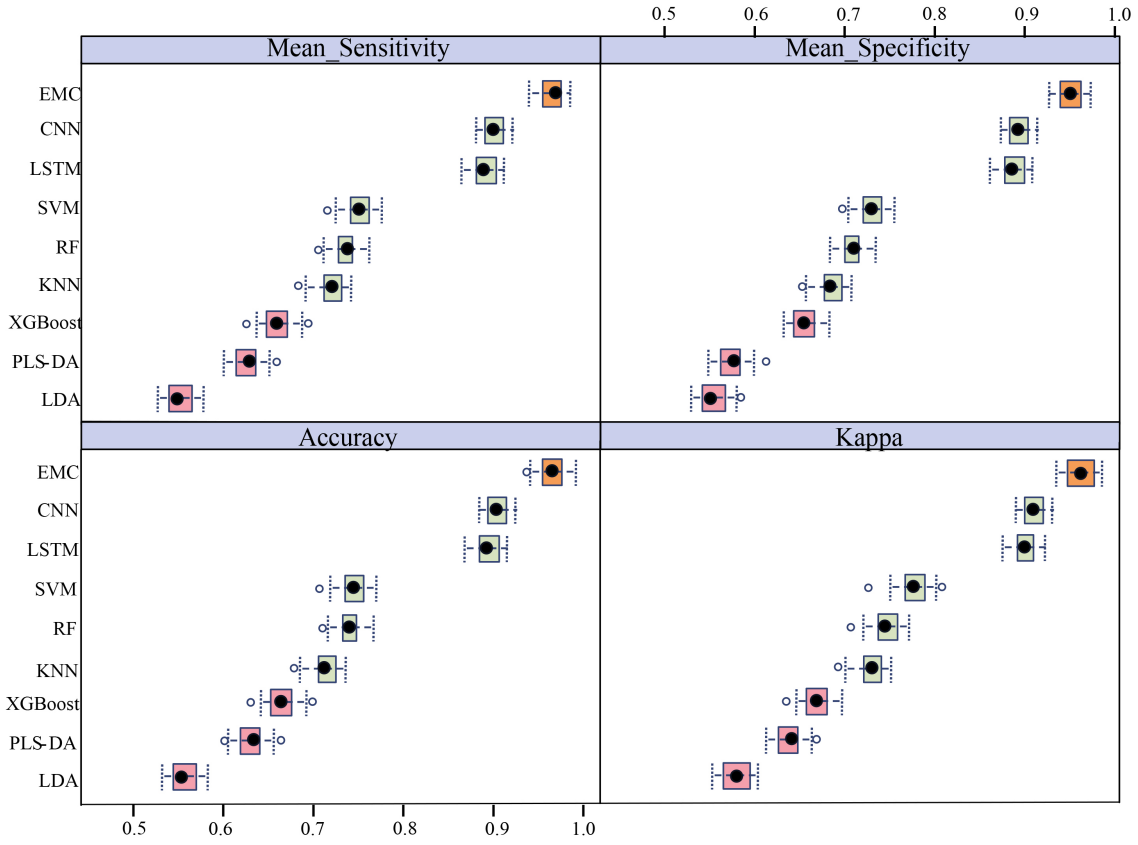

FIGURE 4
Phylogenetic classification of LAB based on machine learning. Results are based on 10 times 10-fold cross-validation. The box plots illustrate the distribution of the 100 resamples, with the central dot showing the median and the whiskers representing the quartiles. The orange box plot represents the EMC model, green box plots represent the first five classifiers with the best performances that were included in the EMC model, and pink box plots represent the three classifiers that performed poorly.

TABLE 3 Comparison of the predictive abilities of the nine supervised learning algorithms at LAB species/subspecies level.

Metrics	LDA	PLS-DA	XGBoost	KNN	RF	SVM	LSTM	CNN	EMC
Accuracy (%)	70.41	73.11	76.59	80.76	82.45	84.32	92.07	93.64	97.3
Mean sensitivity (%)	70.78	74.85	76.33	81.23	81.94	83.97	91.89	93.32	97.54
Mean specificity (%)	69.07	73.37	75.46	80.31	81.07	83.03	91.77	93.14	96.96
Kappa	0.6871	0.7194	0.7532	0.8066	0.8184	0.8437	0.9265	0.9416	0.9836

accuracy of a single classifier of the dataset as a measurement index; (2) took the accuracy rate as the weight of the model and normalized it, where the specific calculation formula was as follows:

$$W_{mn} = \frac{Accuracy_{mn}}{Sum\left(Accuracy_{m1}, Accuracy_{m2}, \ldots, Accuracy_{mn}\right)}$$

with W_{mn} representing the weight of the nth classification models (m), $Accuracy_{mn}$ representing the accuracy rate of the nth classification model, and *Sum* representing the sum; (3) calculated the class probability of each classifier and carried out weighted summation, with P_{cj} representing the estimated probability of class j. Each branch model calculates the confidence (prediction probability) for each category and considers the category with the highest confidence as the final prediction category; (4) considered the category with the greatest probability as the final prediction result. This method of model integration helps reduce the prediction bias of individual models, improves the stability and accuracy of the overall classification, and provides strong support for classification tasks in practical applications. This choice was based on the classification of different models into different categories based on comprehensive considerations. Next, we used the ensemble learning method to fuse the predictions of the five models and obtain a stronger classification performance. In the ensemble process, a voting strategy was adopted to combine the prediction results of each model. This method improved the stability and accuracy of the overall classification. The average accuracy of EMC was 97.3%, which was 3.66% higher than the highest accuracy of the single-model CNN (93.64%). The sensitivity, specificity, and kappa coefficient increased to 97.54,

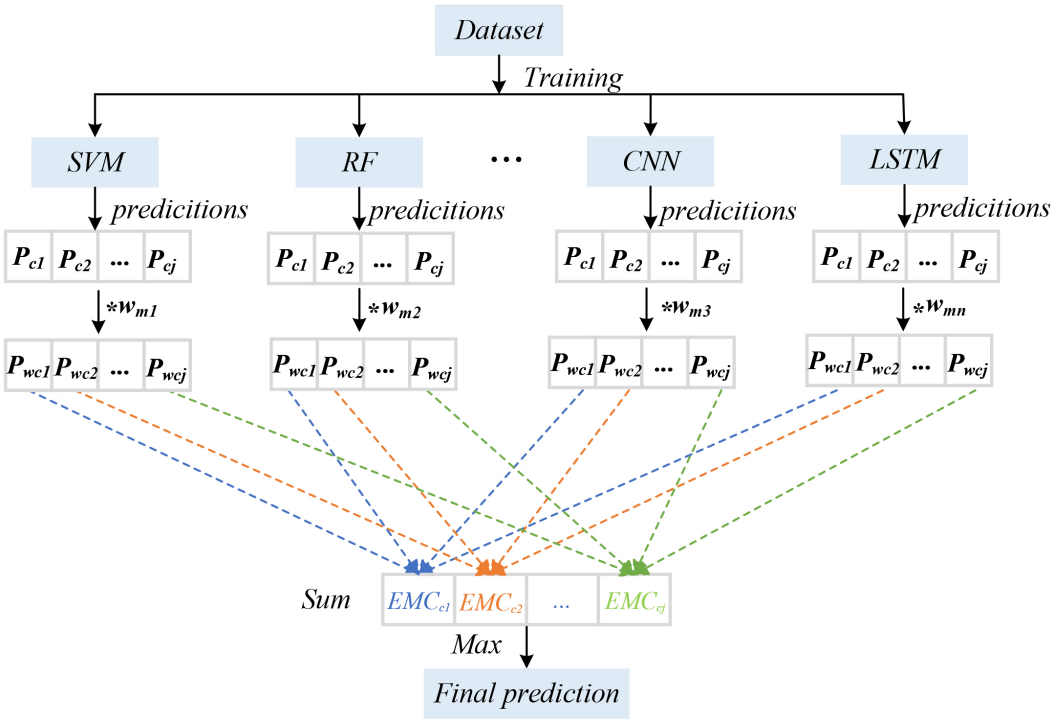

FIGURE 5
The diagram of EMC. We evaluated the performance of each classification model and used the accuracy of a single classifier for the dataset as a measurement index; took the accuracy rate as the weight of the model and normalized it; calculated the class probability of each classifier and carried out weighted summation; and considered the category with the greatest probability as the final prediction result. W_{mn} represents the weight of the nth classification model; P_{cj} represents the estimated probability of class *j*.

96.96%, and 0.9836, respectively (Table 3). Hence, the EMC of the best-performing base classifiers was built, which performed better than each base classifier.

3.3 Confusion matrix analysis

A confusion matrix is a quantitative visualization method used in machine-learning analysis to summarize the prediction results of a classification model (Liang et al., 2022). A confusion matrix describes the relationship between the real attributes of the sample data and the predicted results. Therefore, it is an efficient method for evaluating the performance of machine learning classifiers. In this study, we chose the best-performing model (EMC) to calculate the confusion matrix for LAB species/subspecies. The results of cross-validation may be presented in the form of a confusion matrix, where the true class (rows of the matrix) corresponds to the identification of species/subspecies based on 16S rRNA and biochemical methods, and the predicted class (columns) corresponds to the identification suggested by the Ramanome (Figure 6). The correctly identified spectra (in red) are diagonal. Off-diagonal spectra (in black font) correspond to incorrectly identified spectra (and their suggested classification by the Ramanome). The sensitivity of the method (true-positive rate (True)) and false-negative rate are presented in the rightmost columns, which represent the relative counts of SCRS data of the given species/subspecies that were incorrectly identified. The two bottom rows represent the positive predictive values (Predict), the relative counts of correctly identified spectra and spectra from different species falsely identified as a given species in each column, and the false discovery rate. In an ideal case, the prediction should be 100%. The results for the testing set at the single-spectrum level are shown in Figure 6 (Katarina et al., 2023).

As shown in Figure 6, for the nine different LAB species/subspecies, the EMC model achieved good prediction ability, and the classification accuracy for each strain was greater than or equal to 97%. As an example, 96.55% of *Lacticaseibacillus rhamnosus* were correctly predicted, whereas 2.3% of *Lacticaseibacillus rhamnosus* were misclassified as *Lacticaseibacillus* paracasei, and 1.15% of *Lacticaseibacillus rhamnosus* were misclassified as *Lacticaseibacillus paracasei* subsp. *paracasei*. In addition, 1.15% of *Lactiplantibacillus plantarum* and 1.92% of *Lactiplantibacillus pentosus* were predicted to be *Lactiplantibacillus argentoratensis*. Notably, 1.43% of *Lactiplantibacillus plantarum* subsp. *plantarum* and 0.48% of *Lacticaseibacillus paracasei* subsp. *paracasei* was predicted to be *Pediococcus pentosaceus*.

4 Discussion

The core characteristics of LAB products depend on the state of live bacteria, quantity, and bacterial health function, and their function and safety are strain-specific. Therefore, the precise identification of LAB at the strain level has become increasingly important domestically and internationally. The species/subspecies of LAB can be identified by means of colony morphology, physiological and biochemical characteristics, molecular biological analysis, and

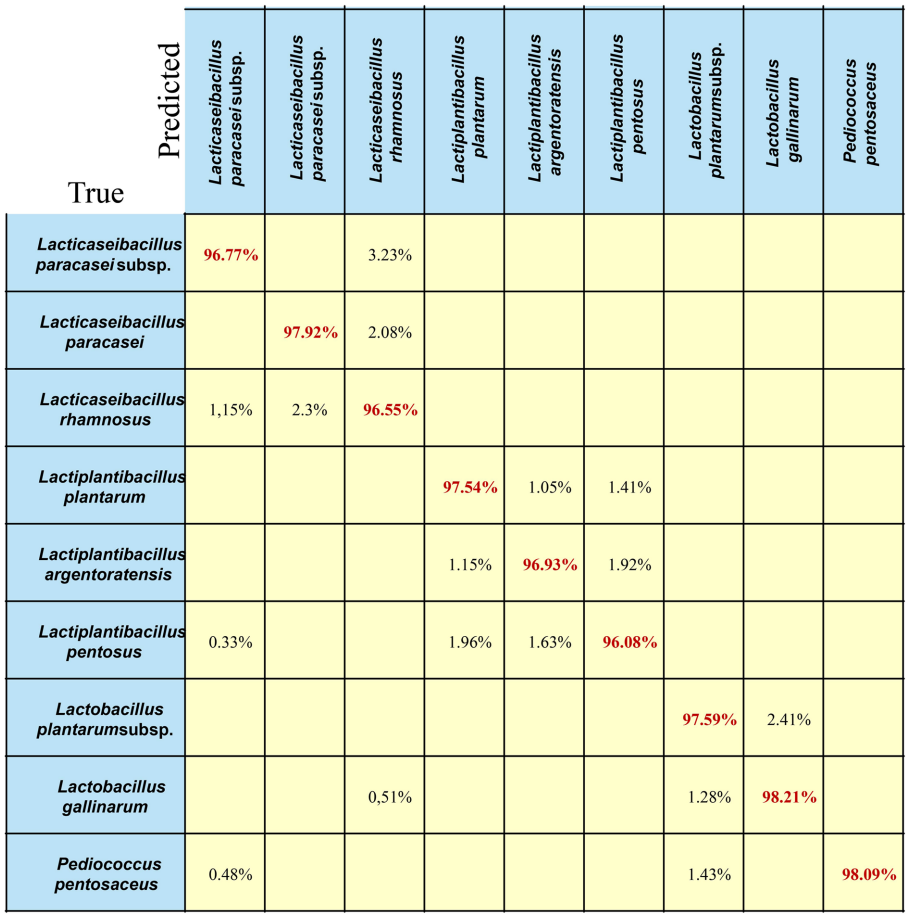

FIGURE 6
Confusion matrix of the EMC model for LAB species. Each row in the matrix represents an instance in the true class, and each column in the matrix represents an instance in the predicted class. The diagonal line represents the prediction accuracy of the EMC model on different bacterial strains. The average prediction accuracy of the EMC model is 97.3%.

MALDI-TOF-MS. Although the Ramanome has the potential for the rapid detection of bacterial pathogens and microalgae, little work has been done on LAB in both species and subspecies identification (He et al., 2019; Heidari et al., 2021). In addition, owing to the sophistication of Ramanome data, classical statistical methods are insufficient for spectral data analysis. Thus, Ramanome data with further EMC analysis is necessitated. The EMC in this study exhibited improved training performance metrics; the accuracy and sensitivity increased to 97%, while specificity and Kappa increased to 97 and 98%, respectively. Hence, the EMC of the best-performing base classifiers was built, which performed better than each base classifier. As expected, ensemble learning augmented the training performance for the classification of the nine LAB species, in which both accuracy and mean sensitivity increased to 97.3% and mean specificity increased to 97.54%.

Many previous studies have explored the possibility of combining the Ramanome technique with machine learning algorithms for the rapid detection of bacteria (Rodriguez et al., 2017; Lu et al., 2020; Liang et al., 2022; Rebrosova et al., 2022; Zhang et al., 2023). For example, Zhang et al. built a reference database of Ramanome from 21 pure-cultured probiotic strains that represent the standard statutory strains for human consumption (including 14 *Lactobacillus* spp., 6 *Bifidobacterium* spp., and 1 *Streptococcus* sp.), finding that the CNN classification algorithm showed that the best classification accuracy (93.02 ± 1.39%), indicating that Ramanome combined with machine learning is capable of discriminating probiotic bacteria (Zhang et al., 2023). However, little work on ensemble learning algorithms has been employed for SCRS analysis until recently, where Baladehi et al. combined EMC learning and Ramanome to rapidly identify and metabolically profile microalgal single cells and found that the accuracy of classifying species and states can be above 97%, further highlighting the promising potential of the technique for classification and identification of microorganisms (Heidari et al., 2021). It is interesting to note that in recent studies, the deep learning algorithm LSTM did not perform well as did CNN, and in some cases, it was not as good as classical algorithms, such as RF and KNN (Yu et al., 2021; Tang et al., 2022). However, in our study, the LSTM method was almost as accurate as the CNN method, with an accuracy of 92.07%. A recent study by Yu et al. (2021) showed that the LSTM model was faster and more accurate than the CNN model, achieving an average isolation level accuracy of more than 94%, which is worthy of further exploration (Yu et al., 2021).

In this study, we aimed to reveal the intrinsic differences between the Ramanomes of nine LAB species/subspecies using a novel EMC learning model. In addition, the prediction abilities of nine machine learning algorithms (one ensemble learning algorithm, EMC; two deep

learning algorithms, CNN and LSTM; and six classical machine learning algorithms, LDA, PLS-DA, XGBoost, KNN, RF, and SVM) were thoroughly compared. A comparison of the evaluation indicators (accuracy, sensitivity, specificity, and Kappa) for all the machine learning algorithms (Table 3) clearly showed that the performance of the EMC algorithm was the best (97.3%), which improved by 3–4% compared with the other two deep learning models, CNN and LSTM. The results indicate that when data complexity increases, the novel EMC learning algorithm displays better robustness than classical machine learning algorithms, which is consistent with a previous report (Heidari et al., 2021). Therefore, the EMC learning algorithm combined with the Ramanome can classify and predict LAB at the species/subspecies level with high accuracy and computational efficiency.

Altogether, this novel approach could significantly accelerate the identification of LAB species/subspecies, leading to the timely and accurate treatment of similar LAB species. However, this study had some limitations. Only nine LAB species/subspecies were included. This small number might affect the robustness of the model and cause data overfitting. Based on this pilot test, we plan to perform a more extensive study to include more LAB strains/species, as well as more microbial species, in the testing of the method. For example, in our next experiment, we mocked the microbiota by combining *Escherichia coli* and a mixture stock containing equal amounts of the nine LAB strains in different ratios (1:99, 10:90, 50:50, 95:5, 99:1). For each of the five mocking LAB samples, 10 randomized SCRS-based identifying experiments were performed using the EMC model. Then, the proportion of *Escherichia coli* in each reconstructed simulated community was observed. Finally, the reliability of the model to distinguish *Escherichia coli* from other LAB strains was evaluated. These initial results can be improved by adding more samples to the database to increase the robustness of the model, which is especially important for the EMC to avoid overfitting the data. Expanding the number of species and subspecies in the database would allow us to further evaluate the capacity of the Ramanome to identify LAB at the species/subspecies level or even the strain level. Other possibilities to improve classification rates include selecting key wavelengths associated with differences in species and subspecies and building multilayered models to assess the species and subspecies. Developing a panel for inclusivity and exclusivity studies would help estimate the sensitivity and specificity of the method. Parallel analysis of panel strains with a gold-standard diagnostic tool would also help assess the potential of this method as an alternative to conventional techniques (Treguier et al., 2019).

Despite the limitations mentioned above, the Ramanome has numerous advantages, proving its high potential as an *in situ* diagnostic tool. It is non-invasive and non-destructive; therefore, samples (cells) can be used for further or complementary testing. It has a broad spectrum of applications across various scientific fields for numerous *in situ* diagnostics purposes. Moreover, the method does not require expensive consumables. Furthermore, the sample preparation is easy and quick. Therefore, we believe that the Ramanome can become an assistive tool in the future, significantly improving the accuracy of LAB identification.

5 Conclusion

This study presents a highly effective approach for the identification of LAB species/subspecies using Ramanome combined with EMC at the single-cell level. We demonstrated the ability of our method to distinguish closely related LAB species and subspecies with a high degree of accuracy. Moreover, this is the first time that the EMC algorithm has been used to analyze LAB. This tool may be useful for further investigations into the identification of different microorganisms. Hopefully, the miniaturization and automation of the Raman instrument, accumulation of more Ramanome data for different LAB species/subspecies, and fast cloud data services will promote the utilization of this technique in various applications for accurate LAB identification.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

YR: Writing – original draft, Writing – review & editing. YZ: Investigation, Methodology, Writing – review & editing. XW: Investigation, Methodology, Writing – review & editing. SQ: Investigation, Methodology, Writing – review & editing. LS: Data curation, Methodology, Visualization, Writing – review & editing. CS: Data curation, Methodology, Visualization, Writing – review & editing. JD: Data curation, Methodology, Visualization, Writing – review & editing. YJ: Data curation, Methodology, Visualization, Writing – review & editing. GW: Data curation, Methodology, Visualization, Writing – review & editing. PZ: Conceptualization, Project administration, Supervision, Writing – review & editing. LC: Conceptualization, Project administration, Supervision, Writing – review & editing.

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Conflict of interest

CS, JD, YJ, and PZ were employed by the company Qingdao Single-Cell Biotechnology Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1361180/full#supplementary-material

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Metagenomics profiling of the microbial community and functional differences in solid-state fermentation vinegar starter (seed *Pei*) from different Chinese regions

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Introduction: The starter used in solid-state fermentation (SSF) vinegar, known as seed *Pei* is a microbial inoculant from the previous batch that is utilized during the acetic acid fermentation stage. The seed *Pei*, which has a notable impact on vinegar fermentation and flavor, is under-researched with comparative studies on microorganisms.

Methods: Herein metagenomics was employed to reveal the microbes and their potential metabolic functions of four seed *Pei* from three regions in China.

Results: The predominant microbial taxa in all four starters were bacteria, followed by viruses, eukaryotes, and archaea, with *Lactobacillus* sp. or *Acetobacter* sp. as main functional taxa. The seed *Pei* used in Shanxi aged vinegar (SAV) and Sichuan bran vinegar (SBV) exhibited a higher similarity in microbial composition and distribution of functional genes, while those used in two Zhenjiang aromatic vinegar (ZAV) differed significantly. Redundancy analysis (RDA) of physicochemical factors and microbial communities indicated that moisture content, pH, and reducing sugar content are significant factors influencing microbial distribution. Moreover, seven metagenome-assembled genomes (MAGs) that could potentially represent novel species were identified.

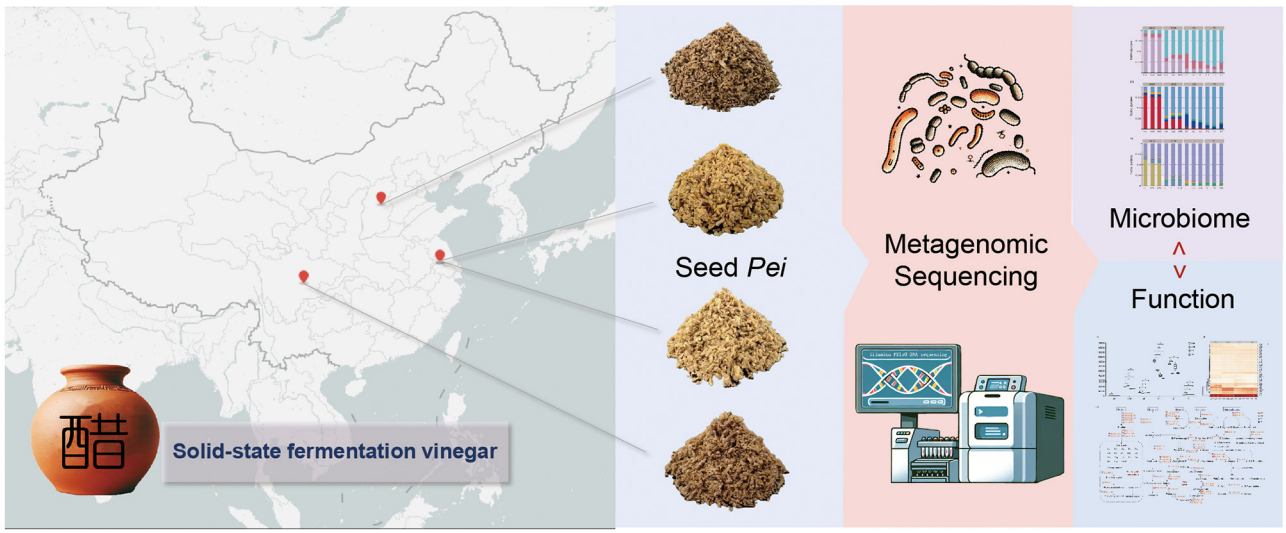
Conclusions: There are distinctions in the microbiome and functional genes among different seed *Pei*. The vinegar starters were rich in genes related to carbohydrate metabolism. This research provides a new perspective on formulating vinegar fermentation starters and developing commercial fermentation agents for vinegar production.

KEYWORDS

metagenomic features, microbial diversity and function, microbiome, seed *Pei*, solid-state fermentation vinegar

1 Introduction

Vinegar is an essential sour condiment in diets worldwide. Traditional vinegar brewing in China has predominantly favored solid-state fermentation (SSF) (Liu et al., 2004; Nie et al., 2017), by using grain-based substrates. This technique yields a richer flavor in vinegar than liquid-state fermentation, despite its controllability and struggles to match (Liu et al., 2018). Compared to the liquid-state fermentation of rice vinegar, SSF generally results in a higher production of compounds derived from microbial metabolism



GRAPHICAL ABSTRACT

(Li et al., 2015). Renowned Chinese vinegars such as Zhenjiang aromatic vinegar (ZAV), Shanxi aged vinegar (SAV), and Sichuan bran vinegar (SBV) are all produced by SSF, although their processes are uniquely tailored (Hu et al., 2023). These vinegars use an open fermentation process with grains (rice, sorghum, wheat bran, or malt) as the main raw materials (Ho et al., 2017). The process involves multiple stages of saccharification, alcohol fermentation, and acetic acid fermentation and typically takes nearly 1 month to complete. ZAV is derived from glutinous rice, while SAV is derived from sorghum (Xia et al., 2019); both are steamed and fermented in two distinct pools. In contrast, SBV, made from raw wheat bran (Zhu et al., 2021), is completely fermented in a single tank. In the entire process, most of the raw materials are not sterilized (Wu et al., 2012). *Daqu* is used as the starter for both saccharification and alcohol fermentation. During acetic acid fermentation, vinegar *Pei* from the previous batch is used as a starter (Chen et al., 2015; Nie et al., 2015; Wang et al., 2016). In traditional food fermentation processes, it is a common practice to inoculate fresh substrates with solid or semi-solid cultures from the previous batch to initiate fermentation or modulate the ecological niche. Thus, for vinegar brewing, vinegar *Pei* represents both an intermediate stage in the fermentation process and a starter. Due to the variability in raw materials and the fermentation volume of different vinegar types, the actual fermentation times of seed *Pei* are primarily chosen based on experience.

The microorganisms used in fermentation are key in conferring unique flavors and nutritional value to fermented foods, with the starter studies on microbial communities across different SSF vinegar starter cultures, ensuring a smooth start of the fermentation process (Li et al., 2015). Due to its unique process characteristics, the seed *Pei* represents a self-assembled and stable microbial community (Li et al., 2016b; Lu et al., 2018). The microbial community succession occurs during each batch of fermentation, resulting in the production of substances that impart flavor to vinegar through microbial metabolism (Wang et al., 2016). Although extensive studies have elucidated microbial succession and its correlation with the synthesis of flavor compounds during a particular vinegar fermentation (Li et al., 2016b, 2024; Huang et al., 2022), there is a lack of comparative studies on microbial communities across different SSF vinegar staters. Isolating and culturing microorganisms from these substrates pose significant challenges as they are difficult to grow on existing culture media (Milanović et al., 2018). Additionally, these bacterial colonies can exist in a viable but non-culturable (VBNC) state, which can sometimes affect the results of culture-dependent methods (Vegas et al., 2013). To overcome this limitation, culture-independent methods such as denaturing gradient gel electrophoresis (PCR-DGGE), amplicon sequencing, and metagenomics have been the mainstay methodologies in investigating microbial communities. Metagenomics, in particular, offers an unprecedented resolution by quantifying the microbial relative abundance to a more detailed taxonomy, elucidating functional gene landscapes, and potentially uncovering novel groups that have not been cultivated (Zhang et al., 2021). It has become an instrumental technology in decoding the microbial and functional landscapes of fermented foods such as vinegar, *Daqu*, and fermented vegetables (Li et al., 2016b; Yang et al., 2021; Liu et al., 2023).

In this study, we focus on the acetic acid fermentation of SSF vinegar, using metagenomic sequencing to gain an insight into the composition and functionality of microbiomes within seed *Pei*. Furthermore, our research aims to identify marker microorganisms that are indicative of the vinegar starter cultures for various types of vinegar. We collected vinegar *Pei* from three different regional products (ZAV, SAV, and SBV) originating from four separate enterprises. First, we used metagenomic analysis of the seed *Pei* to decode its microbial constituency and functional characteristics. Second, we measured some physicochemical properties of the seed *Pei* and conducted correlation analyses with microbial species. Finally, metagenome-assembled genomes (MAGs) originating from disparate grains were comparatively analyzed. The findings

are significant for elucidating regional variations in vinegar starter cultures and providing a knowledge base that could spearhead advancements in vinegar fermentation techniques.

2 Materials and methods

2.1 Sample collection

Each sample was produced by SSF during July to August 2023. ZAVa and ZAVb were collected from two vinegar manufacturers in Zhenjiang, Jiangsu Province, China (32.10 N 119.48 E, 32.12 N 119.46 E, respectively). They were both collected on the sixth day of fermentation. SAV was collected from Taiyuan, Shanxi Province, China (37.76 N 112.68 E) on the second day of fermentation. SBV was obtained from Meishan, Sichuan Province, China (30.20 N 103.81 E) on the tenth day of fermentation. ZAVa, ZAVb, and SBV were collected from the long fermentation tank. Each sample was taken from the top to the bottom from the front, center, and back of the tank and then mixed into one sample. SAV was produced in the fermentation vat, and the sample was taken from the center and the surrounding area, from the top to the bottom, and then mixed as one sample. Three replicates were taken from a mixture of three fermentation resources. Samples were placed in sterile bags and sealed and stored in a cooled environment. They were then transferred to the laboratory within 24 h for subsequent preservation at −80°C.

2.2 Physicochemical analysis

The water content was determined using the gravimetric approach, where vinegar *Pei* were subjected to desiccation at 105°C until they reached a stable mass. The water content was quantified by the proportion of dry to wet mass. The pH value, total acid, acetic acid, lactic acid, and reducing sugar content were analyzed as follows: 10 g seed *Pei* was mixed with 30 mL of deionized water and agitated at room temperature with a rotational speed of 100 rpm for 3 h. The filtrate was collected for measurement. The pH value was measured with a pH meter (Mettler-Toledo, FE28). The total acid content was gauged by neutralization titration using NaOH (Huang et al., 2022). The acetic acid and lactic acid analysis were carried out by the modifying method (Danova et al., 2023) using high-performance liquid chromatography (HPLC) Agilent 1260 Series (Agilent Technologies). Specifically, 2.5 mL of the filtrate was added with 1 mL of 106 g/L potassium ferricyanide solution and 1 mL of 300 g/L zinc sulfate solution sequentially. The mixture was left to sit for 1 h at room temperature. Before injection, the mixture was centrifuged and filtered through a 0.22-μm filter. The samples were analyzed on an Aminex HPX-87C Column (300×7.8 mm, Bio-Rad) held at 55°C, using 0.005 mol/L H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min. The reduced sugar content was determined through the application of the 3,5-dinitrosalicylic acid (DNS) assay (Huang et al., 2022). For the measurement of starch and total protein content, the seed *Pei* samples were first dried. The total protein content was determined using the spectrophotometric method according to the National Standards of the People's Republic of China GB/T 5009.5-2016 (Zhang et al., 2022). The starch content was determined according to the National Standards of the People's Republic of China GB/T 5009.9-2003, with specific procedures referenced from a previous study (Liu et al., 2020). All measurements were performed in triplicate.

2.3 DNA extraction, sequencing, and assembly

The DNA was extracted from *Pei* using the cetyltrimethylammonium bromide (CTAB) method, as previously reported (Huang et al., 2022). The quality and quantity of the extracted DNA were assessed by using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) and agarose gel electrophoresis. The qualified DNA (> 50 ng/mL, OD_{260}/OD_{280} 1.8∼2.2, OD_{260}/OD_{230} 1.8∼2.2) was sent to Beijing Novozymes Technology Co. for metagenomic sequencing. The genomic DNA was randomly fragmented into 350 bp using ultrasonic disruption. The sequencing libraries were prepared through processes including end repair, A-tailing, adapter ligation, and purification. Libraries were sequenced by using the Illumina PE150 platform (Illumina, USA). More than 10 Gb of raw data were obtained for each sample sequencing. Raw data were filtered by Readfq (https://github.com/cjfields/readfq) to obtain clean data. The reads with low-quality bases (quality ≤ 38), or N bases reaching 10 bp, or overlaps with adapters exceeding 15 bp were removed. Furthermore, the reads were aligned to plant genomes by Bowtie2 software to remove potential host contamination (Langmead et al., 2018). Sequence assembly was performed by MEGAHIT software (Li et al., 2016a) with clean data, and sequences with lengths >500 bp were retained.

2.4 Annotation of microbial species, functional genes, and metabolic pathways

Open reading frame (ORF) prediction of assembled contigs was done using MetaGeneMark (http://topaz.gatech.edu/GeneMark/). De-redundancy of predicted ORFs was performed by CD-HIT software (http://www.bioinformatics.org/cd-hit/). The non-redundant genes obtained through this process are referred to as unigenes. Bowtie2 (Langmead et al., 2018) was used to annotate the abundance of unigenes in reads of clean data. Unigenes were aligned to NCBI's NR database, KEGG database, and Carbohydrate-Active enZYmes (CAZy) database using DIAMOND software (Buchfink et al., 2015) to obtain taxonomic annotations and functional annotations. For taxonomic annotations, each sequence was determined by the LCA algorithm of MEGAN software (Huson et al., 2016) selected from the results with alignment e-value ≤ 10 × minimum e-value. Species abundance and functional gene abundance were determined based on the results of annotation and the abundance of unigenes, respectively.

2.5 Metagenomic assembled genome (MAG) assembly and taxonomy annotation

The clean data of three repetitions in each sample were mixed and assembled into contigs using the default parameters of MEGAHIT (Li et al., 2016a). Each sample sequencing result (~30 Gb of clean data in total) was divided into different bins using MetaBAT 2 (Kang et al., 2019). Each bin was evaluated for quality by CheckM (Parks et al., 2015). Bins with ≥ 80% integrity and ≤ 10% contamination were considered as high-quality MAGs. The taxonomy annotation of high-quality bins was performed using the Genome Taxonomy Database Toolkit (GTDB-Tk) (Chaumeil et al., 2020). If the MAGs were not assigned at the species level by GTDB-Tk, they were defined as novel species. MAGs were phylogenetically analyzed using GTDB-Tk to identify 122 archaeal and 120 bacterial marker genes, and the phylogenetic tree was constructed using IQ-TREE2 (Minh et al., 2020). The tvBOT tool was used for phylogenetic tree visualization (Xie et al., 2023).

2.6 Data analysis and visualization

The Venn diagram illustrating species composition was generated using the online platform available at http://www.ehbio.com/test/venn/#/ (Chen et al., 2021). Alpha diversity was assessed using the vegan package (version 2.5-2) in R software, which can be accessed at https://cran.r-project.org/web/packages/vegan/index.html. Statistical evaluation was conducted using SPSS software, version 23.0, through a two-tailed *t*-test, with significance determined at a $p < 0.05$. Principal coordinate analysis (PCoA) was performed based on Bray–Curtis dissimilarity at the species level, with plotting performed using the principal coordinate combinations that contributed the most variance. The visualization of KEGG functional genes distribution and clustering tree of different samples was done based on Bray–Curtis distance using R software. Differences across the taxonomic levels within seed *Pei* were investigated using the linear discriminant analysis (LDA) effect size (LEfSe) method (Segata et al., 2011). The correlation between microbial species in seed *Pei* was calculated using the iNAP web tool (Feng et al., 2022). The top 1,000 species in abundance were analyzed using Spearman's rank correlation. Only correlations with correlation coefficients $|r| > 0.8$ and $p < 0.01$ were used to construct the co-occurrence network. The co-occurrence network was visualized using Gephi software (version 0.10.1) (Bastian et al., 2009). As for CAZymes, quantitative differential graphs, heatmaps, and MAG quality distribution charts were generated using the online visualization website https://www.chiplot.online/. Redundancy analysis (RDA) was conducted using the online analysis tool available at http://www.cloud.biomicroclass.com/CloudPlatform/SoftPage/CCA.

3 Results

3.1 Metagenomic characterization of the four types of seed *Pei*

Sequencing using the Illumina PE150 platform generated a total of 134.14 Gb of raw sequences. After quality filtration procedures, the dataset was consolidated to 129.05 Gb of high-quality sequences without host data, averaging 32.26 Gb per sample. The ratio of valid data was 96.20%. Among the non-host clean data of all samples, the proportion of base quality greater than Q30 was 93.73%, and the G+C content was 41.21%. The sequencing information of individual samples is shown in Supplementary Table S1. After assembly, a total of 272,988 sequences (length > 500 bp) were obtained, with a sequence average length of 1,194 bp. A total of 176,618 non-redundant ORFs were predicted by MetaGeneMark.

An overview of the taxonomy of the four seed *Pei* samples shows that bacteria were the major taxa (94.48–97.81%, mean 95.94%), followed by viruses (0.35%), eukaryota (0.07%), archaea (0.003%), and others (3.64%). At the phylum level (Figure 1A), the ZAVa was more clearly annotated with only 6.63% of unclassified taxa. The dominant phylum is Bacillota (83.89%), followed by Pseudomonadota (8.90%). As for the other starters, the majority of genes were not annotated to an existing taxonomic phylum (59.41–80.45%). The most dominant phylum in ZAVb, SAV, and SBV was Bacillota or Pseudomonadota. The content of Bacillota in the two samples of ZAV was higher than that in SAV and SBV, while the content of Pseudomonadota in SAV and SBV was higher than that in ZAV. The archaea found in seed *Pei* were Euryarchaeota, "*Candidatus Korarchaeota*", "*Candidatus Bathyarchaeota*", "*Candidatus Thorarchaeota*", and "*Candidatus Woesearchaeota*". Eukaryota in seed *Pei* samples included Ascomycota, Basidiomycota, Mucoromycota, Microsporidia, and Zoopagomycota. Uroviricota, Preplasmiviricota, Nucleocytoviricota, Cossaviricota, Artverviricota, and Pisuviricota were viral phyla found in seed *Pei*.

The microbiological distribution at the genus level (Figure 1B) showed that a total of 598 genera were detected. The dominant genera (abundance > 1%) in ZAVa were *Lactobacillus* (77.79%), *Acetobacter* (6.5%), and *Limosilactobacillus* (3.6%). The dominant genera (abundance > 1%) in ZAVb major genera were *Lactobacillus* (22.33%), *Acetobacter* (5.94%), and *Limosilactobacillus* (2.82%), which differed from ZAVa by also containing *Acetilactobacillus* (4.28%). The dominant genera (> 1% abundance) of SAV and SBV were similarly composed of *Acetobacter* (19.78%, 7.45%), *Acetilactobacillus* (4.71%, 5.8%), and *Lactobacillus* (4.4%, 2.27%).

At the species level (Figure 1C), we detected a total of 1,839 species. The main species (abundance > 1%) in four samples were either *Acetobacillus* sp. or *Lactobacillus* sp. The dominant species in ZAVa were *Lactobacillus acetotolerans* (57. 07%), *Lactobacillus helveticus* (6.95%), *Lactobacillus amylovorus* (2.43%), and *Limosilactobacillus pontis* (1.06%). There dominant species in ZAVb were *Lactobacillus helveticus* (11. 93%), *Acetilactobacillus jinshanensis* (4.28%), *Lactobacillus amylovorus* (2.29%), and *Limosilactobacillus pontis* (1.25%), with the relative abundances exceeding 1%. The dominant species in SAV were *Acetilactobacillus jinshanensis* (4.71%), *Acetobacter pasteurianus* (2.7%), and *Lactobacillus acetotolerans* (2.2%), with a relative abundance above 1%. In SBV, the dominant species were *Acetilactobacillus jinshanensis* (5.8%) and *Lactobacillus acetotolerans* (2.03%). The four starters differed in the composition of the dominant species. More information on the top 60 annotated species is shown in Supplementary Figure S1.

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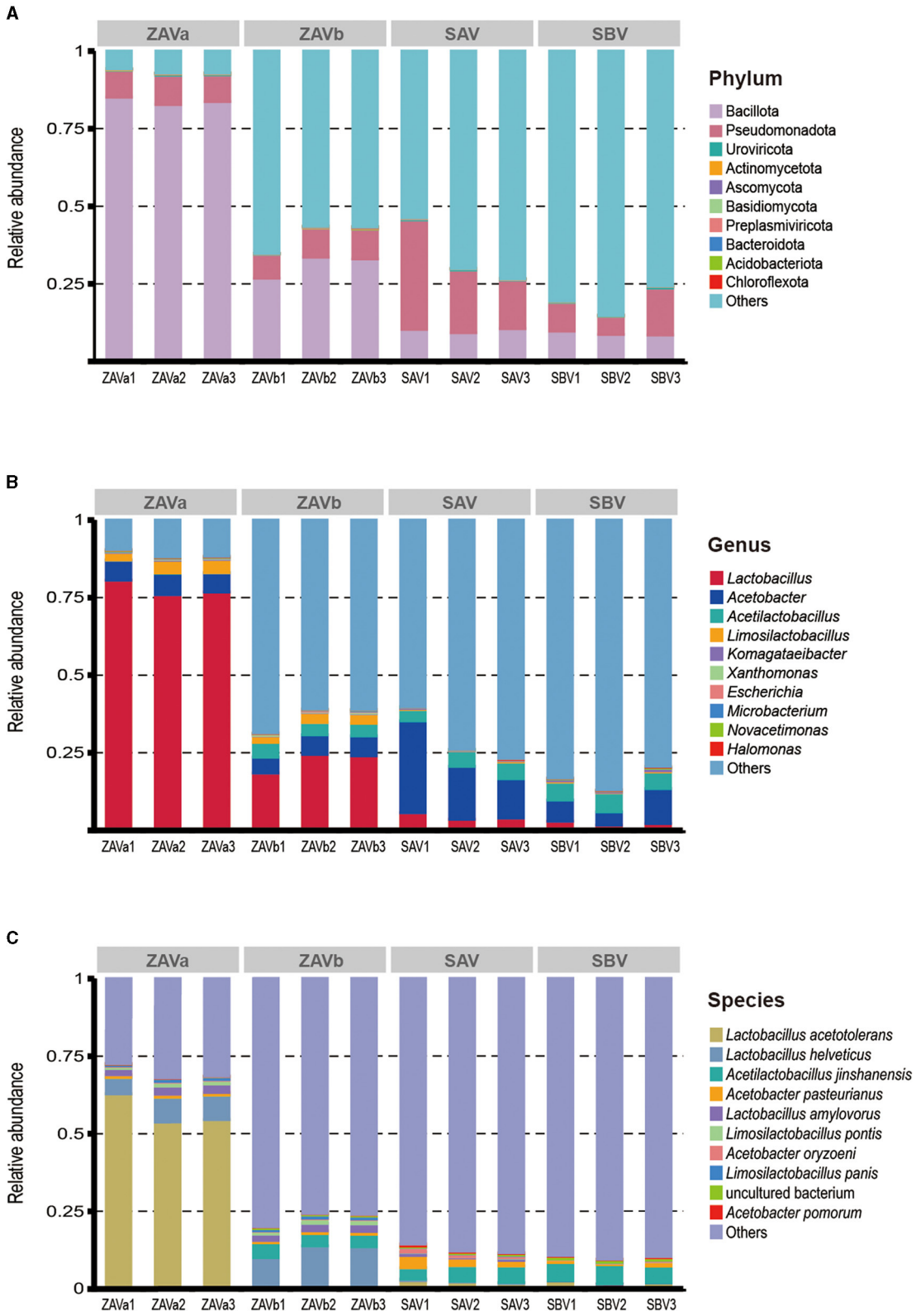

FIGURE 1
Relative abundance of microorganisms in four groups of seed *Pei* at the phylum **(A)**, genus **(B)**, and species **(C)** levels. Group: ZAVa and ZAVb were seed *Pei* samples from two different manufacturers of Zhenjiang aromatic vinegar (Zhenjiang, China); SAV was seed *Pei* from Shanxi aged vinegar (Taiyuan, China); SBV was seed *Pei* from Sichuan bran vinegar (Meishan, China).

The composition of the main acid-producing microbial species, *Lactobacillus,* was found to be 54 species (abundance: 67.63%), followed by 48 (15.25%), 45 (3.33%), and 32 (2.08%) in ZAVa, ZAVb, SAV, and SBV, respectively. *Limosilactobacillus* comprised 28 (3.21%), 27 (2.61%), 27 (0.26%), and 32 species (0.26%). *Acetobacter* consisted of 43 (1.93%), 43 (1.79%), 40 (5.56%), and 42 species (2.41%). It is evident that acid-producing species are highly diverse in seed *Pei*.

3.2 Differences in microbial communities among the four types of seed *Pei*

The Venn diagram (Figure 2A) illustrates the distribution of the four samples in terms of species diversity: ZAVa and ZAVb had a higher number of species, 1,433 and 1,413, respectively, followed by SAV (1,085), and SBV contained the lowest number of species (933). Out of the total 1,839 species, 598 species could be detected in all four types of seed *Pei*. Each *Pei* contained their unique species. ZAVa had the highest number of unique species (115), while SBV had the lowest number of unique species (70).

To obtain an overview of the microbial diversity and richness in the seed *Pei*, we calculated the alpha diversity index (ACE, Chao1, Shannon, and Simpson) (Supplementary Table S2). Further significance analysis ($p < 0.05$) revealed that the ACE and Chao1 indices of ZAVa and ZAVb were significantly higher than those of SBV and SAV. The Shannon and Simpson indices of ZAVa were the highest, followed by those of ZAVb, while the Shannon and Simpson indices of SBV and SAV were the lowest. It can be seen that the diversity and richness of microorganisms in ZAVa and ZAVb species were higher than those in SBV and SAV. The ZAVa species also exhibited the highest degree of dominance.

Beta diversity analysis, specifically the principal coordinates analysis (PCoA), revealed elucidated microbial composition variations among the four seed *Pei* samples (Figure 2B). Principal component 1 accounted for 94.96% of the total community variations, representing the primary explanatory axis, while principal component 2 explained an additional 5.47% variation. The SAV and SBV groups were positioned in close proximity, delineating a separation from the ZAVa and ZAVb groups. This result indicates that, despite differing geographical origins, the seed *Pei* samples from the SAV and SBV groups harbor similar microbial communities. Conversely, the ZAVa and ZAVb groups, although geographically closer, exhibit distinct microbial compositions from each other and also differ from the SAV and SBV groups. Clustering tree analysis of microbiome species based on Bray–Curtis distance also revealed similar sample relationships (Supplementary Figure S2).

LEfSe analysis (Figures 2C, D) revealed the taxon with LDA values > 3.5, indicating the distinctive taxon of seed *Pei*. The indicated species of SBV was an uncultured bacterium, which was significantly different from the species to the kingdom level. There were two distinctive species of SAV: *Acetilactobacillus jinshanensis* and *Enterobacter* sp. BIDMC110. The indicated species of ZAVb is *Vibrio fluvialis*. ZAVa has the highest number of significantly different species, with three different phyla including unclassified viruses, namely, *Preplasmiviricota*, *Pseudomonadota*, and *Bacillota*. There were 11 different genera in ZAVa: *Acetobacter*, *Xanthomonas*, *Gammatectivirus*, *Microbacterium*, *Proteus*, *Limosilactobacillus*, *Lacticaseibacillus*, *Lentilactobacillus*, *Lactobacillus*, *Leuconostoc*, and *Lactococcus*. At the species level, there were 48 different species, mainly the *Acetobacter* sp. (8), *Limosilactobacillus* sp. (11), and *Lactobacillus* sp. (10).

The microbial co-occurrence network analysis revealed that only six species exhibited specific correlations among different seed *Pei* samples (Supplementary Figure S3). *Acetobacter pasteurianus* showed negative correlations with *Lactobacillus helveticus* and *Limosilactobacillus pontis*, whereas *Lactobacillus amylovorus* exhibited negative correlations with *Acetilactobacillus jinshanensis* and other species. *Acetilactobacillus jinshanensis* displayed positive correlations with other species. These species represent the major functional groups responsible for acid production during vinegar fermentation, and they are primarily negatively correlated.

3.3 Investigation of metagenomic function in carbohydrate metabolism and main metabolic pathways of four types of seed *Pei*

Among KEGG gene annotations, carbohydrate metabolism genes were the most abundant in different functional categories (Supplementary Figure S4). The number of genes annotated to the CAZy database accounted for 6.61% of the total number of unigenes. The total number of genes encoding CAZymes in enzyme classes of glycoside hydrolases (GH), glycosyl transferases (GT), carbohydrate-binding modules (CBM), carbohydrate esterases (CE), polysaccharide lyases (PL), and auxiliary activities (AA) was 5,082, 4,810, 1,194, 365, 116, and 107, respectively. The most prevalent identified gene clusters were the GH > GT > CMB > CE > PL > AA families. The differences in the number of the six classes of CAZymes in SAV and SBV were not significant (Figure 3A). There were significant differences in the CAZymes classes between ZAVa and the other samples. Specifically, the relative content of GH, GT, and CE was significantly high, while the relative content of AA and PL was low in all four samples. Additionally, the number of genes in the CBM family in ZAVa was lower than that in ZAVb but higher than that in SAV and SBV.

The heatmap (Figure 3B) of the carbohydrate-active enzyme classes showed that GT2 and GT4 were most prevalent in seed *Pei*. These genes are involved in the formation of polysaccharides in the cell walls of various organisms (Howe et al., 2016) as well as in cellulose synthesis (Stone et al., 2010). The next highest content was of GH13, also known as the α-amylase family, which is one of the largest GH families in CAZy. It is the predominant functional enzyme in the hydrolysis of starch feedstocks (Møller et al., 2016). The content of the GH13 family varied in the four seed *Pei* samples, with the highest content found in ZAVb, followed by SBV. GH23, a peptidoglycan lyase of bacterial and phage origin, and GH1, a peptidoglycan lyase containing a variety of β-glycosidases, were found in higher concentrations in the ZAVa samples than in the other samples. The CBM family with the highest concentration in seed *Pei* was CBM50, which consists of modules that bind to peptidoglycan and chitin (Andrade et al., 2017). The PL family

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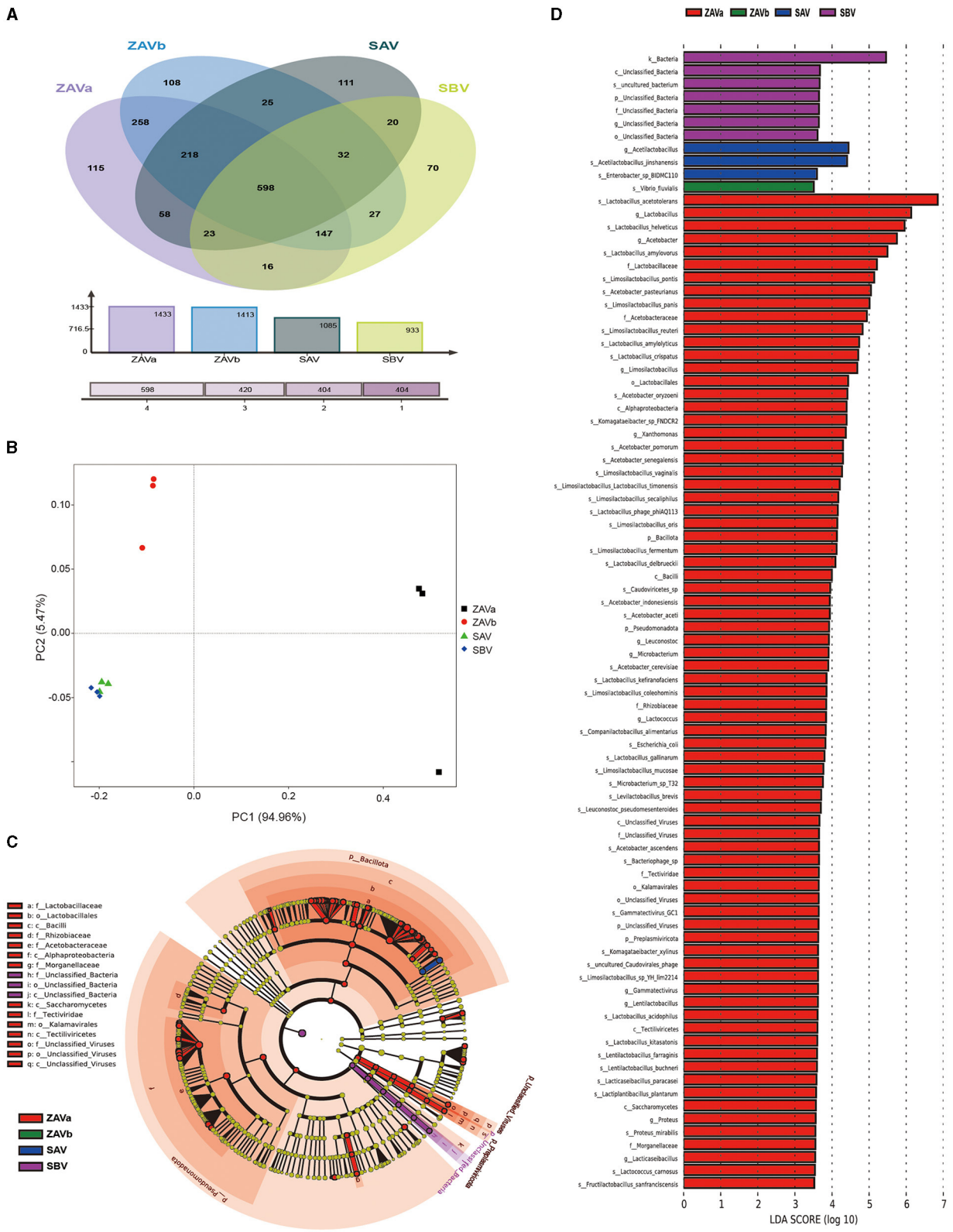

FIGURE 2
Venn diagram of the distribution of species in four groups of seed *Pei* **(A)**; PCoA plot of the distribution of species in four groups of seed *Pei* **(B)**; differential biomarker analysis in four groups of seed *Pei* by linear discriminant analysis effect size (LEfSe), microbiota cladogram **(C)**; LDA effect size analysis **(D)**.

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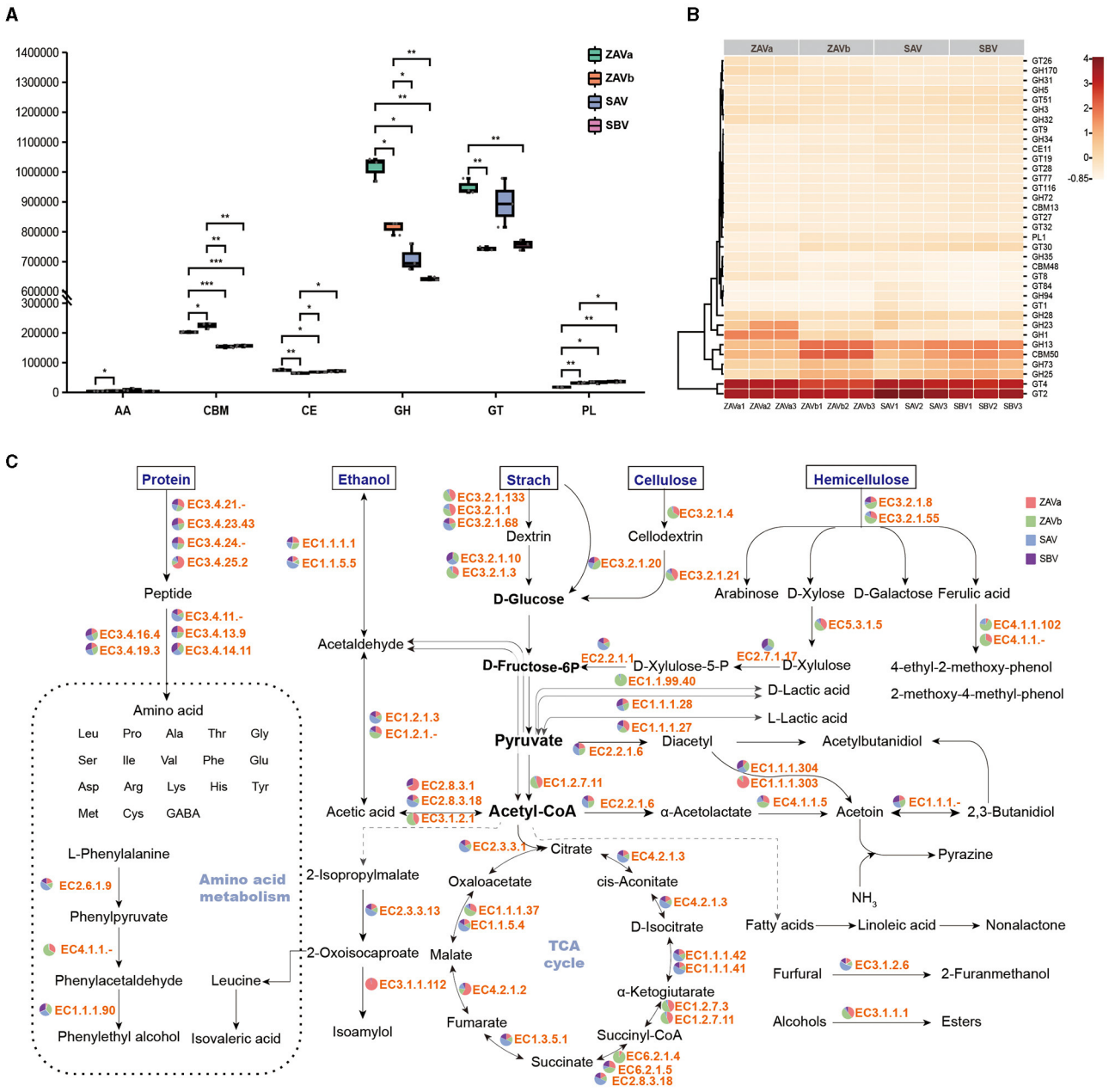

FIGURE 3
Comparative analysis of carbohydrate active enzyme (CAZy) with different composition and diversity in seed *Pei*. Abundance of *six* CAZy families in four groups of seed *Pei* **(A)**; heatmap plot depicting CAZy family abundance in each sample **(B)**; metabolic networks of major flavor substances in SSF vinegar and KEGG-based enzyme abundance **(C)**. Significant differences are presented by ***$p < 0.001$, **$0.001 \leq p < 0.01$, and *$0.01 \leq p < 0.05$.

with the highest concentration was PL1, but the PL family was not prevalent in seed *Pei*. PLs use an elimination mechanism to cleave glycosidic bonds of complex carbohydrates, and PL1 mainly acts in the hydrolysis of pectin. From the analysis of carbohydrate-active enzymes, it can be seen that different sources of seed *Pei* have different carbohydrate enzymes to adapt to different carbohydrate raw materials.

We constructed a metabolic network of the main flavor-producing species in vinegar and compared the proportional relationships of functional genes in the pathway of flavor compound formation across different sample groups (Figure 3C, Supplementary Table S3). Through intergroup comparisons, significant disparities were observed in the absolute levels of genes encoding starch hydrolases, cellulose hydrolases, and hemicellulose hydrolases within the diverse seed *Pei* samples. Notably, the ZAVb sample exhibited the highest expression of these three enzyme genes, indicating a superior capability for efficient grain substrate utilization. This is achieved by effectively converting starch, cellulose, and hemicellulose into pentose and hexose sugars, which serve as a readily accessible carbon source for a wide range of microorganisms. When it comes to the hydrolysis of proteins into free amino acids, the majority of enzyme genes demonstrated

comparable distributions across the different seed *Pei* samples, except for the increased levels of EC 3.4.14.11 in ZAVb and SBV samples, the elevated prevalence of EC 3.4.25.2 in ZAVa, and the highest content of EC 3.4.11 found in SAV. Functionally, EC 3.4.14.11 corresponds to Xaa-Pro dipeptidyl-peptidase, EC 3.4.25.2 corresponds to ATP-dependent peptidase, and EC 3.4.11. encompasses aminopeptidases.

3.4 Correlation between the microbiota and physicochemical properties

The water content, reducing sugar content, pH value, total acid content, acetic acid content, lactic acid content, total starch content, and total protein content of the *Pei* were measured (Figures 4A–H). There were significant differences ($p < 0.05$ in all cases) in the physicochemical properties of four seed *Pei* samples, with protein content showing the least variation among the aforementioned physicochemical parameters. ZAVa had the highest moisture content, lowest pH, lowest total acid, lowest acetic acid, and lowest starch content and also contained the highest reducing sugar content. ZAVb and SAV did not show significant differences in moisture, total acid, starch, and protein content but differed in reducing sugar, acetic acid, lactic acid content, and pH. SBV had the lowest moisture, the highest total acid, and the highest lactic acid content. Moreover, we conducted RDA on the relationship between the physicochemical properties and microbial communities at the species level (abundance > 0.1%) (Figure 4I). We found that three out of the eight environmental factors (Aw, pH, and reducing sugar) explained 99.89% of the variation in the microbial community structure. Among these factors, the reducing sugar content had the greatest impact on the microbial community structure, followed by pH value and then moisture content. It is noteworthy that the reduction in sugar content had a smaller impact on the ZAVb community compared to other types of *Pei*. In contrast, water content had the most significant effect on the ZAVa community, while pH value had the greatest impact on the ZAVb community. The RDA revealed that two species of lactic acid bacteria showed the most significant response to the environmental factors. Specifically, *Lactobacillus acetotolerans* was greatly influenced by Aw and reducing sugar content, while *Lactobacillus helveticus* was more sensitive to Aw and pH value.

3.5 MAGs in seed *Pei*

A total of 98 MAGs were identified from the metagenomes of individual groups with 27 MAGs in ZAVa, 36 MAGs in ZAVb, 21 MAGs in SAV, and 14 MAGs in SBV. The MAGs obtained from the bins were checked for genomic quality, and 22 high-quality MAGs (≥ 80% completeness and ≤ 10% contamination) were selected for subsequent analysis (Figure 5A). A total of 22 high-quality MAGs (ZAVa 10, ZAVb 4, SAV 4, and SBV 4) were taxonomically assigned to two phyla, three classes, five orders, six families, and nine genera. The results showed *Limosilactobacillus* had the highest number of MAGs in the high-quality MAGs dataset for all samples, followed by *Lactococcus* (Figure 5B). A total of seven MAGs were considered potentially novel species.

4 Discussion

The acetic acid fermentation process in SSF of vinegar mainly involves the microbial conversion of ethanol, bran, and other raw materials into acetic acid, lactic acid, and other flavor compounds. In this process, the fermentation starter is one of the main sources of microorganisms (Huang et al., 2022) that significantly influences the fermentation process, the extent of raw material utilization, and the flavor of the final product. Studying the microbial community of vinegar fermentation starters in different regions helps us understand the fundamental reasons for variations in fermentation and flavor and provides insights for constructing artificial fermentation starters.

This study represents the first comparative analysis of microbial species composition, functional genes, and metabolic pathways in different SSF seed *Pei* samples. A total of 598 genera and 1,893 species were identified, surpassing the number of species and genera obtained through amplicon sequencing. Bacteria (> 90%) were found to be the dominant microbial group in the seed *Pei*, consistent with previous research (Wu et al., 2017; Li et al., 2023), indicating their significant contribution during acetic acid fermentation. Compared to previous metagenomic studies (Wu et al., 2017), this study elucidated the annotation of viral groups, revealing their presence in the seed *Pei* and higher abundance compared to eukaryotes and archaea. In a specialized virome study of the fermentation process of ZAV, it was found that viruses are rich in carbohydrate metabolism genes that may be involved in the bacterial fermentation process (Yu et al., 2022). This finding suggests that the presence of viruses (bacteriophages) may have an impact on vinegar fermentation. The samples ZAVb, SAV, and SBV also contained numerous unclassified groups, indicating the presence of "microbial dark matter" that has not yet been isolated, cultured, or characterized genomically. Further research on these "dark matter" organisms is essential to unravel the fermentation mechanisms of vinegar. While the main microbial species composition of the seed *Pei* observed in this study aligns with previous findings (Nie et al., 2017; Huang et al., 2022; Li et al., 2023, 2024), consisting primarily of acetic acid bacteria and lactic acid bacteria such as *Lactobacillus*, *Acetobacter*, *Limosilactobacillus*, and *Acetilactobacillus*, the specific species composition and abundance vary among different regions.

A previous study identified the genera of *Acetobacter*, *Lactobacillus*, *Enhydrobacter*, *Lactococcus*, *Gluconacetobacter*, *Bacillus*, and *Staphylococcus* as crucial microbiota contributing significantly to the synthesis of flavor components of ZAV. Their prevalence and functional roles were outlined based on amplicon sequencing (Wang et al., 2016). However, in the four seed *Pei* samples we collected, *Enhydrobacter* and *Gluconacetobacter* were not detected, while *Bacillus* (> 0.005%), *Staphylococcus* (> 0.02%), and *Lactococcus* (> 0.1%) were found to be present but in low abundance. Another study identified 11 core functional microorganisms in Sichuan Shai vinegar using PacBio sequencing combined with the traditional culture method, namely *Brettanomyces bruxellensis*, *Pichia kudriavzevii*, *Acetobacter*

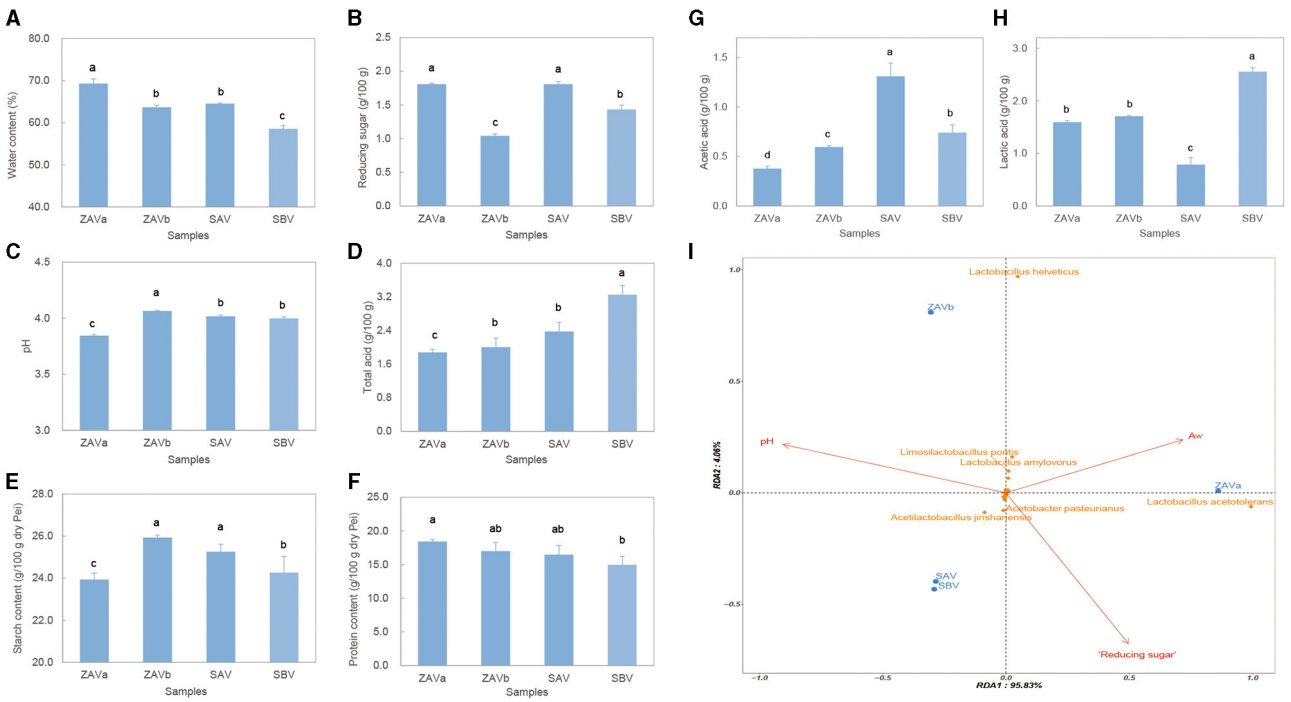

FIGURE 4
Physicochemical properties of seed *Pei*, water content **(A)**; reducing sugar content **(B)**, pH **(C)**; total acid content **(D)**; total starch content of dry *Pei* **(E)**; total protein content of dry *Pei* **(F)**; acetic acid content **(G)**; lactic acid content **(H)**; RDA of main microorganisms and physicochemical properties for different seed *Pei* **(I)**. Columns in **(A–H)** relied on the replicates of three experiments, columns with different superscript letters (a–d) are significantly different ($p < 0.05$).

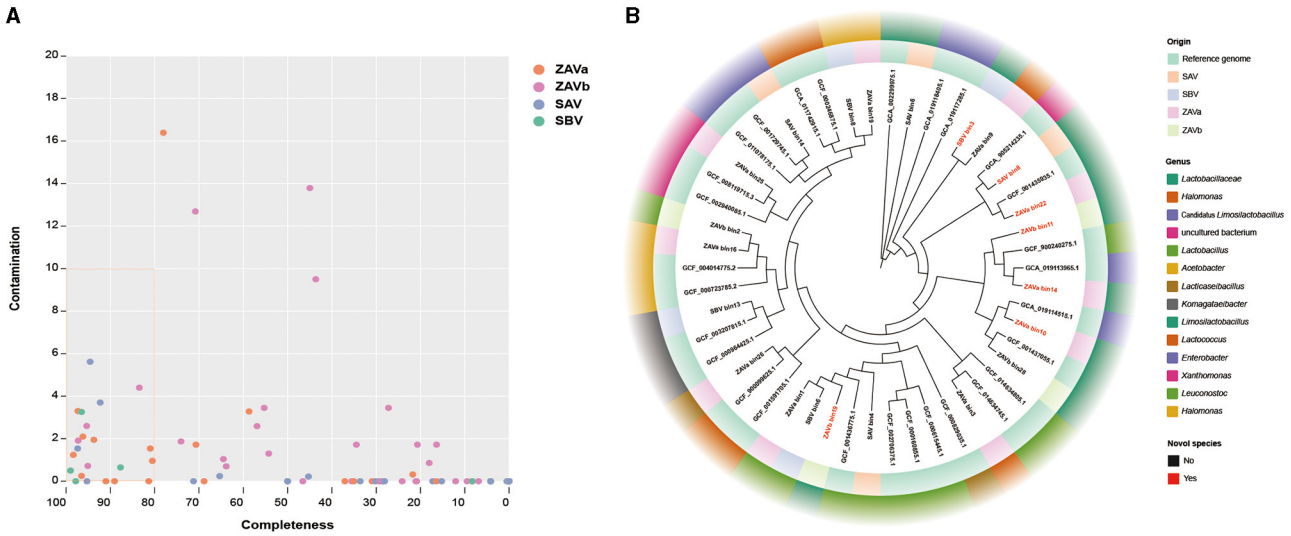

FIGURE 5
The assembly and analysis of metagenome-assembly genomes (MAGs) of four groups of seed *Pei*. MAG quality assessment by CheckM **(A)**. Points within the orange boxed line represent high-quality MAGs (≥ 80% completeness and ≤ 10% contamination). The phylogenetic tree based on 120 bacterial marker genes of high-quality MAGs and reference genomes **(B)**.

pasteurianus, *Acetobacter pomorums*, *Lactobacillus acetotolerans*, *Lactobacillus amylolyticus*, *Lactobacillus amylovorus*, *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Clostridium beijerinckii*, and *Lichtheimia ramose* (Li et al., 2024). The core functional microorganisms identified in this study were also detected in our seed *Pei*, and the effects of the acid-producing microorganisms were consistent with our results. However, *Brettanomyces bruxellensis*, *Pichia kudriavzevii*, *Clostridium beijerinckii*, and *Lichtheimia ramose* were present in low abundance. According to previous studies, some low-abundance taxa in the seed *Pei* were

associated with certain flavor compounds in vinegar. For example, *Bacillus amyloliquefaciens* was found to enhance acetoin and tetramethylpyrazine contents when added to *Daqu* (Zhang et al., 2017). Additionally, *Pantoea*, *Pediococcus*, and *Rhizobium* were found to be associated with esters, particularly propanoic acid-2-hydroxy-ethyl ester, hexanoic acid ethyl ester, and ethyl acetate in SAV (Zhu et al., 2018). Theoretically, these low-abundance taxa may play essential roles in vinegar fermentation, but their specific functions and mechanisms require further investigation.

Furthermore, through LEfSe analysis, we identified characteristic species for each vinegar starter. ZAVa was found to harbor the highest number of characteristic species, including both core and non-core species. Two of the seed *Pei* pairs share similar raw materials, production processes, and geographical proximity. However, compared to seed *Pei* with different raw materials, production processes, and geographical distributions, the differences in microbial community structure and functionality are more noticeable within these pairs (as evidenced by PCoA). Conversely, the other two seed *Pei* samples, with distinct raw materials, processes, and geographical distributions, exhibit more similar species compositions. Therefore, we speculate that the long-term cultivation and preservation practices of unique microbial strains by individual manufacturers are the main factors contributing to microbial community differences in the SSF of vinegar.

In the co-occurrence network analysis, due to significant microbial community variations among the seed *Pei* samples, we only observed correlations between five core microorganisms involved in vinegar fermentation. This finding indicates a negative correlation between the key acid-producing microorganisms, such as *Acetobacter* and *Lactobacillus*, which aligns with previous studies on vinegar microbiota (Hutchinson et al., 2019; Chai et al., 2020; Xia et al., 2022). Furthermore, our review of previous research reveals a consistent trend of competitive dynamics between *Acetobacter* and *Lactobacillus* in different vinegar production processes (Wang et al., 2016; Nie et al., 2017; Huang et al., 2022; Li et al., 2023). In some instances, the abundance of *Acetobacter* decreased with the fermentation process and the abundance of *Lactobacillus* gradually increased (Huang et al., 2022), while in other cases, the opposite scenario is observed (Wang et al., 2016; Nie et al., 2017; Li et al., 2023). Therefore, the distinct composition and ratio of *Acetobacter* and *Lactobacillus* in fermentation starters can support successful vinegar fermentation. However, the specific impacts of these differences on the vinegar fermentation process and resulting flavors require further investigation. The observed negative correlations among the five microorganism groups may be attributed to interspecies competition or environmental factors driving species succession.

Functionality-wise, the most abundant genes were the GH and GT family of carbohydrate metabolism. These abundant genes have also been observed in fermented vegetables and dairy products (Liang et al., 2023; Liu et al., 2023). When compared to other seed *Pei* samples, ZAVa displayed the highest abundance of GH1 genes associated with cellulose degradation, indicating that it may be more effective in breaking down the cellulosic component of lignocellulose. ZAVb exhibited a higher abundance of GH13 family genes associated with starch hydrolysis, suggesting a possible benefit in raw material usage. The gene abundance of GT2 and GT4 was highest in SAV. These findings suggest that the seed *Pei* has a wide variety of carbohydrate-active enzymes that are associated with the rapid utilization of various carbohydrates to start fermentation and provide unique flavor characteristics. Based on KEGG annotation of the main fermentation pathways, the distribution of genes involved in acetic acid production was relatively balanced among the four starters. Notably, ZAVb possessed a higher abundance of genes involved in the decomposition of macromolecular substrates such as starch, cellulose, and hemicellulose. Furthermore, throughout this study, different seed *Pei* samples exhibited variations in the primary enzyme systems responsible for protein degradation during vinegar fermentation.

The RDA was conducted to reveal the correlations between physicochemical factors and microbial distribution in the seed *Pei*. The factors such as water content, reduced sugar content, and pH value were found to have a significant impact on the distribution of key microbial groups. However, further experimental verification is required to determine the actual effects. This result suggests that controlling water content, pH, and reducing sugar content is crucial for ensuring successful vinegar fermentation.

5 Conclusion

In this study, we focused on investigating the microbial distribution and functionality of seed *Pei*, which serve as fermentation starters obtained from four manufacturers in three provinces. The dominant microbial group in the seed *Pei*, according to metagenomic sequencing, was bacteria, followed by viruses, eukaryotes, and archaea. Variations in microbial communities were observed among seed *Pei* from different manufacturers, with *Lactobacillus* sp. and *Acetobacter* sp. being the main functional groups. However, specific species composition and abundance varied. Seed *Pei* from SAV and SBV showed a higher similarity in microbial composition, while significant differences were observed when compared to the two forms of ZAV. The distribution of functional genes revealed a similar pattern to the species composition, with SAV and SBV exhibiting more similarity in functional gene composition than the two forms of ZAV. These findings suggest that the differences in microbial composition and functionality among seed *Pei* are not only geographically driven but are also related to the long-term cultivation and preservation practices of microbial strains by different manufacturers. Additionally, RDA of physicochemical factors and microbial communities revealed that water content, pH, and reducing sugar content are significant factors influencing microbial distribution. Furthermore, we also identified seven MAGs that could potentially represent novel species. This study contributes to our understanding of the properties and functionalities of fermentation starters used in SSF vinegar fermentation, providing insights into the utilization or modification of microbial compositions to optimize vinegar fermentation processes.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://db.cngb.org/cnsa/, CNP0005244.

Author contributions

DH: Conceptualization, Funding acquisition, Writing – original draft. YYa: Data curation, Methodology, Writing – original draft. ZG: Data curation, Formal analysis, Writing – original draft. KC: Data curation, Formal analysis, Writing – original draft. SD: Formal analysis, Visualization, Writing – original draft. YZ: Investigation, Methodology, Writing – original draft. YW: Writing – review & editing. ZY: Visualization, Writing – original draft. KW: Writing – review & editing. PL: Software, Writing – original draft. CR: Writing – review & editing. YYu: Funding acquisition, Project administration, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1389737/full#supplementary-material

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Formic acid enhances whole-plant mulberry silage fermentation by boosting lactic acid production and inhibiting harmful bacteria

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Mulberry has also been regarded as a valuable source of forage for ruminants. This study was developed to investigate the impact of four additives and combinations thereof on fermentation quality and bacterial communities associated with whole-plant mulberry silage. Control fresh material (FM) was left untreated, while other groups were treated with glucose (G, 20 g/kg FM), a mixture of *Lactobacillus plantarum* and *L. buchneri* (L, 10^6 CFU/g FM), formic acid (A, 5 mL/kg FM), salts including sodium benzoate and potassium sorbate (S, 1.5 g/kg FM), a combination of G and L (GL), a combination of G and A (GA), or a combination of G and S (GS), followed by ensiling for 90 days. Dry matter content in the A, S, GA, and GS groups was elevated relative to the other groups ($p < 0.01$). Relative to the C group, all additives and combinations thereof were associated with reductions in pH and NH_3-N content ($p < 0.01$). The A groups exhibited the lowest pH and NH_3-N content at 4.23 and 3.27 g/kg DM, respectively ($p < 0.01$), whereas the C groups demonstrated the highest values at 4.43 and 4.44 g/kg DM, respectively ($p < 0.01$). The highest levels of lactic acid were observed in the GA and A groups (70.99 and 69.14 g/kg DM, respectively; $p < 0.01$), followed by the GL, L, and GS groups (66.88, 64.17 and 63.68 g/kg DM, respectively), with all of these values being higher than those for the C group (53.27 g/kg DM; $p < 0.01$). *Lactobacillus* were the predominant bacteria associated with each of these samples, but the overall composition of the bacterial community was significantly impacted by different additives. For example, *Lactobacillus* levels were higher in the G, A, and GA groups ($p < 0.01$), while those of *Weissella* levels were raised in the L, GL, and GS groups ($p < 0.01$), *Pediococcus* levels were higher in the A and GA groups ($p < 0.01$), *Enterococcus* levels were higher in the G and S groups ($p < 0.01$), and *Lactococcus* levels were raised in the S group ($p < 0.01$). Relative to the C group, a reduction in the levels of undesirable *Enterobacter* was evident in all groups treated with additives ($p < 0.01$), with the greatest reductions being evident in the A, S, GA, and GS groups. The additives utilized in this study can thus improve the quality of whole-plant mulberry silage to varying extents through the modification of the associated bacterial community, with A and GA addition achieving the most efficient reductions in pH together with increases in lactic acid content and the suppression of undesirable bacterial growth.

KEYWORDS

mulberry, silage, glucose, formic acid, lactic acid bacteria, salts, bacterial community

1 Introduction

Mulberry (*Morus alba L.*) is a woody perennial plant belonging to the Moraceae family that is highly adaptable and widely distributed throughout China. Parts of the mulberry plant, particularly the leaves, are frequently used in the practices of traditional Chinese medicine as they are rich in phenols, polysaccharides, alkaloids, and other phytochemicals such that they possess an array of anti-inflammatory, antioxidant, antidiabetic, antihelmintic, and antimicrobial properties (He et al., 2018; Zhang et al., 2018; Shan et al., 2022). Mulberry has also been regarded as a valuable source of forage for ruminants, as it exhibits high protein content, contains high levels of vitamins and minerals, and presents with a high biomass yield (Thaipitakwong et al., 2018). The high moisture content in fresh mulberry plants, however, makes them difficult to preserve, particularly during the rainy season in Southern China. While silage represents an effective approach to conserving fresh material, the fermentation process is complex and subject to numerous influencing factors, resulting in variability in silage quality that may require the incorporation of additives to properly manage the fermentation process (Yitbarek and Tamir, 2014). The generation of mulberry silage of superior quality remains difficult due to its low contents of water-soluble carbohydrates (WSC) and high buffering capacity (Wang et al., 2019a). There is thus a pressing need for the use of rational strategies to select appropriate additives that can be applied during ensiling in order to produce higher quality silage.

A variety of silage additives can be used to enhance fermentation, increase silage nutritional value, minimize losses, protect against aerobic deterioration, and thereby achieve improved aerobic stability (Yitbarek and Tamir, 2014). These additives can be broadly classified into stimulants and inhibitors of fermentation, inhibitors of aerobic deterioration, absorbents, and nutrients (McDonald et al., 1991). Glucose can serve as a fermentation stimulant by increasing the amount of fermentable sugar substrates accessible to lactic acid bacteria (LAB) during ensiling, supporting a rapid decrease in pH and enhanced lactic fermentation through the inhibition of proteolytic and butyric fermentation (Trabi et al., 2017). Li et al. (2014) found that glucose was able to improve king grass ensiling fermentation by facilitating the rapid accumulation of lactic acid during the early period of ensiling. LAB present in silage consist of homofermentative and heterofermentative LAB (Muck et al., 2018). Homofermentative LAB can rapidly and robustly reduce pH levels during ensiling through their conversion of WSC into lactic acid (Zhang et al., 2019). *Lactobacillus buchneri* is the dominant obligate heterofermentative LAB species used as a silage additive, functioning during the later stages of ensiling by the gradual conversion of lactic acid into acetic acid and 1,2-propanediol, thereby leading to enhanced aerobic stability (Muck et al., 2018). In addition, formic acid has been utilized as a fermentation inhibitor through its ability to achieve a rapid decline in pH through direct acidification, suppressing the growth of undesirable spoilage-associated microbes including enterobacteria and aerobic bacteria while allowing an optimal environment for more rapid LAB growth and improved silage preservation (Yitbarek and Tamir, 2014). Jiang et al. (2020) found that treating of whole-plant corn silage with formic acid, acetic acid, and potassium sorbate at a 7:1:2 ratio (6 L/t) was associated with increased lactic acid levels and a reduction in the levels of undesirable microbes including *Klebsiella*, *Paenibacillus*, and *Enterobacter*. Salts including sodium benzoate and potassium sorbate can effectively reduce the growth of yeast, molds, and spoilage-related bacteria in silage, thereby leading to increased aerobic stability. Knický and Spörndly (2009) found that when ensiling grasses with low dry matter (DM) content, sodium benzoate and potassium sorbate treatment were associated with higher lactic acid levels, together with lower levels of butyric acid and NH_3-N relative to untreated silage. This suggests that adding glucose may provide a means of overcoming the lack of WSC content in mulberry plants, while the application of LAB, organic acids, salts, or combinations thereof may help further improve fermentation quality for whole-plant mulberry silage.

Improvements in silage quality through the use of any additive types are ultimately attributable to effects on the activity of beneficial or undesirable microbes (Kung et al., 2018; Liu et al., 2019). The development of high-throughput sequencing has fueled a growing number of studies exploring how various additives impact microbial communities in stylo (He et al., 2021), amaranth (Zhao et al., 2022), paper mulberry (Cheng et al., 2022), and native grass (Li et al., 2022). As far as we know, previous studies mainly used mulberry leaves as raw materials to prepare silage, focusing on the effects of additives on chemical composition, fermentation quality and part of microorganisms, rather than the whole plant (He et al., 2019; Wang et al., 2019a; Dong et al., 2020; Wang C et al., 2020; Cui et al., 2022). Little remains known, however, on the influence of different types of additive on fermentation and variations in microbial communities associated with whole-plant mulberry silages. As such, the present study was conceived with the goal of assessing how glucose, LAB, organic acids, salts and mixtures thereof impact fermentation quality and bacterial communities during mulberry ensiling through a high-throughput sequencing approach. The findings provided by this study can serve as a reference for addressing feed shortages, enhancing the quality of forage feed, and improving the economic management of ruminant husbandry.

2 Materials and methods

2.1 Plant materials and silage treatment

Whole mulberry plants were manually collected from a field in Changle County, Weifang City (118°83′E, 36°69′N), Shandong, China on September 25th, 2023. Harvested materials were manually chopped using a chopper (FS-690, Zili, China) to produce 2 cm segments. Mulberry material chemical composition before ensiling is shown in Table 1. The lactic acid bacteria (LAB), glucose, formic acid, and salts were employed as additives during ensiling. LAB treatment consisted

TABLE 1 Chemical composition of fresh whole-plant mulberry (Mean ± SD, $n = 4$).

Items	Fresh whole-plant mulberry
DM (% FM)	40.69 ± 0.10
CP (% DM)	14.48 ± 0.55
NDF (% DM)	41.26 ± 1.62
ADF (% DM)	18.96 ± 0.67
ADL (% DM)	6.89 ± 0.40
Ash (% DM)	10.70 ± 0.06
EE (% DM)	1.66 ± 0.15
WSC (% DM)	4.96 ± 0.10
Lactic acid bacteria (log cfu/g FM)	3.72 ± 0.24
Yeast (log cfu/g FM)	5.33 ± 0.47

FM, fresh; DM; dry matter; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin; EE, ether extract; WSC, water soluble carbohydrate.

of a 9:1 combination of *Lactobacillus plantarum* and *Lactobacillus buchneri*, which was used to inoculate samples at 10^6 colony-forming units (CFU) per gram of fresh material (FM). Glucose and formic acid were applied to the silage at 2 and 0.5% of FM, respectively. Salts consisted of a 2:1 (m/m) mix of potassium sorbate and sodium benzoate that was applied to silage at 1.5 g/kg of FM. The chopped mulberry material was mixed thoroughly, and separated into eight equal-sized parts for the following treatment conditions: no additive (C), glucose (G), LAB (L), formic acid (A), salts (S), G + L (GL), G + A (GA), G + S (GS). The mixed forage (1,000 g) was packed into plastic bags (20 × 30 cm; Deli Group, China), using vaccum sealing (Deli 14,886, Deli Group) for air removal. Four replicates per treatment were established for 32 total silage samples (8 treatments × 4 replicates), and these samples were stored in the dark for 90 days of ensiling at ambient temperature (21–25°C). The study period was from September 2023 to February 2024.

2.2 Chemical composition and microbial population analyses

A 1-mm screen was used to grind samples following incubation for 48 h at 65°C for chemical analysis. Dry matter (DM) contents were assessed by dying samples to a constant weight for 3 h at 105°C. WSC was analyzed as detailed previously by Murphy (1958). Acid detergent fiber (ADF) and neutral detergent fiber (NDF) were determined as in a prior report (Van Soest et al., 1991). Crude protein (CP), acid detergent lignin (ADL), ether extract (EE), and ash were analyzed as per protocols published by the Association of Official Analytical Chemists (AOAC, 1990).

Microbial population analyses were performed by mixing 20 g of fresh sample with sterile saline solution (0.85% NaCl), followed by serial dilution from 10^{-1} to 10^{-7}. LAB were then counted using the plate count method on de Man, Rogosa, and Sharpe (MRS) agar after anaerobic incubation for 48 h at 37°C. Yeast were counted after aerobic incubation for 48 h at 30°C on potato dextrose agar (PDA). Colony counts were reported as the number of viable microbes, with these values being transformed into log10 cfu/g FM.

2.3 Analyses of fermentation characteristics

Silage samples (20 g) were combined with sterile water (180 mL), homogenized for 60 s using a blender, and filtered by passing the solution through four cheesecloth layers. The resultant filtrate was then immediately tested to determine its pH using a pH meter (HI-9126; Hanna Instruments, United States). Ammoniacal nitrogen (NH_3-N) content was measured as reported previously by Broderick and Kang (1980). The levels of organic acids (lactic, acetic, propionic, and butyric acids) were measured via PLC using a Shodex RSpak KC-811S-DVB gel C column (Shimadzu, Japan), using a mobile phase consisting of 3 mmol/L $HClO_4$ and a 1.0 mL/min flow rate.

2.4 Microbiome analyses

2.4.1 DNA extraction and PCR amplification

Samples (50 g) were collected and immediately frozen for future extraction of bacterial DNA performed with the E.Z.N.A.® soil DNA Kit (Omega Bio-tek, GA, United States) as directed. The concentration and purity of the DNA were analyzed with a NanoDrop 2000 UV–vis spectrophotometer (Thermo Scientific, DE, United States) and 1% agarose gel electrophoresis (AGE), respectively.

The V3-V4 region of the 16S rRNA gene was amplified via PCR with the 338F (5'-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5'-GGACTACHVGGGTWTCTAAT-3′) primers using a GeneAmp 9,700 instrument (ABI, United States). Amplified products were separated via 2% AGE and purified using an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, CA, United States) and quantified using QuantiFluor™-ST (Promega, Madison, WI, United States).

2.4.2 Sequencing analyses

Equimolar amounts of the purified amplicons were pooled. Paired-end sequencing (2 × 300) was performed on an Illumina Miseq PE300 platform (Illumina, CA, United States). Trimmomatic was used to quality filter the resultant paired-end reads, eliminating sequences of low quality (average quality score < 20) or ambiguous bases. Pre-processes reads were merged using FLASH (v1.2.11) with a minimum 10 bp overlap and a 2% mismatch error rate. QIIME v1.8.0 was then used for sequence denoising, with the detection and removal of chimeric sequences. Operational taxonomic units (OTUs) were clustered using UPARSE (v11) with a cutoff level of 97% similarity. OTUs were standardized based on the sample with the lowest sequence number to facilitate further analyses. Taxonomic analyses were conducted using the Silva (SSU138) database to align 16S rRNA sequences with the RDP classifier algorithm (v2.13) at a 70% confidence threshold. Venn diagram analyses were used to detect unique and overlapping OTUs among groups. MOTHUR (v1.30.2) was used for the calculation of alpha diversity indices (Ace, Chao, Shannon, Simpson, and Coverage), with differences among groups being analyzed with the Kruskal-Wallis H test with the Tukey–Kramer *post hoc* test. A weighted unifrac distance-based principal coordinate analysis (PCoA) was performed in QIIME (v1.9.1) to assess beta diversity, while differences among groups were examined using analysis of similarities (ANOSIM) with 999 permutations. Differences in genus-level bacterial abundance among groups were analyzed with Kruskal–Wallis *H*-tests with the Tukey–Kramer *post hoc* test.

2.4.3 Correlation analyses

An RDA approach was used to probe the relationships among fermentation characteristics and samples at the genus level with the "vegan" package in R, utilizing fermentation characteristics as explanatory variables. Prior to modeling, the variance inflation factor (VIF) was used to select explanatory variables, omitting those variables with a VIF > 10. Correlations between microbial genera and fermenation characteristics were assessed through Spearman's rank correlation analyses, and a heatmap was used to present the resultant correlation matrix.

2.4.4 Statistical analyses

Fermentation characteristics and chemical composition data were analyzed with one-way ANOVAs and Duncan's multiple range test using the GLM procedure in SAS (V9.1). Results are presented as least squares means. $p \leq 0.05$ was regarded as significant unless otherwise indicated, while trends were identified using $0.05 < p \leq 0.10$.

3 Results

3.1 Chemical and microbial composition of fresh mulberry

The fresh mulberry samples used for this study had a DM content of 40.69% (Table 1). On the basis of DM, the CP, NDF, ADF, ADL, Ash, EE and WSC of mulberry were 14.48, 41.26, 18.96, 6.89, 10.70, 1.66, and 4.96%, respectively. The respective LAB and yeast counts for these mulberry samples were 3.72 and 5.33 log cfu/g of FM.

3.2 Mulberry silage chemical composition

Similar DM levels were observed in the silage from the A, S, GA, and GS groups, seen in increased values relative to the C, G, and L groups ($p < 0.05$, Table 2). The different treatments had no significant impact on CP, NDF, ADF, or ADL levels ($p > 0.05$). The GL group contained the greatest amount of EE (2.36%), followed by the L group (2.03%), GS group (1.54%), and S group (1.39%), in which the values were greater than those in the all the remaining groups ($p < 0.01$). Relative to the C group, the L and S groups exhibited increased Ash contents ($p < 0.01$), while these levels were reduced in the G and GA groups ($p < 0.01$). The highest WSC content was observed in the GA group (2.83%), followed by the G group (1.75%), A group (1.63%), and GS group (1.59%), with these groups exhibiting values higher than those for all other groups ($p < 0.01$).

3.3 Mulberry silage fermentation characteristics

Samples from the C group presented with the highest pH, propionic acid, butyric acid and NH_3-N levels, together with the lowest level of lactic acid (Table 3). The lowest pH, propionic acid, and

TABLE 2 Chemical composition of whole-plant mulberry silage.

Items	Treatments								SEM	*p*-value
	C	G	L	A	S	GL	GA	GS		
DM (% FM)	38.93^{d}	39.46^{cd}	39.39^{cd}	40.16^{ab}	39.85^{bc}	39.54^{bcd}	40.16^{ab}	40.60^{a}	0.11	<0.001
CP (% DM)	15.39	15.50	15.77	15.93	14.74	15.41	15.73	14.70	0.18	0.649
NDF (% DM)	39.72	39.36	39.30	40.06	40.61	39.09	38.99	38.92	0.42	0.983
ADF (% DM)	18.81	17.69	17.57	18.12	17.91	17.54	17.45	17.53	0.17	0.538
ADL (% DM)	6.12	5.55	5.31	5.85	5.68	5.43	5.69	5.60	0.10	0.681
Ash (% DM)	11.31^{bc}	10.99^{d}	11.56^{b}	11.13^{cd}	12.07^{a}	11.17^{cd}	10.91^{d}	11.08^{cd}	0.07	<0.001
EE (% DM)	0.80^{d}	0.76^{d}	2.03^{b}	0.75^{d}	1.39^{c}	2.36^{a}	0.96^{d}	1.54^{c}	0.11	<0.001
WSC (% DM)	0.75^{c}	1.75^{b}	0.88^{c}	1.63^{b}	0.80^{c}	1.03^{c}	2.83^{a}	1.59^{b}	0.12	<0.001

Means with different lowercase letters in the same row (a–d) differ significantly ($p < 0.05$). C, control; G, glucose; L, lactic acid bacteria; A, formic acid; S, salts; GL, glucose + lactic acid bacteria; GA, glucose + formic acid; GS, glucose + salts; SEM, standard error of means; DM, dry matter; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin; EE, ether extract; WSC, water soluble carbohydrate.

TABLE 3 Fermentation characteristics of whole-plant mulberry silage.

Items	Treatments								SEM	*p*-value
	C	G	L	A	S	GL	GA	GS		
pH	4.43^{a}	4.31^{c}	4.28^{cd}	4.23^{e}	4.35^{b}	4.25^{de}	4.23^{e}	4.30^{c}	0.01	<0.001
Lactic acid (g/kg DM)	53.27^{d}	60.15^{bcd}	64.17^{abc}	69.14^{a}	58.90^{cd}	66.88^{ab}	70.99^{a}	63.68^{abc}	1.23	<0.001
Acetic acid (g/kg DM)	10.81^{bcd}	11.47^{ab}	11.18^{abc}	11.62^{a}	10.63^{cd}	11.19^{abc}	11.23^{abc}	10.37^{d}	0.10	0.017
Propionic acid (g/kg DM)	0.80^{a}	0.73^{b}	0.71^{b}	0.33^{e}	0.38^{d}	0.39^{d}	0.33^{e}	0.49^{c}	0.03	<0.001
Butyric acid (g/kg DM)	0.08^{a}	0.05^{b}	0.05^{bc}	0.02^{d}	0.04^{c}	0.04^{bc}	0.02^{d}	0.04^{bc}	0.00	<0.001
NH_3-N (g/kg DM)	4.44^{a}	3.79^{bc}	3.36^{cd}	3.38^{cd}	3.91^{b}	3.30^{d}	3.27^{d}	3.78^{bc}	0.08	<0.001

Means with different lowercase letters in the same row (a–e) differ significantly ($p < 0.05$). C, control; G, glucose; L, lactic acid bacteria; A, formic acid; S, salts; GL, glucose + lactic acid bacteria; GA, glucose + formic acid; GS, glucose + salts; SEM, standard error of means.

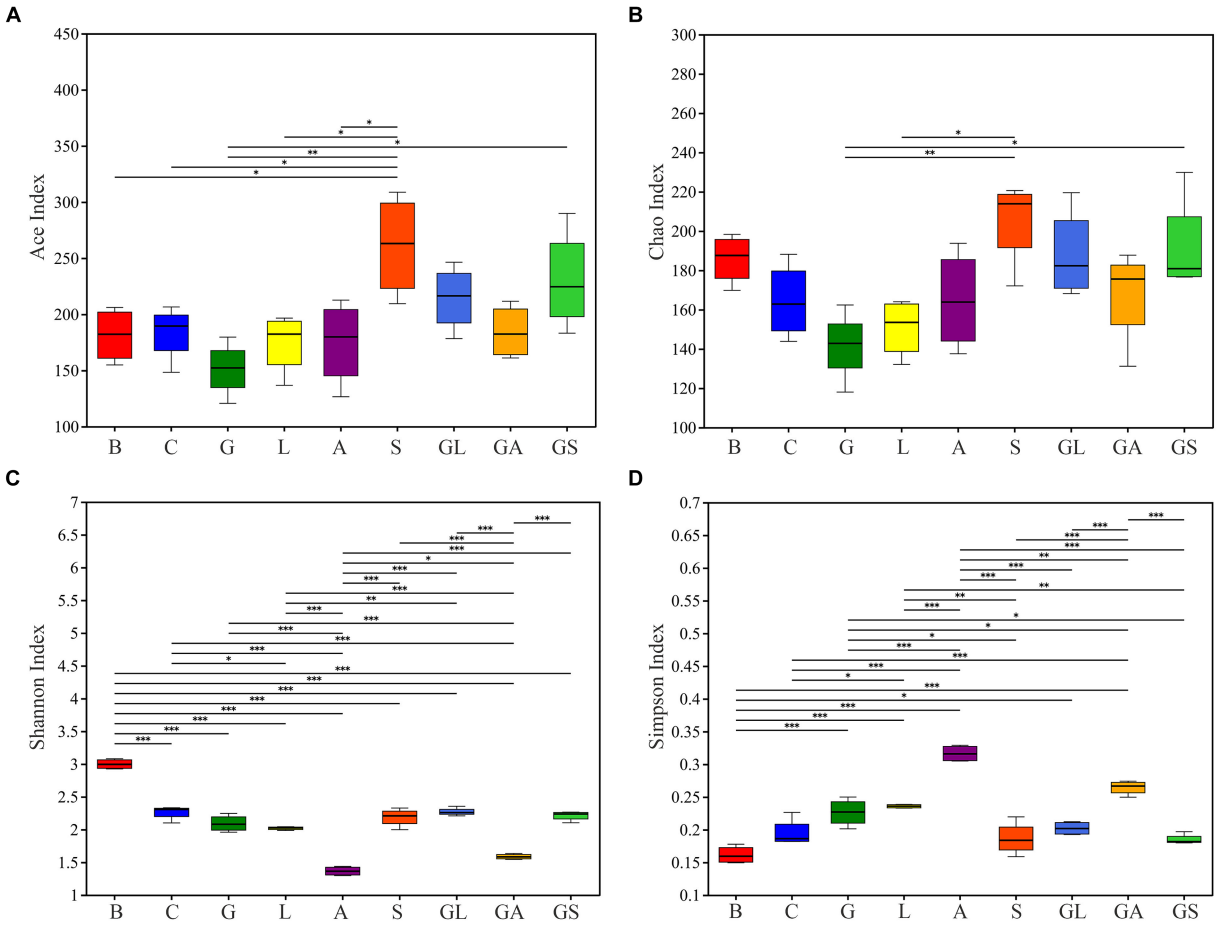

FIGURE 1
Differences in bacterial community diversity and richness between fresh mulberry and mulberry silage. **(A)** Ace index. **(B)** Chao index. **(C)** Shannon index. **(D)** Simpson index. C, control; G, glucose; L, lactic acid bacteria; A, formic acid; S, salts; GL, glucose + lactic acid bacteria; GA, glucose + formic acid; GS, glucose + salts.

butyric acid levels, in contrast, were observed in the A and GA groups, while NH_3-N content was lowest in the GA group. Significantly higher lactic acid levels were observed in the L, A, GL, GA, and GS groups relative to the C group ($p < 0.05$), although no marked differences were seen among these groups ($p > 0.05$). The lowest acetic acid contents were observed in the GS group, with these levels being similar to those in the C and S groups and below those of other groups ($p = 0.017$). Relative to the control groups, all additive groups presented with lower propionic acid, butyric acid, and NH_3-N contents ($p < 0.01$).

3.4 Microbial community of mulberry silage

In total, these analyses entailed the evaluation of 36 samples (4 fresh mulberry, 32 silages), yielding 1,316,664 high-quality reads with an average of 36,574 reads per sample. These reads were clustered into 397 OTUs, using the criterion of 97% similarity. The Good's coverage across these samples was ~0.99 in all cases (Supplementary Table S1). Alpha diversity analyses are presented in Figure 1, revealing similar trends in Ace and Chao1 indices across samples, with these values being highest in the S group, followed by the GS and GL groups, while they were lowest in the G group. Shannon index values declined in all treatment groups following ensiling, whereas Simpson index values rose. Among the silage treatment groups, lower Shannon indices and higher Simpson indices were observed in the A and GA groups.

PCoA analyses revealed clear separation among samples from different groups (Figure 2A). PC1 and PC2 were, respectively, found to explain 58.53 and 23.16% of the total change. Groups A and GA were separated from the C group and the five other groups by the greatest distance, whereas the distance among these remaining five groups was relatively similar. In total, 76 OTUs were shared across all treatment groups, while the B, C, G, L, A, S, GL, GA, and GS groups each exhibited 22, 2, 2, 6, 11, 11, 8, 13 and 15 unique OTUs, respectively (Figure 2B).

The relative phylum- and genus-level bacterial abundance in the analyzed samples is illustrated in Figure 3. The Firmicutes and Proteobacteria phyla were dominant in the fresh samples (53.34 and 22.15%, respectively), while in silage samples the relative abundance of these phyla had shifted to 95.07 and 4.58%, respectively (Supplementary Table S2). In fresh samples, the domainant genera were *Bacillus* (47.56%), *Sphingomonas* (6.34%) and *Hymenobacter* (5.60%) (Supplementary Table S3), while *Lactobacillus* (range: 45.86 to 67.08%) dominated in silage samples. The second most abundant genera in samples from the C group was *Enterobacter* (9.13%), while in samples

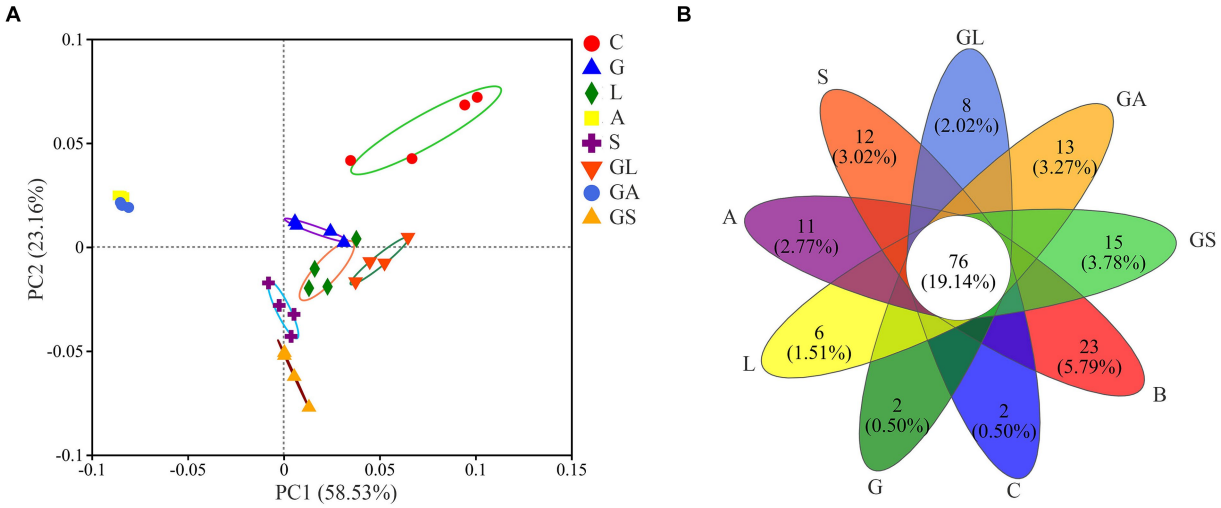

FIGURE 2
(A) Principle coordinate analysis (PCoA) of the bacterial community in silage samples based on Weighted Unifrac distance. **(B)** Venn diagram depicting unique or common bacterial OTUs in silage samples. C, control; G, glucose; L, lactic acid bacteria; A, formic acid; S, salts; GL, glucose + lactic acid bacteria; GA, glucose + formic acid; GS, glucose + salts.

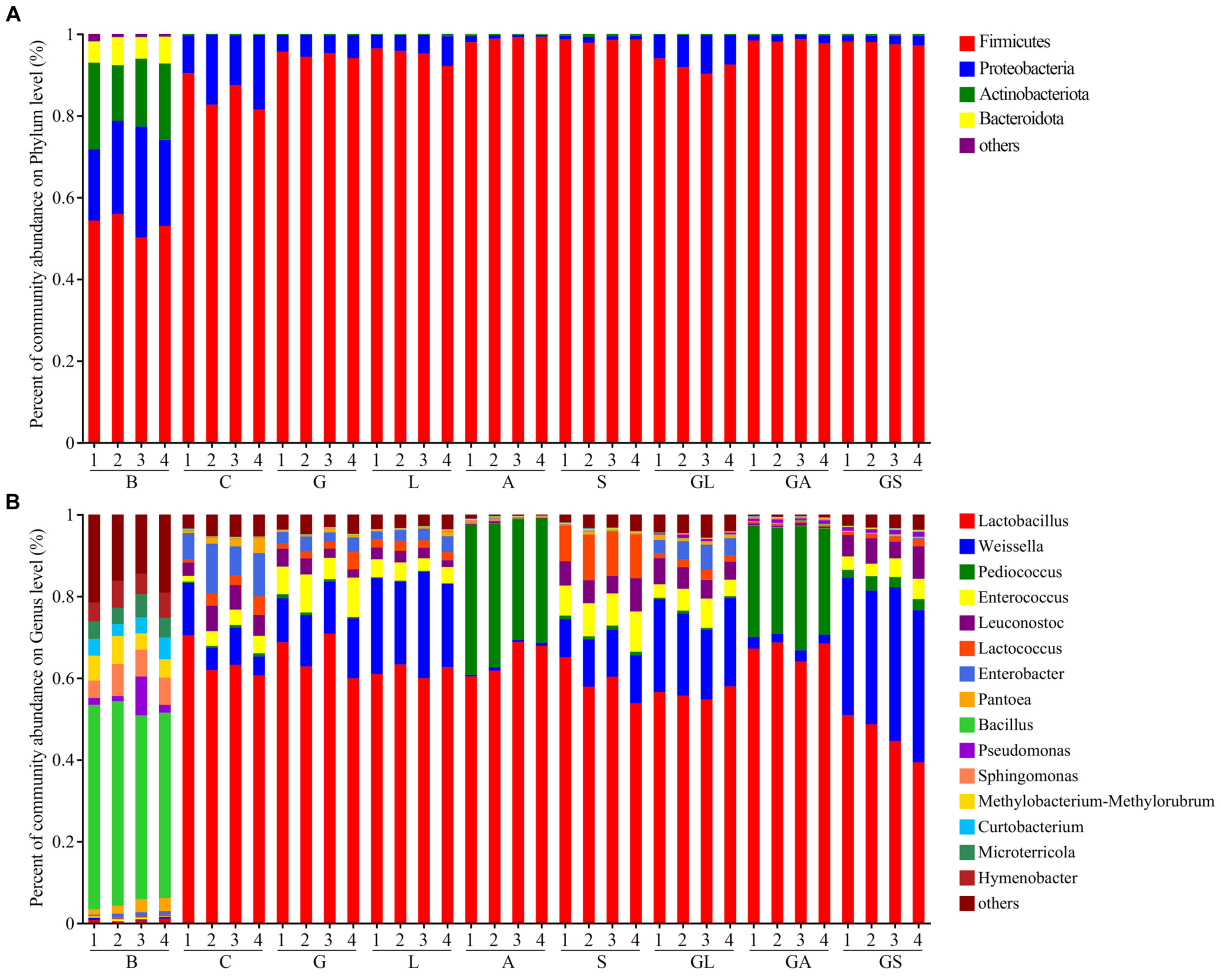

FIGURE 3
Relative abundance of bacterial composition in fresh mulberry and mulberry silage at the **(A)** phylum and **(B)** genus level. C, control; G, glucose; L, lactic acid bacteria; A, formic acid; S, salts; GL, glucose + lactic acid bacteria; GA, glucose + formic acid; GS, glucose + salts.

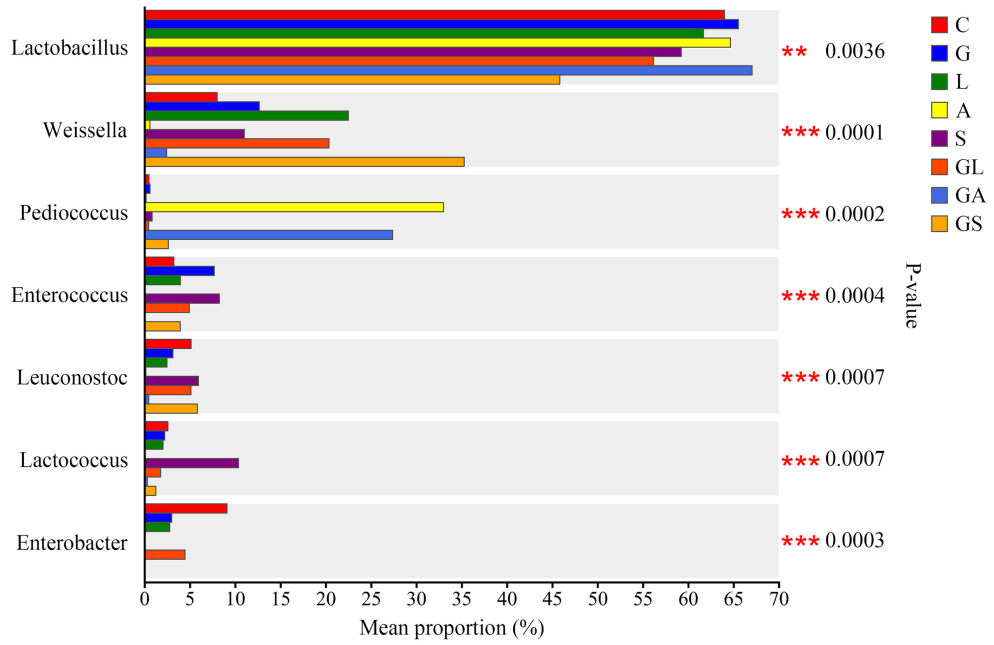

FIGURE 4
Comparison of different bacteria among the eight treatments. C, control; G, glucose; L, lactic acid bacteria; A, formic acid; S, salts; GL, glucose + lactic acid bacteria; GA, glucose + formic acid; GS, glucose + salts. $^{*}p < 0.05$; $^{**}p < 0.01$.

from the G, L, S, GL, and GS groups it was *Weissella* with a relative abundance of 12.70, 22.53, 11.04, 20.39, and 35.31%, respectively. The second most abundant genus in the A and GA treatment groups was *Pediococcus,* with respective relative abundances of 33.02 and 27.41%.

Genus-level comparisons of the different bacteria in this study are presented in Figure 4. The levels of *Lactobacillus* were lower in the GS group compared with the remaining seven groups ($p < 0.01$), which were fairly similar to one another. Increased proportions of *Weissella* were observed in the L, GL, and GS groups relative to the C group ($p < 0.01$). The A and GA groups exhibited increased *Pediococcus* abundance together with decreased abundance of *Weissella*, *Enterococcus, Leuconostoc*, *Lactococcus*, and *Enterobacter* relative to the remaining six groups ($p < 0.01$). The proportion of *Lactococcus* in the S group was elevated relative to that in the other groups ($p < 0.01$), while the proportion of *Enterobacter* in the C group was elevated in comparison with the other groups ($p < 0.01$).

3.5 Correlation analyses

RDA analyses indicated that pH, NH_3-N, and levels of lactic, acetic, and propionic acids markedly affected bacterial community structures ($p < 0.05$, Figure 5A). Samples in groups A and GA were positively associated with both lactic and acetic acids, while most of the other samples showed a negative association with these acids.

Spearman's rank correlation analyses revealed positive correlations between *Leuconostoc*, *Enterococcus*, and *Lactococcus* and pH, NH_3-N, propionic acid, and butyric acid levels ($p < 0.05$, Figure 5B) while the bacteria showed negative relationships with lactic acid levels ($p < 0.01$). *Weissella* was positively associated with propionic acid and butyric acid levels ($p < 0.05$), while a positive correlation was seen between *Enterobacter* and pH, propionic acid, and butyric acid levels ($p < 0.05$) and a negative association with lactic acid levels ($p < 0.05$), while *Pediococcus* exhibited the opposite trend.

4 Discussion

Greater CP and lower cell wall content (NDF, ADF, and ADL) can often serve as indicators associated with high-quality forage (Huang et al., 2019). Fresh whole-plant mulberry exhibited a CP content of 14.48% in this study, in line with values reported for traditional forage such as silage derived from alfalfa and soybean (Ni et al., 2018; Özüretmen et al., 2022). The higher CP contents, together with the low ADF and NDF content observed in whole-plant mulberry, suggests that it may be a valuable protein-rich forage option for ruminants. However, whole-plant mulberry exhibited lower CP content than that for mulberry leaves (Wang et al., 2019a; He et al., 2020), together with higher NDF and ADF content, potentially suggesting that the nutritional composition of mulberry plants varies across various parts of these plants. Relative to the fresh material, mulberry silages exhibited expected reductions in DM and WSC content. This is attributable to oxygen consumption by plant cells and aerobic microorganisms during the early stages of ensiling together with the fermentation of WSC into lactic acid by LAB over the course of ensiling (Dunière et al., 2013). The observed decreases in silage NDF, ADF, and ADL content were likely primarily attributable to the hydrolytic breakdown of cell walls by organic acids and enzymes produced during ensiling (Larsen et al., 2017).

Ensiling entails inevitable changes in chemical composition resulting from soluble carbohydrate conversion into organic acids and the breakdown of proteins and fibers (Wei et al., 2021). The higher levels of DM content in the A, S, GA, and GS groups suggest lower DM loss primarily stemming from the ability of formic acid and salts

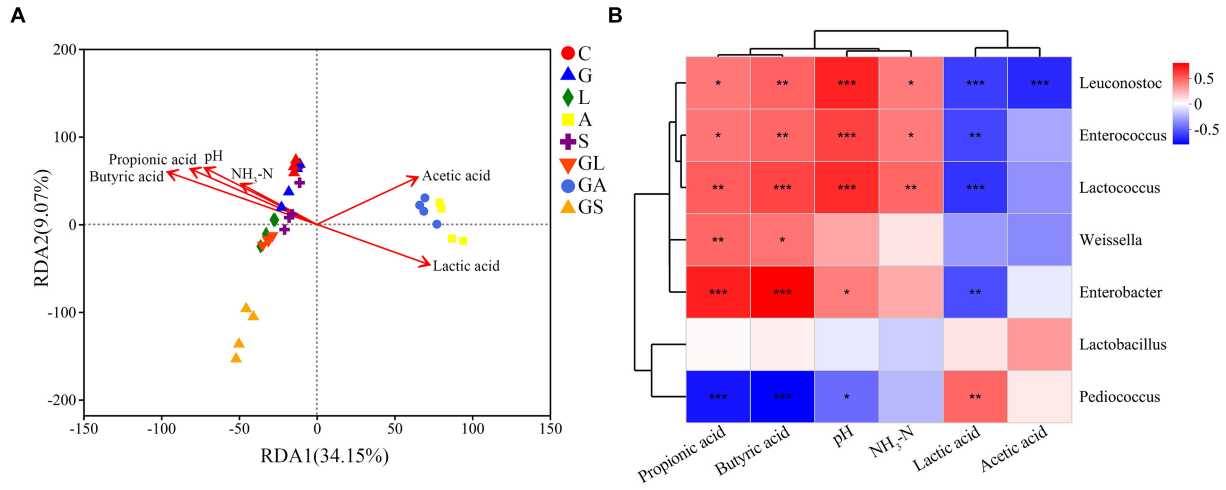

FIGURE 5
(A) Redundancy analysis (RDA) of bacterial data (symbols) and fermentation characteristics (arrows). **(B)** Correlation matrix between fermentation characteristics and microbial composition at the genus level. Positive correlations are shown in red, and negative correlations are shown in blue. Intensity of color is proportional to Pearson correlation coefficient. C, control; G, glucose; L, lactic acid bacteria; A, formic acid; S, salts; GL, glucose + lactic acid bacteria; GA, glucose + formic acid; GS, glucose + salts. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

(sodium benzoate + potassium sorbate) to more efficiently inhibit the growth of undesirable microbes, thereby minimizing nutrient loss (Muck et al., 2018). As reported previously (Li et al., 2022; Wu C et al., 2022; Zhao et al., 2022), additives failed to impact NDF content in this study. Cheng et al. (2022), however, found that the *L. plantarum* inoculation of paper mulberry silage led to an increase in NDF content after ensiling for 60 days, while Jiang et al. (2020) reported no change in NDF content in corn silage after treatment with LAB and organic acid after 45 days but a decrease following 90 days of ensiling. Adding formic acid, molasses, and fibrolytic enzymes also reportedly reduced NDF content in Napier grass samples (Desta et al., 2016). These discrepant results may be attributable to differences in the particular grass species, additives, and ensilage times in individual studies. WSC content was increased in groups treated with glucose relative to other groups in the present study, as expected. However, WSC content changes dynamically over the course of ensiling, with the acid hydrolysis of the fiber fraction leading to the release of WSC that can be used by LAB for the production of organic acids (Jiang et al., 2020).

Analyzing pH, organic acid content, and NH_3-N content can provide valuable insights into silage fermentation quality (Li et al., 2019; Gao et al., 2022). Relative to the C group, the groups treated with additives in the present study exhibited lower pH values and NH_3-N content, together with higher lactic acid content, suggesting that utilizing these additives led to significant improvements in silage fermentation quality. While the lactic acid content in silage found in the current investigation was consistent with that reported for corn silage in past reports (Liu et al., 2021; Wang et al., 2022), the pH (4.28) was substantially higher than in these prior reports (3.50–3.88). This further emphasizes the challenges of directly ensiling whole mulberry plants, potentially owing to their high buffering capacity, inhibiting drops in the pH of the resultant silage (Hao et al., 2021). Epiphytic LAB counts $>10^5$ CFU/g of FM and a WSC content >6% DM are essential for successful fermentation (Oliveira et al., 2017). Adding glucose and LAB can thus overcome the limitations in WSC and epiphytic LAB found in the fresh mulberry material, leading to superior fermentation. Here, the A and GA groups exhibited the best fermentation quality, potentially because the formic acid was able to rapidly lower the pH, thereby inhibiting undesirable microbe growth while providing an environment suitable for the growth of LAB (Lv et al., 2020).

Microbes are vital in the ensiling process such that efforts to monitor the bacterial community during ensiling can inform efforts to better understand and enhance the process of fermentation (Xu et al., 2017). Here, ensiling led to increases in Shannon index values together with decreased Simpson index values, consistent with reduced diversity in bacterial communities and in line with earlier findings (Guan et al., 2018; Wu B et al., 2022). Méndez-García et al. (2015) posited that this may reduction may be linked to the reduction in the pH of the resultant silage, limiting the ability of microbes to grow under acidic conditions. Reductions in unique bacterial OTUs following ensiling were also evident in the present study, consistent with past reports (Ren et al., 2019; Su et al., 2021). The magnitude of this reduction in community diversity was greatest in the A and GA groups, supporting the ability of formic acid to improve the quality of the resultant fermentation more effectively than other additives.

The epiphytic bacterial communities found in the fresh material are closely associated with the type of plant (Jiang et al., 2020). Fresh native grasses, for example, are frequently dominated by *Pantoea*, *Pseudomonas,* and *Erwinia* (Li et al., 2022), whereas *Moringa oleifera* leaves are dominated by *Exiguobacterium*, *Acinetobacter,* and *Pseudomonas* (Wang et al., 2018). In the present study, the dominant genera associated with fresh material were *Bacillus*, *Sphingomonas*, and *Hymenobacter*. *Bacillus* species are capable of producing antifungal compounds and facilitating the degradation of anti-nutritional factors and macromolecular nutrients through exoenzyme secretion (Chi and Cho, 2016). Phyllosphere microbiomes including those associated with fruits and flowers are often rich in *Sphingomonas* and *Hymenobacter* (Olimi et al., 2022). Epiphytic bacteria also exhibit a high degree of sensitivity to climatic, regional, and environmental factors. Guan et al. (2018), for instance, found that epiphytic bacterial

communities were significantly correlated with temperature, humidity, and rainfall. Interestingly, despite the variations in epiphytic bacteria reported in the above studies, all of these species decreased sharply in abundance during the ensiling process together with prominent *Lactobacillus* outgrowth.

Additives can have a profound impact on silage quality through their effects on microbial communities present therein. LAB are the primary microorganisms that shape the process of fermentation, and these species can be morphologically classified into bacilli including *Lactobacillus* species, as well as cocci such as *Enterococcus*, *Lactococcus*, *Leuconostoc*, *Pediococcus,* and *Weissella* species (Yang et al., 2022). As expected, greater *Lactobacillus* abundance was evident in the G, A, and GA groups in the present study, likely owing to the addition of glucose as a source of WSC and the ability of formic acid to suppress harmful microbe growth, thereby generating conditions favorable to LAB growth. Higher *Lactobacillus* abundance has also been reported in native grass silage after treatment with molasses (Li et al., 2022) or in corn silage to which formic, acetic, and propionic acids were added at a 7:1:2 ratio (Jiang et al., 2020). Strikingly, the addition of LAB in the L and GL groups led to a decrease in *Lactobacillus* levels relative to the C group while increasing the proportion of *Weissella* in the resultant silage. This coincided with enhanced fermentation quality in these L and GL groups, as determined by the observed reduction in pH and elevation of lactic acid levels. In line with these results, Mu et al. (2021) also determined that adding *Lactobacillus plantarum* and molasses to mixed rice straw and amaranth silage led to a reduction in relative *Lactobacillus* abundance together with an increase in *Weissella* abundance. The mechanisms underlying this observation remain uncertain and warrant further study. The proportions of *Lactobacillus* and *Weissella* in the S and GS groups tended to exhibit similar trends in their variability to those observed in the L and GL groups. This is likely attributable to the ability of potassium sorbate and sodium benzoate to inhibit undesirable microbe growth while also limiting *Lactobacillus* growth, ultimately establishing conditions conducive to *Weissella* growth. Strikingly, all of the additives employed in this study resulted in decreased *Enterobacter* abundance, which is important given that these undesirable bacteria can induce fermentation of lactic acid to produce succinic acid, acetic acid, and certain endotoxins, thereby compromising the nutritional quality of the silage and leading to its contamination (Wang et al., 2022). Relative to other tested additives, the formic acid (A and GA) and salt (S and GS) groups exhibited more effective inhibition of harmful bacterial growth. Adding formic acid also increases the relative abundance of *Pediococcus*, which is frequently utilized to enhance silage quality due to its ability to produce lactic acid and to tolerate acidic conditions (Wang et al., 2019b). Relative to other additives, formic acid thus provides the most effective approach to enhancing *Lactobacillus* and *Pediococcus* abundance while limiting the growth of potentially harmful microbial species.

Using an RDA approach, the relationships between bacterial community composition and fermentation parameters were explored in greater detail. Across all groups, longer arrows were observed for lactic acid and acetic acid, indicating that these two organic acids had the greatest impact on the bacterial communities present in silage, in line with what has been reported in paper mulberry silage (Wu C et al., 2022) and alfalfa silage (Bai et al., 2020). Lactic acid and acetic acid were also found to be positively associated with the samples in groups A and GA, suggesting that adding formic acid led to more effective increases in the content of these organic acids. In line with expectations, lactic acid concentrations were negatively correlated with pH, butyric acid levels, and NH_3-N concentrations, as lactic acid can reduce pH values and suppress the growth of spoilage-related bacterial species including *Clostridia*, thereby preventing protein degradation and the generation of butyric acid (Wang et al., 2019). Lactate-producing bacteria tended to be positively linked to the concentrations of lactic acid in these samples. However, *Lactobacillus* abundance was not significantly correlated with lactic acid concentrations in the present study, while a negative relationship was reported in alfalfa silage by Wang B et al. (2020). The contributions of *Lactobacillus* to increases in lactic acid levels may be less significant than the contributions of other silage bacteria. *Leuconostoc*, *Enterococcus*, and *Lactococcus* were also found to be negatively associated with concentrations of lactic acid while they were positively associated with pH, butyric acid, and NH_3-N contents, in contrast to the overall findings. This may suggest that the growth of these beneficial species of bacteria coincided with the increased growth of certain undesirable species. Li et al. (2020) determined that when initial lactic acid fermentation-induced acidification failed to prevent *Clostridia* growth in silage, *Clostridia* fermentation can occur, leading to the production of butyric acid or to ammonia accumulation. Formic acid, in contrast, exhibits a greater capacity for acidification such that it can more effectively suppress the outgrowth of *Clostridia* and certain other undesirable species of bacteria (Lv et al., 2020). Consistent with this possibility, the pH and community diversity indices in the A and GA groups were lower. Additionally, *Pediococcus*, which was more abundant in samples from the A and GA groups, was positively associated with lactic acid concentrations but negatively associated with pH, butyric acid, and NH_3-N levels, in line with what has previously been described in corn silage by Guan et al. (2018).

5 Conclusion

In summary, the present results revealed an increase in DM content in the A, S, GA, and GS treatment groups. Decreased pH and NH_3-N levels were observed in all additive treatment groups. The A and GA groups presented with the lowest pH values and the greatest lactic acid contents. The various tested additives modified bacterial community structures through increases in the abundance of beneficial bacteria including *Weissella* and *Pediococcus*. A and GA addition was also sufficient to profoundly inhibit *Enterobacter* growth. As such, the additives utilized in this study offer promise as tools to improve whole-plant mulberry silage to varying degrees. A and GA treatments were found to be the most efficient approach to reducing pH levels, increasing lactic acid content, and preventing the growth of undesirable bacteria in ensiled mulberry samples.

Data availability statement

The 16S rRNA sequence data were submitted to the NCBI Sequence Read Archive (SRA; https://submit.ncbi.nlm.nih.gov/subs/sra/) database with the accession number of PRJNA1024408 for open access.

Author contributions

LH: Writing – review & editing, Writing – original draft, Conceptualization. FJ: Writing – review & editing, Writing – original draft, Conceptualization. YW: Writing – review & editing, Project administration. HW: Writing – review & editing, Resources, Data curation. HH: Writing – review & editing, Project administration, Data curation. WY: Writing – review & editing, Formal analysis, Data curation. XH: Writing – review & editing, Formal analysis, Data curation. HC: Writing – review & editing, Formal analysis, Data curation. CW: Writing – review & editing, Writing – original draft, Investigation, Funding acquisition. ES: Writing – review & editing, Writing – original draft, Investigation, Funding acquisition.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1399907/full#supplementary-material

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Metagenomic analysis of core differential microbes between traditional starter and Round-Koji-mechanical starter of Chi-flavor *Baijiu*

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Xiaoqu starter serves as the saccharifying and fermenting agent in the production of Cantonese soybean-flavor (Chi-flavor) *Baijiu*, and the complex microbial communities determine the flavor and quality of the product. Round-Koji-mechanical starter (produced by using an automated starter-making disk machine) is advantageous as it decreases operator influence, labor costs, and fermentation time, but the product quality is lower compared to traditional starter. Thus, two types of starters (traditional and Round-Koji-mechanical starter) from a Cantonese *Baijiu* factory were compared in a metagenomic analysis to investigate the differences in microbial community composition and core microbes. The results showed that several core microbes related to carbohydrate metabolism, amino acid metabolism and lipid metabolism, were differentially enriched in the traditional starter. *Mucor lusitanicus* and *Rhizopus delemar* were significantly positively correlated with the three key metabolic pathways. *Saccharomyces cerevisiae, Cyberlindnera fabianii, Kluyveromyces marxianus, Lactobacillus fermentum, Mucor ambiguous, Rhizopus microspores, Rhizopus azygosporus, Mucor circinelloides,* and *Ascoidea rubescens* were significantly positively correlated with two of the three key metabolic pathways. The results of this study provide a basis for understanding the differential core microbes in traditional and Round-Koji-mechanical starters of Chi-flavor *Baijiu*, and they also provide guidance for improving Round-Koji-mechanical starter.

KEYWORDS

metagenomic, core differential microbes, traditional starter, Round-Koji-mechanical starter, Chi-flavor *baijiu*

1 Introduction

Chinese *Baijiu*, a long-standing alcoholic beverage, is considered one of the remarkable creations of ancient China (Xu et al., 2017). In the past five years, the total output of Chinese *Baijiu* has reached 37.85 million kiloliters. Chinese *Baijiu* is typically produced through solid-state fermentation using various grains as the initial ingredients. *Jiuqu* is a specific kind of

starter used in the fermentation process of Chinese *Baijiu*. It is made up of various raw materials, microflora, enzymes, and aromatic precursor substances (Tu et al., 2022). The taste of Chinese *Baijiu* is derived from the existence of volatile and non-volatile compounds, primarily generated through microbial metabolism during the fermentation process. Volatile substances found in Chinese *Baijiu* include esters, alcohols, acids, aldehydes, nitrogen-containing compounds, sulfur-containing compounds, and terpenes. These compounds play a significant role in determining the aromatic characteristics and overall quality of Chinese *Baijiu*. Currently, over 2,400 chemicals have been identified in Chinese *Baijiu* that contribute to its flavor profile. Some of these chemicals, such as short-chain fatty acids, peptides, and phenols, have been found to have potential health benefits for humans (Sun et al., 2015; Fang et al., 2019; Xu et al., 2020; Jiang et al., 2021). The various types of Chinese *Baijiu* can be distinguished based on factors such as the production processes, raw materials, flavors, and region. According to the *Jiuqu* starter used, Chinese *Baijiu* can be classified as *Xiaoqu Baijiu*, *Daqu Baijiu*, *Maiqu Baijiu*, etc. (Zheng and Han, 2016). *Xiaoqu Baijiu* accounts for a sixth of Chinese *Baijiu*, and is mainly distributed in the southern region of China, including in Guizhou, Sichuan, and Hubei (Zhao et al., 2021). Rice, sorghum, and wheat are used in the production of *Xiaoqu Baijiu* (Su et al., 2010). These raw materials are cooked, mixed with microbes in a starter, and brewed by solid-state fermentation. *Xiaoqu* starter is the fermentation and saccharification agent for *Xiaoqu Baijiu* production. Compared to *Daqu* starter, it has fewer microbial species (mainly genera such as *Rhizopus*, *Trichoderma*, *Lactobacillus*, and yeast) (Gou et al., 2015) and a shorter fermentation cycle, and the flavors are not as rich (Xu et al., 2022).

Based on the production processes, raw materials, and other factors such as edaphoclimatic condition in different regions, there are many different types of *Xiaoqu Baijiu*, such as *Huaxia Xiaoqu*, *Hubei Xiaoqu*, *Dazhou Xiaoqu*, and *Jiujiang Xiaoqu* (Wu et al., 2017; Wang et al., 2018). The production of traditional *Xiaoqu* starter mainly involves manual processes, including steaming, spreading, and cooling the raw material (rice), adding crushed cake seeds, pressing the mixture into a disk shape, and incubating the product at 28–37°C for 3–5 days. As the traditional *Xiaoqu* starter production process is under non-asseptic conditions, the quality can vary greatly depending on the environment (Zheng and Han, 2016). In addition, the process entails high labor costs, involving many workers and high labor intensity.

With the progress of automated technology, the mechanical production of starter has gradually been developed, which includes automated mechanical disk-forming technologies and automated fermentation facilities, with the aim of reducing labor costs, standardizing the quality of starter, and achieving pollution-free production (Wang et al., 2018). However, the quality of *Jiujiang Xiaoqu* (Chi-flavor) *Baijiu* produced by Round-Koji-mechanical starter failed to reach the quality of *Baijiu* produced by traditional starter (Fei et al., 2023). Starter, which is a combination of many microbes and many enzymes (Shen, 1998; Yu, 2010), promotes simultaneous saccharification and fermentation during *Xiaoqu Baijiu* production. The quality and flavor composition of starter largely depend on the microbial community composition and metabolic functions (Jin et al., 2019). Exploring the differences in microbial community composition between mechanical and traditional starters, which can help for improving the quality of mechanical starters. An amplicon sequencing study compared the bacterial (but not fungal) diversity between traditional starter and the Round-Koji-mechanical starter of Chi-flavor *Baijiu*. The results showed that *Lactobacillus* and *Pediococcus* were dominant in both starters, *Weissella* was dominant in the traditional starter, and *Bacillus*, *Acetobacter*, *Acinetobacter*, and *Klebsiella* were dominant in the mechanical starter (Wang et al., 2018). Additionally, our team compared the bacterial diversity in the prophase of Chi-flavor *Baijiu* fermentation between the two starters, and we found that *Lactobacillus* and *Saccharomyces* were the dominant genera in both starters, but *Pediococcus* and *Weissella* were enriched in the traditional starter compared to the mechanical starter (Fei et al., 2023). However, the core microbial species in traditional starters remain unknown.

Amplicon sequencing involves using PCR technology to amplify the target in the samples. It is often used to analyze the 16S rRNA and ITS sequences of bacterial or fungal communities, but it is only accurate to the genus level (Caporaso et al., 2011). Metagenomic analysis based on amplicon or high-throughput sequencing involves collecting genome-wide data from diverse organisms in a given sample (Walsh et al., 2017). Metagenomic analysis based on high-throughput sequencing can achieve species-level analysis, and various analytical methods can be used to process and analyze metagenomic data for targeted microbial research (Franzosa et al., 2015).

A comparative analysis of physical factors in our team showed that the saccharifying, esterification, and fermentation capacities were significantly higher in traditional starter than Round-Koji-mechanical starter (Wang et al., 2023). In the current study, the differences in microbial community composition between traditional and mechanical starters were analyzed by metagenomic analysis. In addition, the core differential genes related to carbohydrate, lipid, and amino acid metabolism (based on the KEGG database) were identified, and a correlation analysis of the core differential genes was conducted using the NR database, which was mined to determine the core differential microbes between traditional and mechanical starters. The core advantageous differential microbes between the traditional and mechanical starters were identified, and the results provide a basis for improving the quality of Round-Koji-mechanical starter and promoting the industrialization and mechanization of Chi-flavor *Baijiu* production.

2 Materials and methods

2.1 Sample collection

Two types of starters contained traditional starter (BQ) and mechanical starter (Round-Koji-mechanical starter, SQ), were collected from *Jiujiang* Distillery in Foshan City, Guangdong, China. Regarding BQ, a batch with a starter was used for three-point sampling (for example, three pieces from the top, middle and bottom of the starter). Regarding SQ, a batch with a starter was used for five-point random sampling.

2.2 Genomic DNA extraction

The collected samples were sent to Shanghai Meiji Biotechnology Company (Shanghai City, China) for DNA extraction. Briefly, each sample was weighed at −80°C to obtain 10 g. DNA was extracted

using an E.Z.N.A.® Soil DNA Kit according to the manufacturer's instructions. DNA quality was measured by a microspectrophotometer (NanoDrop2000, Thermo Fisher Scientific) and 1% agarose gel electrophoresis.

2.3 High-throughput sequencing, quality control, and assembly

The extracted DNA was sent to Shanghai Meiji Biological Company for sequencing and data processing. Briefly, raw metagenomic data were obtained using an Illumina HiSeq 4000 sequencing system. The raw data were subjected to quality control using Fast Software Version 0.12.0 (Babraham Institute, UK), which removed the adapter sequences from the 3′ and 5′ ends and the reads that were < 50 bp, had a mean quality <20, or contained N bases, retaining the high-quality paired- and single-end reads. To obtain high-quality clean data, using BWA software (Li and Durbin, 2009), the reads were compared to the DNA sequences of the raw brewery materials, and reads with high similarity (contaminants) were removed. Contigs were obtained using the Multiple MEGAHIT splicing strategy, and the contigs ≥300 bp were selected as the final assembly results. Open reading frame (ORF) prediction of the contigs was performed using Prodigal software (Hyatt et al., 2010). Next, clustering was performed using CD-HIT software, and the longest gene in each cluster was selected as the representative sequence to construct a non-redundant gene set. Finally, the high-quality reads in each sample were compared to the non-redundant gene set (default criterion: 95% identity) using SOAPaligner/soap2 Version 2.21 (Beijing Genomics Institute, China).

2.4 Taxonomic assignment

The DIAMOND software (Buchfink et al., 2015) was used to compare the non-redundant gene set to the NR database,[1] and species annotations were obtained from the corresponding Taxonomy database of the NR database. Based on this information, relative abundances at the domain, kingdom, phylum, class, order, family, genus, and species levels in each sample were calculated. Next, the abundance of the species in each sample was counted at each taxonomic level to construct an abundance table at the corresponding taxonomic level and complete species annotation.

2.5 Kyoto encyclopedia of genes and genomes (KEGG) functional annotation

The KEGG database[2] was used to identify sets of genes related to the three major metabolic pathways, i.e., carbohydrate, lipid, and amino acid metabolism. Next, for gene annotation, DIAMOND was used to compare the abovementioned non-redundant gene set to the KEGG database, including the carbohydrate, lipid, and amino acid metabolism-related genes.

2.6 Identification of core differential microbes based on core differential genes

The functional composition and clustering of the starter samples, based on genes related to carbohydrate, lipid, and amino acid metabolism, were analyzed by Circos Analysis, Clustering Analysis, and Principal Component Analysis (PCA). Functional genes unique to either starter were identified by constructing Venn Diagrams and by conducting significant difference tests. Linear Discriminant Analysis (LDA) was used to analyze the effect sizes. Based on these analyses, core differential genes related to carbohydrate metabolism, amino acid metabolism and lipid metabolism, were identified. Correlation analysis was conducted using the data on these core differential genes and the NR database. The Network Complex Analysis Toolkits (Python package) were used to calculate the correlations between the genes and microbial species. Consequently, the differential core microbial species in traditional starter (BQ) were determined based on the strength of the correlations.

3 Results and discussion

3.1 Overview of metagenomic data

After sequencing the two starters (Supplementary Table S1), there were 263,860,016 raw reads (39,842,862,416 bp) and after quality control, there were 2,586,116,640 clean reads (38,934,390,577 bp). There were 941,901 contigs >300 bp (1,080,460,208 bp). The mean N50 and N90 were 2,152 and 425 bp, respectively. 1,462,862 ORFs were predicted, with a total length of 641,017,419 bp. The distribution of non-redundant gene lengths in Supplementary Figure S1 indicates that the number of sequences decreased with gene length.

3.2 Taxonomic analysis

A total of 3 domains, 5 kingdoms, 16 phyla, 30 classes, 46 orders, 83 families, 134 genera, and 321 species were annotated by NR species annotation of the two starters. The 3 domains were Bacteria, Eukarya and Viruses.

In BQ, the relative abundance of fungi accounted for 72% and that of bacteria accounted for 28%. In SQ, both accounted for about 50%. Environmental factors such as temperature and humidity changed more slowly in SQ than BQ. It was hypothesized that the reason for the different community composition between the two starters was the environment during fermentation. These results regarding relative abundance of domains is consistent with the results of Hu Y. L. et al. (2021).

The results of phylum level were showed in Supplementary Figure S2A. There are 10 phylas with relative abundance greater than 0.1%. The relative abundance of *Mucoromycota* accounted for 65.4% in BQ and that for 46.4% in SQ. The relative abundance of *Firmicutes* accounted for 25.8% in BQ and that for 46.7% in SQ. Thus, the *Mucoromycota* and *Firmicutes* were dominant

1 https://www.ncbi.nlm.nih.gov/refseq/about/nonredundantproteins/
2 http://www.genome.jp/kgee/

in both starters. In a previous studies of *Xiaoqu* (Su et al., 2010; Wu et al., 2017), the dominant phyla were *Firmicutes, Actinobacteria, Proteobacteria, Ascomycota, Mucoromycota, Basidiomycota*, which is similarity with the results of our study.

The results of genus level were showed in Supplementary Figure S2B. There are 10 genus with relative abundance greater than 0.1%. The relative abundance of *Rhizopus* accounted for 50.3% in BQ and that for 31.1% in SQ. The relative abundance of *Lactobacillus* accounted for 22.5% in BQ and that for 18.3% in SQ. The relative abundance of *Mucor* accounted for 8.5% in BQ and that for 4.9% in SQ. Thus, the *Rhizopus*, *Lactobacillus,* and *Mucor* were dominant in both starters. However, the abundance of *Bacillus* accounted for 21.1% and only showed in SQ. The mechanical process uses a large blower to control humidity, a large amount of air can be blown into the starter. This condition was good for growthing of aerobic microorganisms, such as *Bacillus*. These results were consistent with the results of a previous study about mechanical Daqu (Zuo et al., 2020).

Metagenomic sequencing technology allowed species-level analyses of the two starters. According to the Venn diagram, there were 1,574 unique species in BQ, 599 unique species in SQ, and 2,578 common species (Figure 1A). According to the PCA (Figures 1B,C), the confidence intervals were far apart, indicating that the two starters were significantly different.

In the species composition analysis of bacteria (Figure 2A), the abundance of *Lactobacillus fermentum* was higher in BQ (47.3%) than SQ (27.8%). This species is associated with volatile flavors such as 3-methyl-1-butanol, 2-methyl-1-propanol, and phenylethanol in yellow *Baijiu* (Wenhong, 2018). The abundance of *Lactobacillus plantarum* was also higher in BQ (5.8%) than SQ (1.1%). This species can produce certain flavor precursors and can survive at low pH and high alcohol concentrations in *Baijiu* fermentation (Todorov and Franco, 2010). Moreover, *Weissella confusa* and *Weissella paramesenteroides* were not present in SQ, whereas *Bacillus amyloliquefaciens*, *Bacillus subtills, Acetobacter Bacillus amyloliquefaciens, Bacillus subtills, Acetobacter pasteurianus,* and *Bacillus velezensis* were not present in BQ. The dominant species in SQ were *Bacillus amyloliquefaciens* and *Bacillus subtills*. These two species can produce α-amylase and glucoamylase for raw material saccharification and hydrolysis (Li et al., 2014). *Bacillus subtills* and *Bacillus velezensis* inoculation of macroalgae (for biofortification) enhanced saccharification, ethanol fermentation, and aroma formation (He et al., 2019). However, massive reproduction of *Bacillus* in starter inhibiting growth of *Lactobacillus*, *Pediococcus* and *Weissella* (Wang et al., 2015).

In the species composition analysis of molds (Figure 2B), there was no significant difference between the two starters. Yeast is important in *Baijiu* fermentation, so a species composition analysis of yeast (Figure 2C) was conducted. The abundance of *Kluyveromyces marxianus* was higher in BQ (51%) than SQ (32%). This species can produce many enzymes such as *β*-galactosidase, lipase, protein phosphatases, carboxypeptidase, and *β*-glucosidase (Karim et al., 2020). Therefore, it can catalyze the conversion of large molecules into small molecules and the bioproduction of flavor compounds. In addition, the abundance of *Saccharomyces cerevisiae* was also higher in BQ (18%) than SQ (10%). This species can produce ethanol and esters in fermented foods (Annan et al., 2003).

3.3 Distribution of genes associated with KEGG pathways in the two starters

Annotating the metagenomic data with KEGG level 1 pathways showed that the metabolism pathway had the highest abundance of functional genes, while the environmental processing pathway had the lowest (Supplementary Figure S3A). Annotating the metagenomic data with KEGG level 2 metabolism pathways showed that there was a high abundance of functional genes related to carbohydrate metabolism, amino acid metabolism, energy metabolism, lipid metabolism, metabolism of other amino acids, metabolism of cofactors and vitamins, and so on (Supplementary Figure S3B). Among these pathways, carbohydrate metabolism maintains microbial viability during fermentation, providing important compounds for cell structure, providing energy, producing ethanol, etc. Therefore, it is quite reasonable that the abundance of functional genes is highest in this pathway. These results regarding high abundance of functional genes related to carbohydrate metabolism were similar to previous results concerning *Guizhou Xiaoqu* (Liu et al., 2019) and *Dazhou Xiaoqu* (Xie et al., 2020). Amino acids are important for production of specific flavors, and have a large impact on brewery quality. Cofactors provide redox carriers for biosynthesis and catabolism and play an important role in energy transfer in microbial cells (Wang et al., 2013). Vitamins are often involved in metabolic processes in the

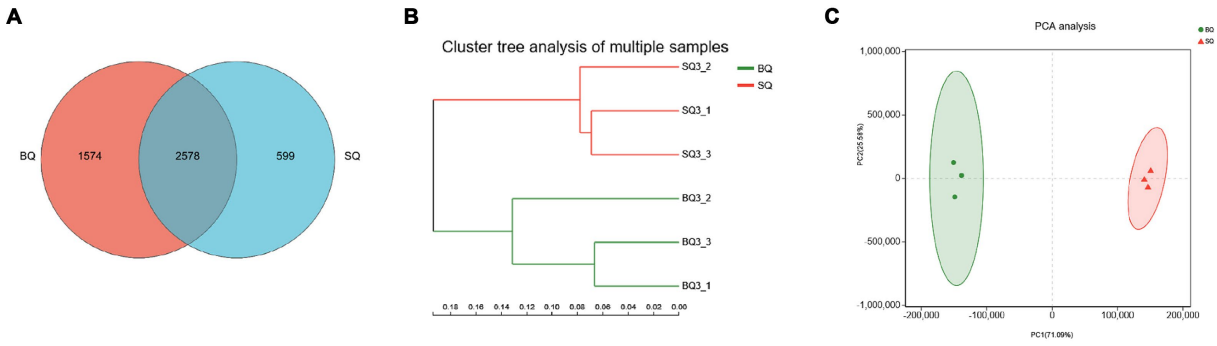

FIGURE 1
Analyses of microbial communities at the species level in the two starters. **(A)** Venn diagram, **(B)** PCA clustering results, and **(C)** PCA score plot.

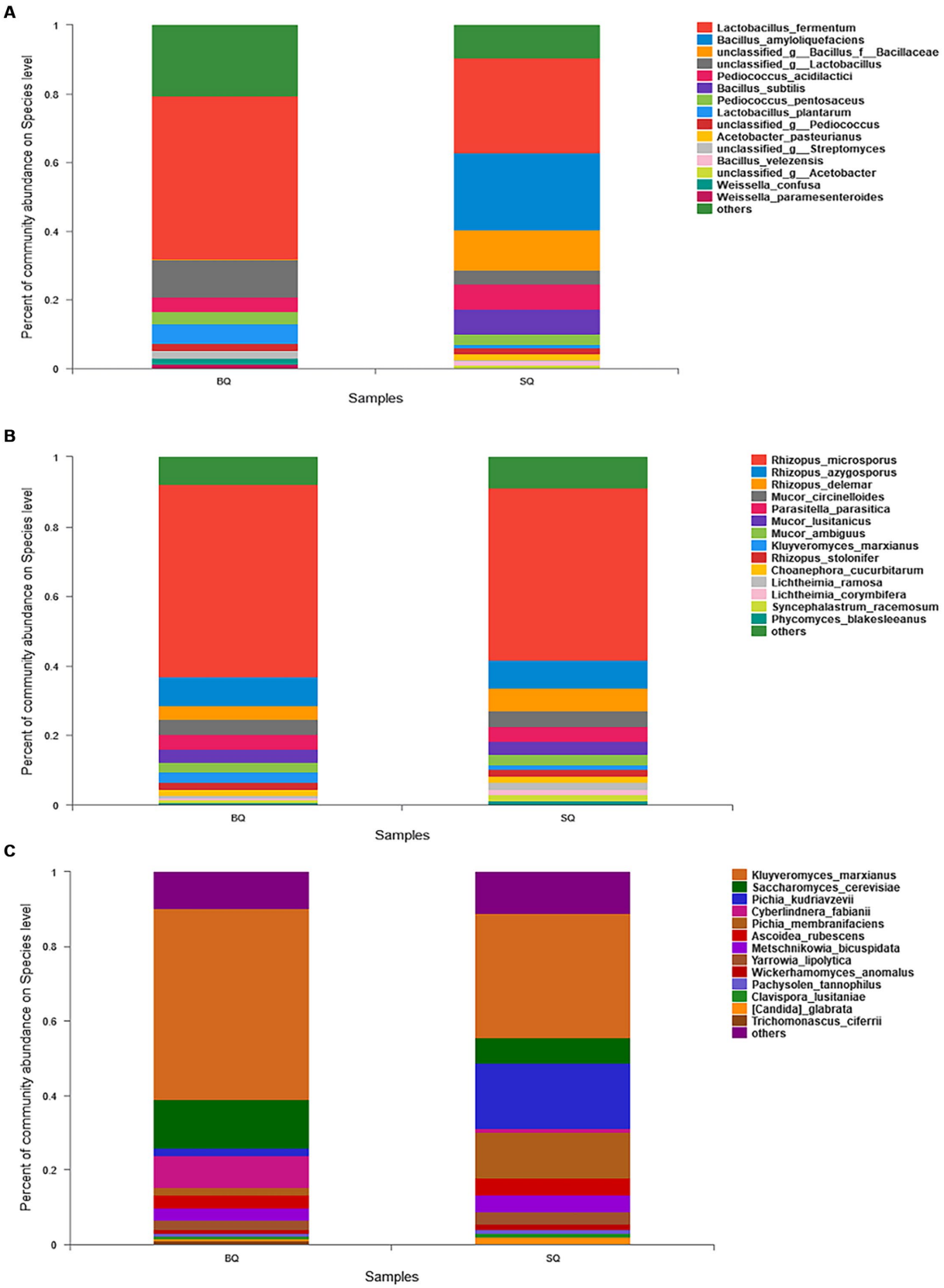

FIGURE 2
Relative abundances of **(A)** bacteria, **(B)** molds, and **(C)** yeast at the species level between the two starters.

form of cofactors. Therefore, there was a high abundance of functional genes related to cofactor and vitamin metabolism.

Regarding the KEGG level 1 pathway annotation, some genes were found to be associated with human diseases. Similar results have been found in other studies of various fermented foods, such as Korean Rice-flavor *Baijiu* (Kim et al., 2015), fermented sweet wort (Menz et al., 2010), and traditional fermented foods from northeastern India (Keisam et al., 2019). However, the presence of these genes does not imply that these foods are pathogenic to humans (Olano et al., 2001). In the case of yellow *Baijiu* fermentation (Liu et al., 2019), the genes associated with human diseases increased and then decreased with fermentation time, which may imply that the microbes or raw materials with genes associated with human diseases are affected by ethanol, *Streptococcus*, yeasts, *Saccharopolyspora, Aspergillus*, and other environmental factors (Vara and Hutchinson, 1988; Flewelling et al., 2015; Phongphakdee and Nitisinprasert, 2015). In addition, a wide variety of starter and *Baijiu* have been used safely for more than 9,000 years (McGovern et al., 2004), so the safety of *Xiaoqu* starter can be guaranteed to a certain extent.

3.4 Differential functional genes between the two starters

Genes related to carbohydrate, amino acid, and lipid metabolism were annotated using the KEGG database.

According to the Venn diagram of genes related to carbohydrate metabolism, there were 61 unique genes in BQ, 19 unique genes in SQ, and 532 common genes (Figure 3A). The PCA confidence intervals were far apart, indicating that the two starters were significantly different (Figure 3B). The LDA identified differential carbohydrate metabolism-related genes. Among the genes that were unique to BQ, >2% were functional genes, including 4-olyl-4-methyl-2-oxoglutarate aldolase (K10218), 2-dehydro-3-deoxygalactose phosphokinase (K00883), D-alpha-alpha-alpha-alpha-6-phosphate differential isomerase (K017195), butanol dehydrogenase (K00100), N-acetylglucosaminoglucosan kinase (K00884), 1,3-propanol dehydrogenase (K00086), succinate dehydrogenase (K00246), α-1-phospho-maltose synthase (K16148), starch synthase (maltosyltransferase) (K16147), and alditol oxidase (K00594). In BQ, the one gene with LDA ≥4 was chitin synthase (K00698), the genes with LDA ≥3 were β-glucosidase (K05349), 1,3-β-glucan synthase (K00706), galactosidase (K07407), malate dehydrogenase (K00029), glycogen synthase (K00693), and 1,3-β-glucosidase (K01210), and the genes with LDA ≥2.5 were glycosylase (K01178), malate dehydrogenase (K00026), glycogen debranching enzyme (K01196), fructose-6-phosphate-2-kinase (K19029), isocitrate decomposing enzyme (K01637), isocitrate dehydrogenase (K00031), malate synthase (K01638), pectinase (K01184), pyruvate kinase (K00873), β-phosphoglucan translocase (K01838), succinate dehydrogenase (K00234), acetyl coenzyme A hydrolase (K01067), (K00830), succinate CoA synthase (K01900), lactate dehydrogenase (K00101), pyruvate dehydrogenase (K21618), ethanol dehydrogenase (K00001), glycogenin (K00750), and 6-phosphofructo-2-kinase (K00900). The set of differential carbohydrate metabolism-related genes in BQ were related to pathways such as the glycolysis pathway, citric acid cycle pathway, glyoxylate pathway, starch synthesis and catabolism pathway, fructose and maltose metabolism pathway, and lactose metabolism pathway. Cellulose, starch, dextrin, maltose, sucrose and other carbon source substances of raw materials can be metabolized into glucose or enter the glycolytic pathway. Therefore, it can be inferred that BQ has a better ability to utilize carbon sources than SQ, with higher levels of saccharification, liquefaction and fermentation. This is consistent with our previous research (Wang et al., 2023).

According to the Venn diagram of genes related to amino acid metabolism, there were 3 unique genes in BQ, 1 unique gene in SQ, and 49 common genes (Figure 3C). The PCA confidence intervals were far apart, indicating that the two starters were significantly

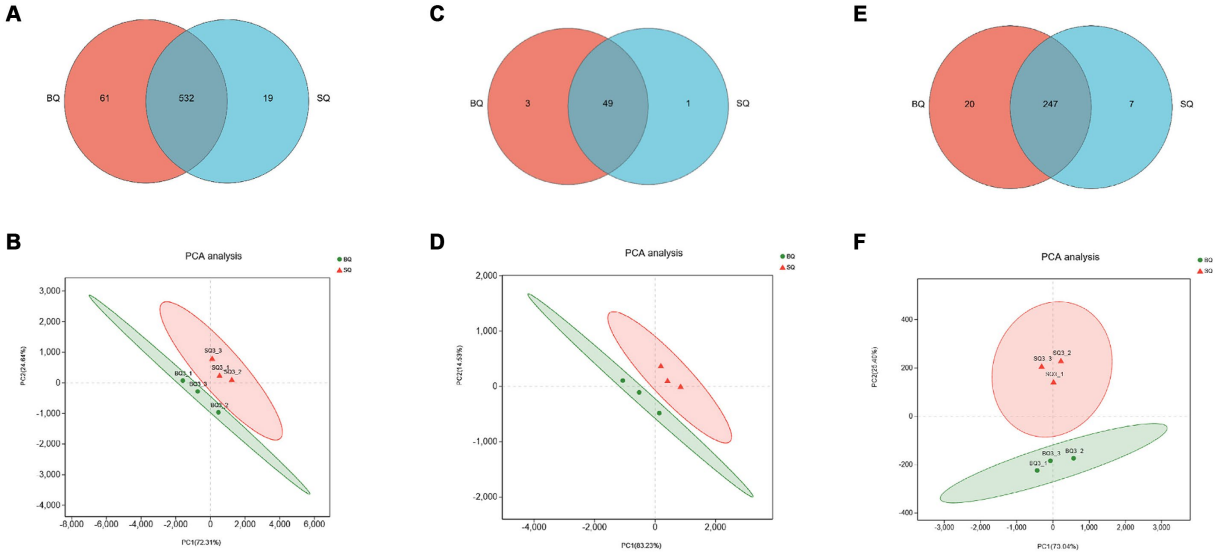

FIGURE 3
Analyses of genes related to three key metabolic pathways in the two starters. Venn diagrams of genes related to **(A)** carbohydrate, **(C)** amino acid, and **(E)** lipid metabolism. PCA score plots of starter samples based on genes related to **(B)** carbohydrate, **(D)** amino acid, and **(F)** lipid metabolism.

different (Figure 3D). The LDA identified differential amino acid metabolism-related genes, including asparaginase (K13051), 2,4-diaminovaleric acid dehydrogenase (K21672), 3-hydroxyacyl-coenzyme A dehydrogenase (K01825), 3-hydroxyisobutyl-CoA hydrolase (K05605), glutamate decarboxylase (K01580), primary amine oxidase (K00277) and glutamine oxidase (K00279), oxidase (K00276), cytoplasmic aminopeptidase (K11142), 4-aminobutyric acid aminotransferase (K13524), glutamate cysteine ligase catalytic subunit (K11204), and cystathionine gamma-cleaving enzyme (K01758). The set of differential amino acid metabolism-related genes in BQ were related to the generation of proline, glutamic acid, cysteine, and glutathione. Amino acids undergo Maillard reaction with reducing sugars, producing various compounds such as furans, pyrans, pyrazines, aldehydes, ketones, etc., which can increase the content of flavor substances in *Baijiu* (Huang et al., 2024). In addition, relevant reports have found that glutathione can reduce the loss of esters and terpenes in *Baijiu*, thereby increasing the content of aroma components (Webber et al., 2014).

According to the Venn diagram of genes related to lipid metabolism, there were 20 unique genes in BQ, 7 unique genes in SQ, and 247 common genes (Figure 3E). The PCA confidence intervals were far apart, indicating that the two starters were significantly different (Figure 3F). The LDA identified differential lipid metabolism-related genes, including fatty acid synthase (K11533), acyl coenzyme A dehydrogenase (K06445), stearoyl coenzyme A desaturase (K22770), 3-phosphoglycerol dehydrogenase (K00112, K00113), glycerol kinase (K00864), acyl coenzyme A oxidase (K00234), cysteine gamma-cleaving enzyme (K01758), acyl coenzyme A oxidase (K00232), fatty acid synthase (K00667), acetyl CoA carboxylase (K11262), and acyl coenzyme A dehydrogenase (K00249). The set of differential lipid metabolism-related genes in BQ were related to pathways such as the fatty acid biosynthesis pathway and fatty acid oxidation pathway. Fatty acids and acetyl CoA can serve as precursors for ester synthesis. Therefore, it can be inferred that BQ has a better esterification ability than SQ. This is consistent with our previous research (Fei et al., 2023; Wang et al., 2023).

A metabolic network of these differential genes is shown in Figure 4. There are a total of 24 dominant differential functional genes, 28 dominant differential genes, and 6 unique functional genes in BQ, respectively.

3.5 Identifying core differential microbes based on core differential genes

The core differential microbes related to carbohydrate metabolism between the two starters are shown in Table 1. Correlation analysis of carbohydrate metabolism-related differential genes, using the NR

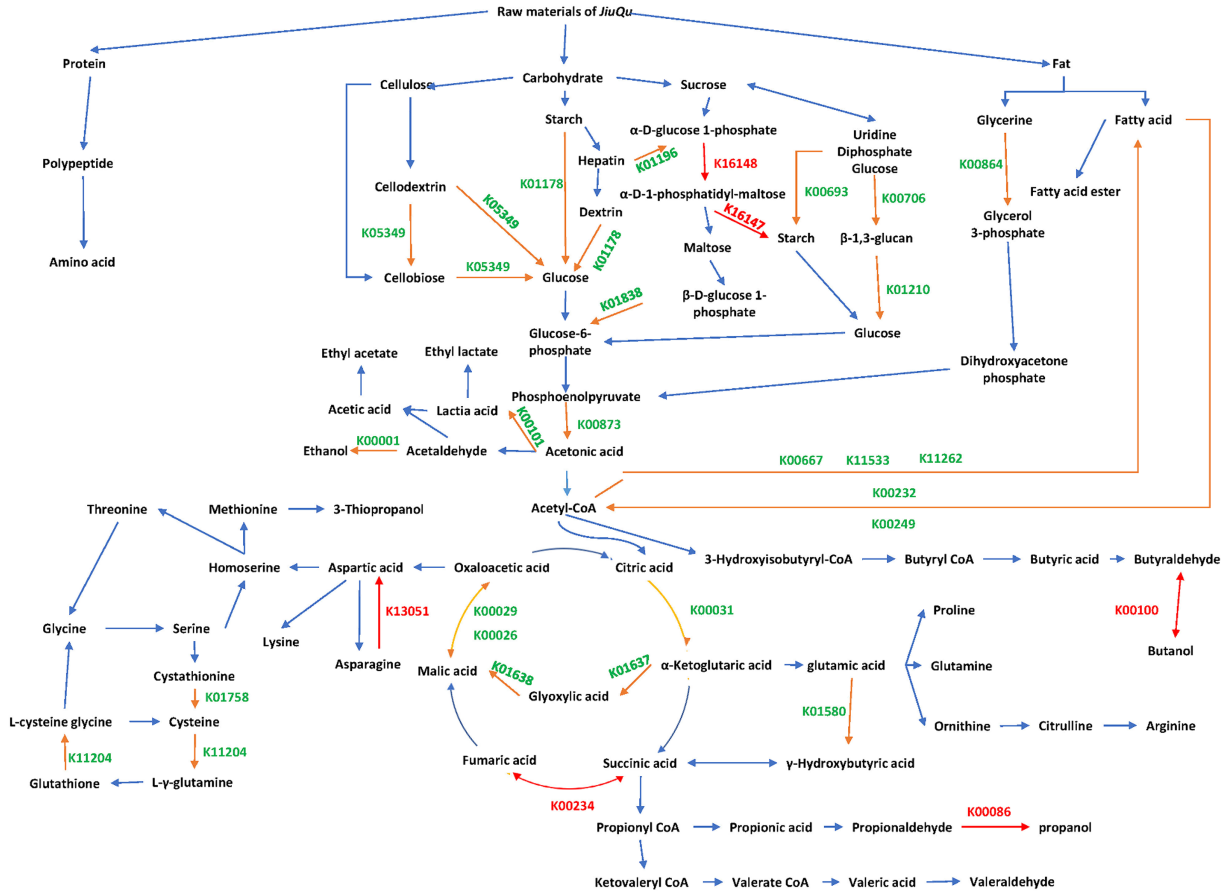

FIGURE 4
Metabolic network of differential genes between the two starters. Orange, green, and red KEGG orthology entries indicate dominant differential functional genes, dominant differential genes, and unique functional genes, respectively.

TABLE 1 Core differential microbes related to carbohydrate metabolism in BQ.

KO	Name	Core microbes
K00693	Glycogen synthase	*Saccharomyces cerevisiae* *Cyberlindnera fabianii* *Mucor lusitanicus* *Mucor ambiguous*
K00706	1,3-β-Glucan synthase	*Cyberlindnera fabianii* *Saccharomyces cerevisiae* *Rhizopus microsporus*
K01196	Glycogen debranching enzyme	*Cyberlindnera fabianii* *Mucor lusitanicus* *Saccharomyces cerevisiae* *Kluyveromyces dobzhanskii*
K01210	1,3-β-Glucosidase	*Rhizopus azygosporus* *Rhizopus microsporus* *Lactobacillus mucosae*
K01178	Glycosylase	*Saccharomyces cerevisiae* *Cyberlindnera fabianii*
K01838	β-Phosphoglucan translocase	*Lactobacillus fermentum*
K05349	β-Glucosidase	*Rhizopus microsporus* *Rhizopus azygosporus* *Lactobacillus mucosae* *Kluyveromyces marxianus*
K16147	starch synthase (maltosyltransferase)	*Microbacterium Indicum*
K16148	α-1-Phospho-maltose synthase	*Brevibacterium* sp. *CS2*
K00029 K00026	Malate dehydrogenase	*Rhizopus microsporus* *Mucor ambiguous* *Rhizopus delemar* *Mucor circinelloides*
K01637	Isocitrate decomposing enzyme	*Diutina rugosa*
K00031	Isocitrate dehydrogenase	*Lactobacillus fermentum* *Kluyveromyces marxianus*
K01638	Malate synthase	*Trichomonascus ciferrii*
K00873	Pyruvate kinase	*Lichtheimia ramosa*
K00234	Succinate dehydrogenase	*Rhizopus azygosporus* *Rhizopus microsporus*
K01900	Succinate CoA synthase	*Ascoidea rubescens*

database, was performed using a *p*-value of 0.05 (Supplementary Figure S4). Regarding carbohydrate utilization, there were 4 yeast species (*Saccharomyces cerevisiae, Cyberlindnera fabianii, Kluyveromyces dobzhanskii,* and *Kluyveromyces marxianus*), 4 bacterial species (*Puccinia cinerea CS2, Microbacterium Indicum, Lactobacillus fermentum,* and *Lactobacillus mucilaginosus*), and 4 mold species (*Mucor lusitanicus* and *Mucor ambiguus, Rhizopus microsporus,* and *Rhizopus azygosporus*). Of these, *Saccharomyces cerevisiae, Cyberlindnera fabinaii, Rhizopus microsporus, Rhizopus azygosporus,* and *Mucor lusitanicus* were positively correlated with genes of multiple enzymes. *Saccharomyces cerevisiae, Cyberlindnera fabinaii* and *Rhizopus microsporus* were shown to be the core microbes for saccharification and liquefaction of starter, as they were associated with genes of K00693, K00706 and K01196. Regarding glycolysis, citric acid cycle, and glyoxylate cycle, *Ascoidea rubescens*, *Lichtheimia ramosa*, *Rhizopus azygosporus*, *Rhizopus microsporus*, *Trichomonascus ciferrii*, *Kluyveromyces marxianus*, *Lactobacillus fermentum*, *Mucor ambiguus*, *Diutina rugosa*, *Rhizopus delemar*, *Mucor circinelloides* were positively correlated with functional genes of these metabolism. *Rhizopus microsporus, Kluyveromyces marxianus, Lactobacillus fermentum and Mucor ambiguus* were consistent with the core microbes discovered in carbohydrate utilization, which indicating that these microorganisms may play a key role in carbohydrate metabolism in BQ. However, there were fewer positively correlated microbes in energy metabolism than in the carbohydrate utilization. Although energy metabolism is necessary for all microbes, some of the microbes were negatively correlated, which may be related to mutual antagonism among the microbes.

The core differential microbes related to amino acid metabolism between the two starters are shown in Table 2. As the variability of amino acid metabolism was lower than that of carbohydrate metabolism, the *p* value for the correlation analysis was increased to 0.5 (Supplementary Figure S5). The core differential microbes related to amino acid metabolism were 6 mold species (such as *Rhizopus microsporus*, *Rhizopus azygosporus*, *Rhizopus delemar*, *Actinomucor elegans*, *Mucor lusitanicus,* and *Lichtheimia ramosa*). *Rhizopus microsporus*, *Rhizopus azygosporus*, *Rhizopus delemar* were consistent with the core differential microbes related to carbohydrate metabolism, suggesting that these microbes play a key role in BQ. *Mucor lusitanicus,* and *Lichtheimia ramosa* were consistent with the core differential microbes related to energy metabolism in the carbohydrate correlation analysis, so these microbes were also important. In addition, *Lactobacillus fermentum, Saccharomyces cerevisiae*, and *Kluyveromyces marxianus* were the core differential microbes related to carbohydrate metabolism, so these microbes may play a key role in BQ.

The core differential microbes related to lipid metabolism between the two starters are shown in Table 3. As the variability of lipid metabolism was lower than that of carbohydrate metabolism, the p value for the correlation analysis was increased to 0.1 (Supplementary Figure S6). The core differential yeast species related to lipid metabolism were *Lachancea kluyveri* and *Cyberlindnera fabianii*, *Cyberlindnera fabianii* was also related to carbohydrate metabolism. The core differential mold species related to lipid metabolism were *Mucor ambiguous*, *Ascoidea rubescens*, *Mucor lusitanicus*, *Rhizopus stolonifer*, *Mucor circinelloides*, *Lichtheimia ramose*, and *Rhizopus delemar*. The first four abovementioned mold species were correlated with ≥2 key lipid metabolism-related genes, suggesting that these molds may play an important role in lipid metabolism in BQ. In addition, the first three abovementioned mold species plus *Cyberlindnera fabianii* were related to carbohydrate metabolism.

The *Mucor lusitanicus* and *Rhizopus delemar* were differential core microbes related to all three metabolic pathways. *Mucor is* dominant fungi and known to be saccharification and esterase production in *Xiaoqu Baijiu* (Xiong et al., 2014; Jin et al., 2017). *Rhizopus is* dominant fungi and known to be instrumental in the aroma enhancement in *Daqu Baijiu* and *Xiaoqu Baijiu* (Hu Y. et al., 2021; Tu et al., 2022). Thus, they may be the most important microbes in BQ. The *Saccharomyces cerevisiae, Cyberlindnera*

TABLE 2 Core differential microbes related to amino acid metabolism in BQ.

KO	Name	Core microbes
K13051	Asparaginase	*Serratia rubidaea*
K05605	3-Hydroxyisobutyl-CoA hydrolase	*Rhizopus microsporus*
K01580	Glutamate decarboxylase	*Rhizopus azygosporus* *Lactobacillus fermentum* *Saccharomyces cerevisiae* *Rhizopus microsporus*
K11142	Cytoplasmic aminopeptidase	*Rhizopus azygosporus* *Rhizopus microsporus* *Rhizopus delemar* *Actinomucor elegans* *Mucor lusitanicus*
K01758	Cystathionine γ-cleaving enzyme	*Rhizopus microsporus* *Unclassified Streptomyces* *Kluyveromyces marxianus*
K11204	Glutamate cysteine ligase catalytic subunit	*Rhizopus microsporus* *Lichtheimia ramosa*

TABLE 3 Core differential microbes related to lipid metabolism in BQ.

KO	Name	Core microbes
K11533	Fatty acid synthase (Bacteria)	*Corynebacterium variabile* *Corynebacterium nuruki*
K00864	Glycerol kinase	*Lichtheimia ramosa* *Lactobacillus helveticus* *Pediococcus acidilactici*
K11262	Acetyl CoA carboxylase	*Rhizopus stolonifer* *Rhizopus delemar* *Ascoidea rubescens*
K00667	Fatty acid synthase (Fungus)	*Rhizopus stolonifer* *Mucor lusitanicus* *Lachancea kluyveri*
K00232	Acyl coenzyme A oxidase	*Rhizopus delemar* *Ascoidea rubescens* *Mucor ambiguous* *Mucor lusitanicus* *Cyberlindnera fabianii* *Mucor circinelloides*
K00249	Acyl coenzyme A dehydrogenase	Not found

fabianii, Kluyveromyces marxianus, Lactobacillus fermentum, Mucor ambiguous, Rhizopus microspores, Rhizopus azygosporus, Mucor circinelloides, and *Ascoidea rubescens* were significantly positively correlated with two of these three metabolic pathways. *Saccharomyces cerevisiae* and *Lactobacillus* was dominant microbes in *Daqu Baijiu* and *Xiaoqu Baijiu* (Jin et al., 2017; Zhu et al., 2024). *Cyberlindnera fabianii* and *Kluyveromyces marxianus* was non-conventional yeast and has greater ester synthesis ability than *Saccharomyces cerevisiae* (Van Rijswijck et al., 2017; Karim et al., 2020). Therefore, they may be also important microbes in BQ. At present, there is no relevant report on *Ascoidea rubescens* in Chinese *Baijiu*. To be verified through future experiments.

4 Conclusion

In this study, the differences in microbial communities and core metabolism genes between traditional and mechanical starters of Chi-flavor *Baijiu* from a Cantonese *Baijiu* factory were investigated using metagenomic technology. The core differential microbes were identified based on a correlation analysis using the NR database. Several core microbes related to carbohydrate, amino acid, and lipid metabolism were differentially enriched in the traditional starter. *Mucor lusitanicus* and *Rhizopus delemar* were significantly positively correlated with all three key metabolic pathways, i.e., carbohydrate, lipid, and amino acid metabolism. *Saccharomyces cerevisiae, Cyberlindnera fabianii, Kluyveromyces marxianus, Lactobacillus fermentum, Mucor ambiguous, Rhizopus microspores, Rhizopus azygosporus, Mucor circinelloides,* and *Ascoidea rubescens* were significantly positively correlated with two of these three metabolic pathways. The results of this study provide a basis for understanding the differences in core microbes between traditional and Round-Koji-mechanical starters, and they also provide guidance for improving Round-Koji-mechanical starter. For example, to increase the level of core microbes in the Round-Koji mechanical starter by optimizing the making conditions of starter. Further core microbes screening and identification will be conducted to gain deeper insights into the two starter types.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Author contributions

JL: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. LD: Data curation, Formal analysis, Investigation, Methodology, Software, Writing – original draft. ZL: Investigation, Methodology, Writing – original draft. YF: Supervision, Writing – review & editing. WB: Supervision, Writing – review & editing. WZ: Supervision, Writing – review & editing. SH: Supervision, Writing – review & editing. RC: Supervision, Writing – review & editing.

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Conflict of interest

SH and RC are employed by Guangdong Jiujiang Distillery Co. Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1390899/full#supplementary-material

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Bacterial composition and physicochemical characteristics of sorghum based on environmental factors in different regions of China

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The fermentation process for Jiang-flavored baijiu using sorghum as the raw material involves a variety of microorganisms. However, the specific physicochemical characteristics of sorghum and microbial composition on its surface have not been fully elucidated. We aimed to perform a comprehensive comparative analysis of the variations in physicochemical properties and surface microflora in waxy sorghum samples from three prominent production regions in China (Renhuai, Jinsha, and Duyun). Multivariate statistical assessments were conducted that incorporated local soil and climate variables. The results showed that Cyanobacteria, unclassified bacteria, Proteobacteria, Firmicutes, and Bacteroidota were the dominant bacteria in these regions. These bacteria were associated with ethyl acetate, ethyl caprylate, ethyl lactate, and butyl groups, which synergistically produce flavorful compounds. The surface bacterial communities were affected by soil total phosphorus, altitude, diurnal temperature range, monthly mean temperature, precipitation, and effective accumulated temperature. The findings of this study provide a new perspective on microorganisms related to Jiang-flavored baijiu and can help establish a reference for the stability of liquor quality.

KEYWORDS

Jiang-flavored baijiu, sorghum, physicochemical indexes, bacterial community, environmental factors

1 Introduction

Jiang-flavored baijiu is one of the world's highest-selling distilled alcoholic spirits made from waxy sorghum (*Sorghum bicolor* L. Moench) as the main raw material (Niu et al., 2022; Wang, 2022). It is produced by solid-state fermentation and distillation, resulting in a strong soy sauce aroma and a long-lasting fragrance (Yan, 2019; Gao et al., 2024). Regional variations in the sorghum and baijiu quality characteristics are critical features of the perceived product identity, with significant consequences for consumer preference and economic appreciation (Liu et al., 2023). These variations may be associated with local climatic and soil conditions that play important roles in sorghum quality and microbiology (Niu et al., 2022).

The liquor quality depends on the physicochemical properties of the sorghum (Liu et al., 2021a). Starch, protein, fat, and other components of sorghum can not only be used as substrates

that provide energy for microorganisms during the fermentation process but can also generate many flavor compounds or participate in subsequent metabolic reactions as flavor precursors (Li E. et al., 2021; Li H. et al., 2021). Numerous studies have reported differences in the physicochemical indices and regional characteristics. It is generally agreed that southern Chinese sorghum has a higher content of amylopectin, fat, and tannins than sorghum from northern China. In addition, differences in the amino acid metabolism, phenylpropanoid biosynthesis and flavonoid biosynthesis pathways result in a higher production of acid ester flavoring substances, making southern Chinese sorghum more suitable for brewing Jiang-flavored baijiu with optimal flavor and quality than sorghum from the north (Zhang et al., 2022). However, the same variety of sorghum grown in different areas may also be affected by environmental factors, such as soil and climate, resulting in different physicochemical properties.

The brewing process of Jiang-flavored baijiu is a complex natural multispecies solid fermentation process in which bacteria, actinomycetes, yeasts, and molds work together to establish a complex, dynamic, and balanced microbial ecosystem (Tu et al., 2022; Xu et al., 2022). Theoretically, many microorganisms inhabiting the sorghum surface cannot survive at high temperatures but their metabolic activities can have long-term effects (Bokulich et al., 2014; Gao et al., 2019). Sorghum is one of the main sources of bacteria during the fermentation process. During the production of Jiang-flavored baijiu, grain wetting, steaming, cooling, mixing, and bacterial cultivation are all conducted in the same semi-open plant so that the microorganisms on the surface of the sorghum raw material can become airborne and integrate into the brewing environment (Dai et al., 2020; Fan and Xu, 2023). In addition, during the grain-turning process, sorghum may be exposed to uneven heat, which can result in the survival of some microorganisms in the form of spores that can survive and enter the subsequent fermentation processes. The structure of the microbial communities on crop surfaces is often influenced by environmental factors. Research has shown that habitat heterogeneity shapes the distribution of fungi and bacteria in brewing environments (Ren et al., 2023; Yuan et al., 2023), while climatic conditions and geographic factors may play a role in controlling microbial community composition (Ding et al., 2021). However, it is unclear whether there is a non-random geographical distribution pattern of microorganisms on the surface of waxy sorghum and understanding their distribution patterns and mechanisms requires further exploration.

Guizhou Province is the core production area for Jiang-flavored baijiu owing to its unique geography and climate. Improvements in the efficiency of the liquor industry have effectively stimulated demand for brewing sorghum (Liu et al., 2021b). Starting in 2020, Guizhou Province prioritized the waxy sorghum planting industry as a key commercial opportunity, with the authorities in Guizhou establishing sorghum planting demonstration sites in addition to Renhuai (e.g., a prominent production region) and gradually forming suitable production areas in the north, southwest, and south of Guizhou. Although the fermentation characteristics of waxy sorghum production in various regions have been reported, there is a lack of information on the physicochemical properties and microbial community composition on the surface of the same variety sourced from different locations. Therefore, the aim of this study was to analyze the physicochemical properties and obtain information on the structure of the microbial communities and dominant functional genes in sorghum from production regions in Renhuai, Jinsha, and Duyun using high-throughput sequencing. To explore the factors that influence surface microorganisms, we collected soil from different production areas and downloaded the relevant meteorological data. This study provides information that supports the improvement of baijiu enterprise product stability and competitiveness through differential development. This study also provides an important scientific basis for evaluating microbial resources for brewing.

2 Materials and methods

2.1 Sample source

The sorghum variety 'Hongliangfeng No. 1' [registration number: GPD Sorghum (2017) 520029] was used in this study. The sorghum was sourced from a factory that obtained waxy sorghum from three supply bases: Renhuai (RH), Duyun (DY), and Jinsha (JS). All three bases are in the key waxy sorghum industrial belt in Guizhou Province, China (Figure 1). In August 2022, six experimental sample plots were randomly selected at each base and the latitude, longitude, and elevation of the sample plots were recorded using a handheld GPS.

Soil samples were collected alongside the sorghum samples. Sorghum batches from all three supply bases were harvested and numbered to indicate their origin (e.g., RH sorghum, DY sorghum, or JS sorghum). Fermented grain samples were collected from the same distillery and workshop. Sorghum from the three regions was first cooked and fermented in piles for 48 h. Then, we collected three portions from the top, middle, and bottom layers of each pile and mixed them well. Finally, we analyzed the samples to determine their volatile compound content.

2.2 Sample collection

Sorghum samples were collected from five randomly selected healthy sorghum plants with the same length from each experimental plot. Sorghum plants of a uniform size and similar color without obvious pests or diseases were selected, placed in sterilized bags and transported to the laboratory via a cold chain at 4°C. Sorghum collected from the nine sample plots was thoroughly mixed and divided into three portions for testing. Each sample was split in half, with one half used for sorghum grain quality analysis and the other half used for high-throughput sequencing after extracting the surface bacterial DNA. Soil samples were collected from the inter-root soil (0–30 cm) of each mature sorghum kernel. Debris, such as litter and gravel, were removed and the soil samples were passed through a 2-mm sieve. The samples were then brought back to the laboratory to determine their chemical properties. Fermented grain samples were taken from the top, middle, and bottom of three fermented grain piles (20 cm); six sampling points and three replicates of the samples were collected, placed in Ziplock bags and transported to the laboratory via a cold chain at 4°C to determine the volatile components.

2.3 Climate data

Sorghum at the three bases (RH, DY, and JS) was planted at the end of March and harvested at the beginning of September 2022. Therefore, climate observation data from March to September 2022

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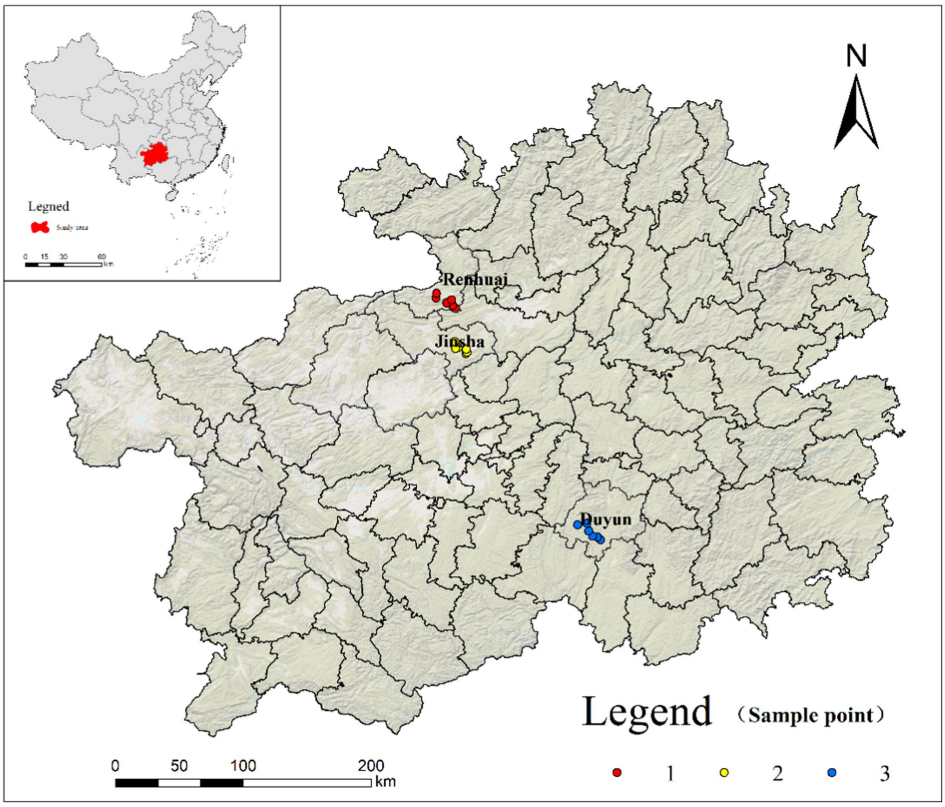

FIGURE 1
Geographic locations of sampling sites.

were selected. These data included the average monthly temperature, effective accumulated temperature, diurnal temperature variation, precipitation, and relative humidity, which were obtained from the daily value dataset of surface climate data provided by the China Meteorological Science Data Sharing Service Network.

2.4 Chemical analysis

The starch content in sorghum was measured as previously described (McCleary et al., 1994). Amylose and amylopectin content were determined using the ISO 6647-2-2007, protein content was determined using the ISO 11085:2015, fat content was determined using the ISO 20483:2013 standard and tannin content was determined using the ISO 9648:1988.

Soil samples were analyzed using agricultural chemical analysis of soil protocols (Bao, 2000) for the indicators, pH, organic matter, total nitrogen, total phosphorus, total potassium and rapidly available nitrogen, phosphorus, and potassium.

2.5 Volatile compound analysis

Volatile compounds were analyzed using headspace solid phase microextraction. The pretreatment method was used as previously described (Ren et al., 2023) with some modification. We added 2.00 g fermented grains into a 20 mL headspace vial, to which 5 mL saturated NaCl solution was added. Then, 6 μL 4-octanol (0.5 mg/mL) was added as an internal standard and the sample was sonicated for 10 min at 90 W. The volatile compounds were extracted using a 50/30 μm DVB/CAR/PDMS fiber (Supelco, Bellefonte, PA, USA) and analyzed using HS-SPME-GC–MS (TSQ 8000 Evo, Trace MS/GC; Thermo Fisher Scientific, Waltham, MA, USA) equipped with a TG-5MS column (30 m × 0.25 mm × 0.25 μm; J&W Scientific, Folsom, CA, USA) and a flame ionization detector. The gas chromatography–mass spectrometry had a starting temperature of 40°C held for 3 min, which was then increased to 100°C at a rate of 2°C/min and held at 100°C for 5 min before it was increased to 150°C at a rate of 2°C/min and held at 150°C for 2 min, and finally increased to 230°C at a rate of 10°C/min and held at 230°C for 5 min. Mass spectra were generated in electron ionization mode at 70 eV. The full-scan mode ranged from 28 to 500 amu. Quantitative analysis of the volatile substances was performed by matching the standard mass spectra with the NIST11. al spectral database (Agilent Technologies, Inc., Santa Clara, CA, USA).

2.6 High-throughput sequencing analysis

Sterile water (450 mL) was added to 50 g sorghum and the mixture was then sealed before being shaken at a constant temperature for 30 min. A 0.22 μm filter membrane was used to extract the bacterial suspension and the membrane was sent to Beijing Biomarker Biotechnology Company Limited at 4°C for

sequencing. The 338F (5′-ACTCCTACGGGAGGCAGCAG3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) primers were used to amplify the 16S rRNA gene of the bacterial V3–V4 variable region. The amplification procedure was as follows: 94°C pre-denaturation for 4 min, 30 cycles of 94°C denaturation for 60 s, 53°C annealing for 60 s, 72°C extension for 60 s and 72°C extension for 10 min. The reaction was composed of 10 × 2.5 μL FastPfu buffer, 0.25 μL 2.5 mmol/L dNTPs, 0.4 μL primers (5 μmol/L), 0.4 μL FastPfu polymerase and 10 ng DNA template. The PCR products were detected on 2% (w/v) agarose gels and the size of the paired-end sequence was no less than 550 base pairs. The PCR products were analyzed using the Illumina MiSeq platform (San Diego, CA, USA). Raw data were filtered to remove joints and low-quality sequences through Quantitative Insights into Microbial Ecology (QIIME2 v2020.6). Operational taxonomic units (OTUs) were clustered with a threshold of 97% sequence similarity using UPARSE software (version 10) and diversity indices, including Shannon, Simpson, Chao 1, and phylogenetic diversity (PD) tree indices, were calculated using QIIME2 2020.6 (Wang, 2022). The metabolic function prediction tool for the flora was passed through the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt). The 16S rRNA gene sequences from the samples were used for metabolic function prediction with the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Louca et al., 2016).

2.7 Statistical analysis

One-way analysis of variance (ANOVA) was used to evaluate the effects of the soil chemical properties, bacterial community diversity, and relative abundance of predicted functional genes in different production areas. Pearson's correlation was used to analyze the relationship between bacterial community composition and flavor matter before the significance of the results was corrected for the false discovery rate (FDR) by p-adjustment. These analyses were performed using Origin 2021 software[1]. Key environmental factors affecting the bacterial community structure were assessed by redundancy analysis (RDA) using Canoco 5.0[2].

3 Results

3.1 Differences in the physicochemical properties of sorghums

We found that JS sorghums exhibited the highest total starch content at 66.54%, whereas RH sorghums had the lowest total starch content (64.94%) but the highest amylopectin content (61.88%), indicating significant differences among the three producing areas, while the content of amylopectin in DY was significantly lower than that in other areas (54.46%) (Figure 2 and Supplementary Table S1). Across all the regions, amylopectin accounted for over 96% of the total starch content. A comparison of the tannin, protein, and fat content across the three regions indicated that the order of their content levels was DY > RH > JS, which underscored the variations in the physicochemical characteristics of sorghum sourced from the three production regions.

3.2 Comparison of bacterial communities

We next examined the variations in sorghum-surface bacterial community composition across the three distinct production regions at both the phylum and genus levels. The predominant bacterial phyla identified were Cyanobacteria, unclassified bacteria, Proteobacteria, Firmicutes, and Bacteroidota (Figure 3A). Each exhibited a relative abundance that exceeded 1%, with the Cyanobacteria phylum more abundant in the DY samples (66.29%) than in the RH samples (45.77%). This difference in phyla composition represented a statistically significant difference. The percentage of Proteobacteria in the samples was highest in RH samples at 19.20%, when compared with that in the JS (15.23%) and DY (2.83%) samples. The abundance of Acidobacteria was 1.15% in JS, 0.74% in DY, and 0.39% RH samples. At the bacterial genus level, the dominant bacteria included unclassified Cyanobacteriales, unclassified bacteria, Pseudomonas, Pantoea and Kosakonia (Figure 3B). The relative abundance of Pseudomonas in the RH sample was significantly higher (17.49%) than that in JS (2.52%) or DY (0.44%). The relative abundance of Pantoea (4.36%) and Kosakonia (2.49%) was significantly higher in the JS production area than in the other two regions. However, no statistically significant differences were observed in the Shannon–Winner and Simpson diversity indices across the three production areas (Figure 4). Furthermore, the Chao 1 and PD tree indices showed no significant variation between the DY and JS samples, whereas both indices were notably higher in the RH production area.

Venn diagrams were used to depict the distribution of shared and unique OTUs between samples (Figure 5). In the present study, 4,939 bacterial OTUs were identified across the samples from the three

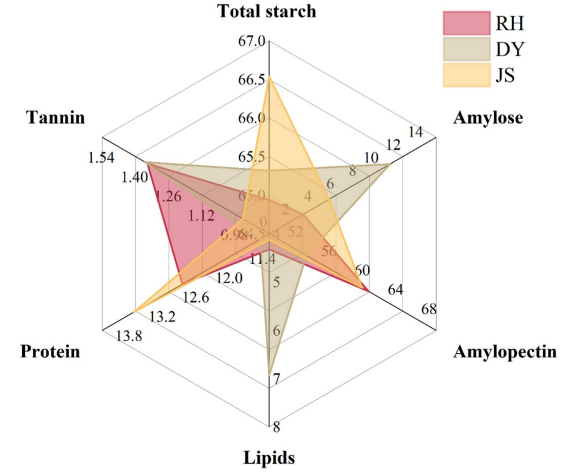

FIGURE 2
Characteristics of physicochemical quality of sorghum grain in different production areas. RH, Renhuai; JS, Jinsha; DY, Duyun.

1 https://www.originlab.com/2021
2 http://www.canoco5.com/

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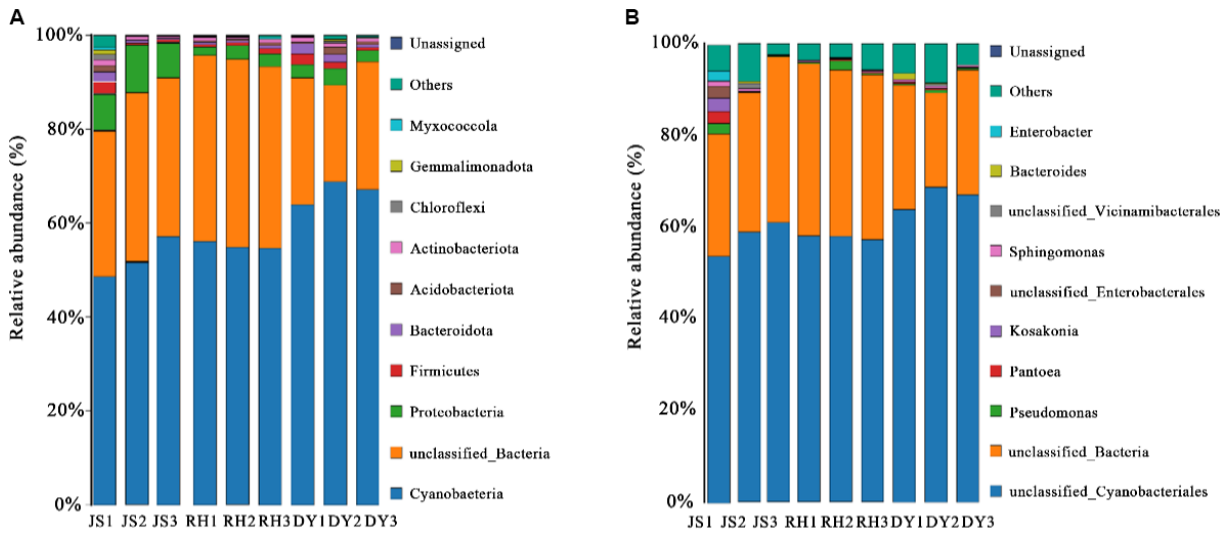

FIGURE 3
Microbial community composition among sorghum samples from the three production areas. Diagrams for bacteria **(A)** phylum and **(B)** genus abundance. RH, Renhuai; JS, Jinsha; DY, Duyun.

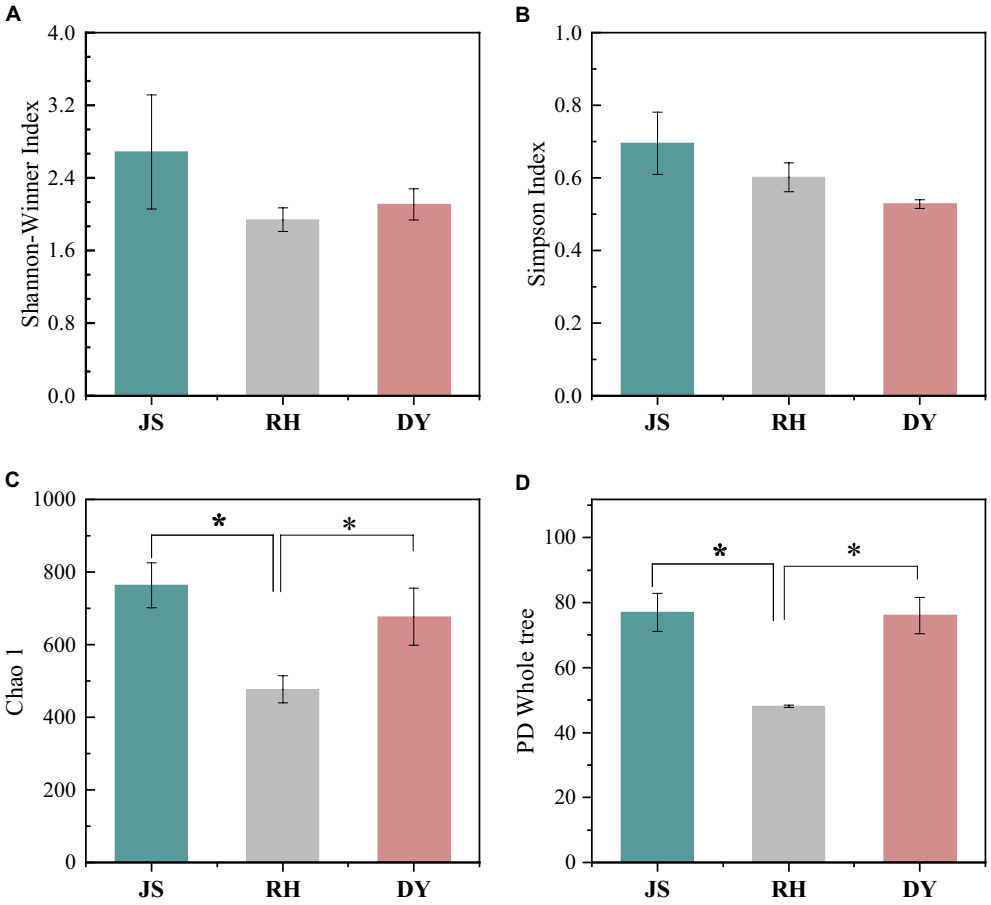

FIGURE 4
Species alpha diversity among the three regional sorghum samples. **(A)** Shannon–Winner index; **(B)** Simpson index; **(C)** Chao 1 index; **(D)** PD tree index. RH, Renhuai; JS, Jinsha; DY, Duyun.

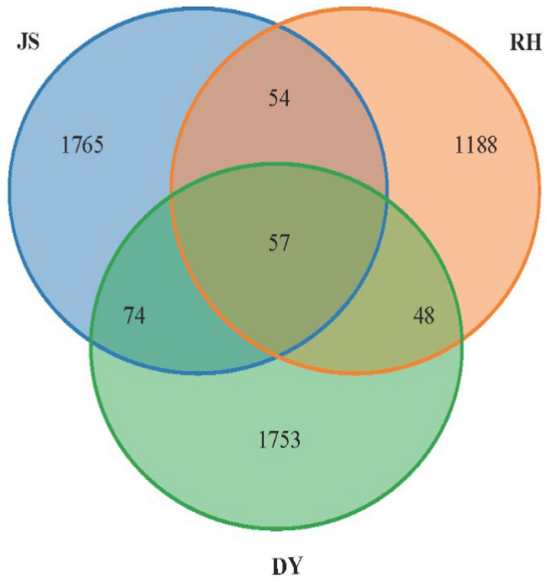

FIGURE 5
Venn diagram of the OTUs. RH, Renhuai; JS, Jinsha; DY, Duyun.

different regions, with 1950 OTUs in JS, 1347 in RH and 1923 in DY. The proportion of 57 shared OTUs accounted for only 1.15% of the overall OTUs. Conversely, the number of OTUs exclusive to each origin far exceeded the number of shared OTUs, indicating substantial differences in the bacterial community composition among the three sorghum production regions.

3.3 Environmental differences among sorghum production regions

Throughout the growing season, the mean monthly temperature was highest in RH, followed by DY and then JS. This pattern was primarily driven by the topographical differences; as the RH area is situated at a lower elevation it experiences progressively higher temperatures, particularly in the month of June. The diurnal temperature variation significantly impacts crop quality, with higher variations leading to increased levels of photosynthetic products, such as sucrose and starch (Tu et al., 2022). The diurnal temperature variation of the regions was in the descending order of DY > JS > RH during the growing seasons (Supplementary Figure S2B). However, the soil physicochemical properties, including pH and total nitrogen and phosphorus content, did not show significant differences among the three production areas ($p > 0.05$).

When comparing the organic matter content (Supplementary Figure S3) of the various production areas, the RH production area exhibited the highest content, followed by the DY production area. The levels of total phosphorus and rapidly available nitrogen and phosphorus were significantly higher in the DY production area than in the RH and JS production areas ($p < 0.05$). Analysis of the rapidly available potassium content indicated a descending ranking of JS > DY > RH, with a highly significant difference observed among the three production areas ($p < 0.001$). The analysis of bacterial taxa and their abundance revealed a first axis gradient of 0.785 that was below the threshold of 3.0, suggesting that bacterial taxa and environmental factors were conducive to RDA. Subsequent RDA demonstrated that the cumulative explanatory rates of the first and second ordination axes were 41.01 and 24.42%, respectively, indicating statistical significance (Figure 6).

The impact of environmental factors on bacterial communities was assessed and ranked in descending order as follows: ELE > EAT > AMT > RH > Pr > DTV > TP > TN. Stronger correlations were observed between Pseudomonas, Pantoea and Kosakonia and soil TP, ELE, and DTV at smaller angles. The unclassified Cyanobacteriales showed stronger correlations with soil TD and relative humidity when the angles were smaller. Notably, a strong correlation was observed between unclassified Cyanobacteriales and soil TD, ELE, and DTV, with small correlation angles, suggesting a significant increase in their abundance with higher levels of these soil properties. Conversely, Bacteroides, unclassified Vicinamibacterales and Sphingomonas were primarily influenced by the monthly mean temperature, precipitation, and effective accumulative temperature.

3.4 Functional gene prediction

PICRUSt compares microbial community abundance with a marker gene sequencing profile database to predict bacterial community function. By using high-throughput sequencing technology and comparing it with the KEGG database (Figure 7), we observed that the bacterial genes were associated with six types of metabolic pathways at the primary functional level: cellular processes (2.65%), environmental information processing (6.83%), genetic information processing (7.06%), metabolism (78.39%), human diseases (3.15%), and organismal systems (1.92%). Metabolism was the predominant functional component (Supplementary Figure S2) while at the secondary functional level, 41 categories of metabolic pathways were identified, 16 of which exhibited relative abundances greater than 1% (Supplementary Figure S3). Notably, global and overview maps, carbohydrate metabolism, amino acid metabolism, energy metabolism and the metabolism of cofactors and vitamins emerged as the major metabolic functions in bacterial communities. However, there was no significant difference in the number of functional genes within the bacterial communities across the different sorghum production regions at either the primary or secondary functional levels, indicating that various strains of sorghum-surface bacteria have unique functions irrespective of regional differences. The bacterial communities at the phylum level, which are closely related to secondary functions, are shown in Figure 7. Proteobacteria, Bacteroidota, Firmicutes, Acidobacteriota, Actinobacteriota, Chloroflexi, Myxococci, and Gemmatimonadota had higher relative abundances in metabolic pathways, such as carbohydrate, amino acid, and energy metabolism, as well as the metabolism of cofactors and vitamins. Thus, these phyla may be important in the production of the alcohols, aldehydes, acids, and esters that act as flavoring substances in baijiu. The bacterial communities on the sorghum surface are involved in processes, such as saccharification and fermentation during the brewing process and may be key players in metabolite production.

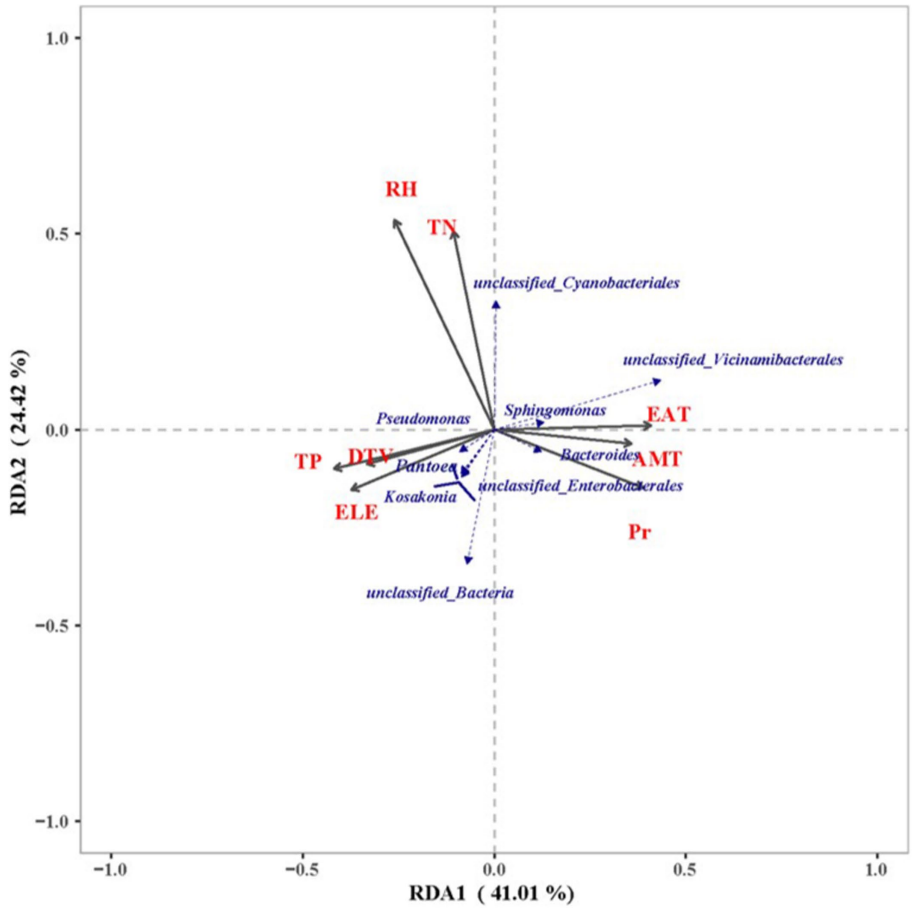

FIGURE 6
Ordination biplot showing the first two-axis results from redundancy analysis (RDA) of microbial communities. TN, total nitrogen; TP, total phosphorus; AMT, average monthly temperature; EAT, effective accumulated temperature; DTV, diurnal temperature variation; Pr, precipitation; RH, relative humidity; ELE, elevation.

3.5 Correlation of flavor substances with microorganisms

Correlation analysis between bacterial communities and flavor compounds indicated a strong positive correlation between ethyl acetate and Acinetobacter ($p < 0.01$) and significant negative correlations between ethyl acetate and Staphylococcus and Microbacterium ($p < 0.05$) (Figure 8). Furthermore, Acinetobacter was significantly and negatively correlated with benzaldehyde and ethyl caprylate ($p < 0.05$), while ethyl lactate synthesis was significantly positively correlated ($p < 0.05$) with Kosakonia, Enterobacter, and Methylobacterium and significantly negatively correlated ($p < 0.05$) with Sodalis and Pantoea. Butyl caproate showed a significant negative correlation ($p < 0.05$) with Idiomarina, whereas unclassified Lachnospiraceae showed a significant negative correlation with these bacteria ($p < 0.05$). This correlation pattern was very similar to the production of 2,3-butanediol, although it was negatively correlated with Alphaproteobacteria and Cyanobium. These results showed that there is a correlation between these flavor compounds and the diversity of the bacterial community on the sorghum surface.

4 Discussion

Variations in the flavor profile of Jiang-flavored baijiu within a single batch are attributed to differences in the raw materials, with particular emphasis on the impact of the chemical properties of sorghum. Numerous studies have investigated the influence of factors such as starch type, starch content, protein content and tannin content on the flavor profiles of baijiu (Li et al., 2021; Ucella-Filho et al., 2022; Wang et al., 2023). Starch, as the primary carbon source for brewing microorganisms, plays a crucial role in the production of ethanol and generation of flavor precursors (Niu et al., 2022; Pang et al., 2023). The waxy sorghum used for brewing must possess a total starch content within the range of 50–75%, as stipulated by the brewing guidelines for Jiang-flavored baijiu (Cheng et al., 2022). Notably, many factors influence baijiu yield, with a positive correlation between the total starch content and yield (Ren et al., 2023). Our investigation revealed that the total starch content of the Hongliangfeng No. 1 waxy sorghum complied with the brewing specifications irrespective of where it was produced; however, variations in the chemical parameters among the sorghum obtained from the different production regions were observed. The JS sorghum exhibited elevated levels of total starch, RH

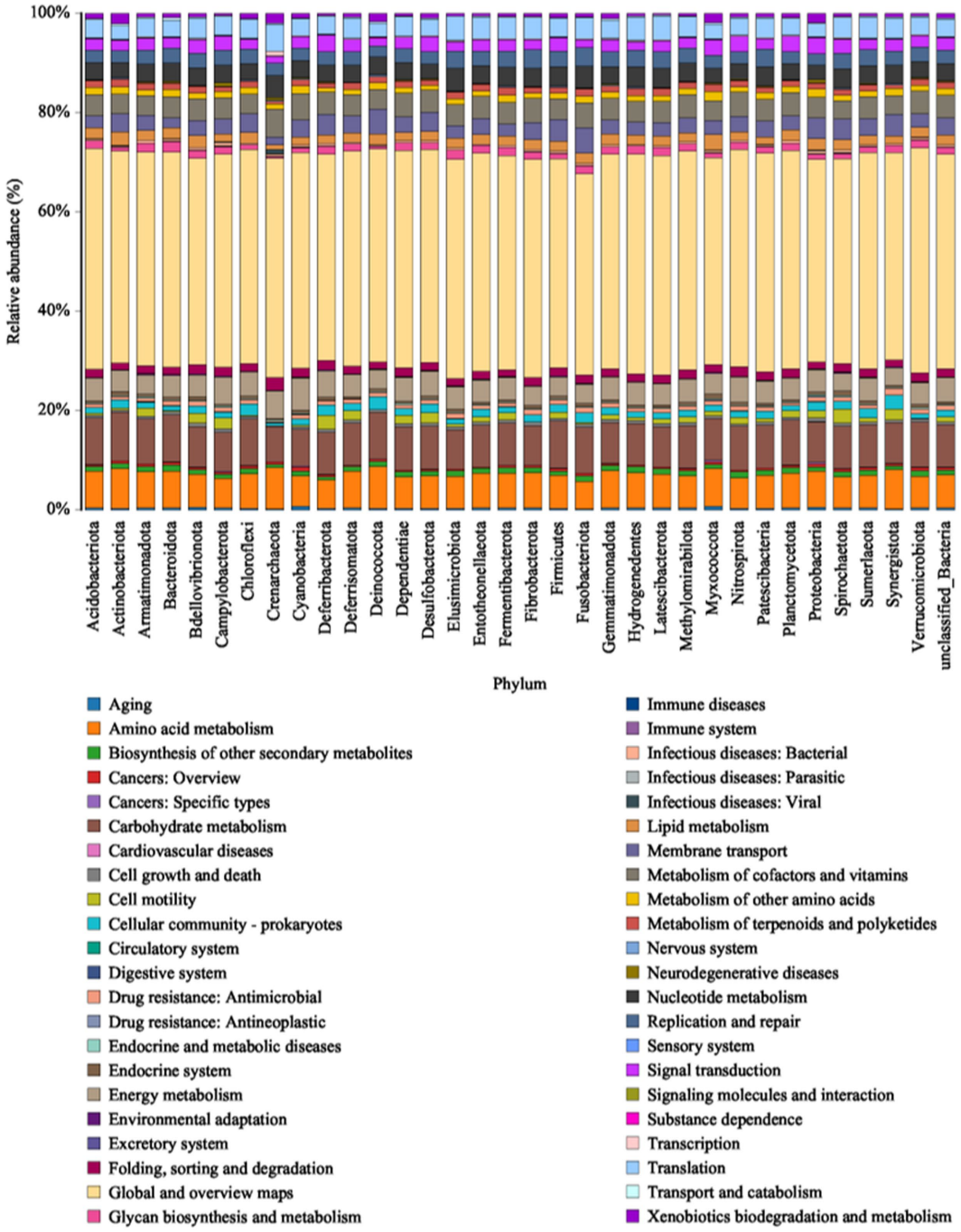

FIGURE 7
Histogram of the KEGG metabolic pathways associated with microbial communities.

sorghum had a notable abundance of amylopectin and DY sorghum had the highest concentrations of tannin, protein, and fat of the three varieties. The fermentation of Jiang-flavored baijiu requires nine rounds of stewing, eight rounds of fermentation, and seven rounds of extraction. Aroma substances are gradually released into fermented grains during the decomposition process (Li et al., 2021; Xu et al., 2022). Therefore, the amylopectin content of sorghum is a crucial factor in determining the quality of Jiang-flavored baijiu. Proteins, fats, and tannins are also important components in baijiu production; however, the inverse relationship between tannin content and yield can be attributed to the detrimental effect of excessive tannins on microbial activity, which thereby hinders fermentation (Ucella-Filho et al., 2022). The flavor of sorghum-brewed Jiang-flavored baijiu from different production areas is influenced by the chemical properties of the raw materials of the original plant.

Although many articles have been published on the effects of different sorghum varieties on baijiu flavor (Zhang et al., 2022; Liu M. et al., 2023; Liu M.-K. et al., 2023), little attention has been paid to how differences in the microbiota on the sorghum surface affects baijiu flavor. Baijiu production involves both positive and negative

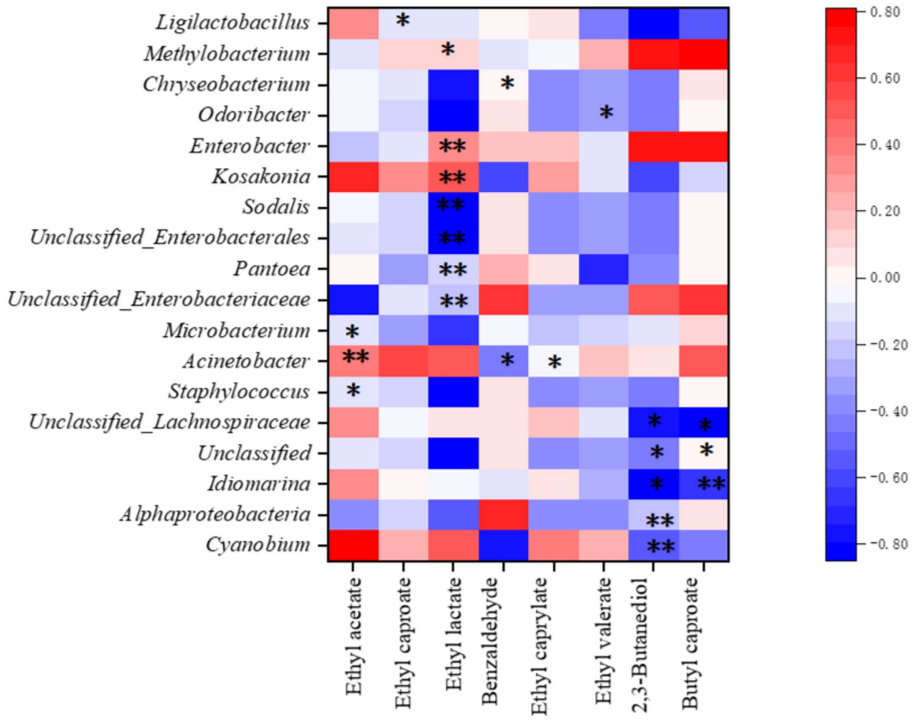

FIGURE 8
Correlation among the microbial community and baijiu flavor substances. * Significantly different ($p < 0.05$); ** highly significantly different ($p < 0.01$).

interactions among bacteria, yeasts, and molds, with the final metabolic products of the continuous adaptation and domestication of microorganisms in anaerobic or aerobic fermentation environments functioning as flavor substances (Xu et al., 2022; Pan et al., 2023). Our analysis of microorganisms on the sorghum surface showed that Cyanobacteria, unclassified bacteria, Proteobacteria, Firmicutes, and Bacteroidota were the dominant bacterial phyla. Firmicutes, Proteobacteria, and Cyanobacteria are the dominant bacterial phyla in the subsequent stacking fermentation of Jiang-flavored baijiu and have a positive effect on the production of both ethanol and other flavor components (Yan, 2019; Wang, 2022). Exploring the sources of these dominant flora has been a popular research topic in recent years, which has highlighted that microorganisms for stack fermentation may originate from tools, air, and drying halls (Lu et al., 2022; Wang, 2022). Our results indicated that the microorganisms introduced via the raw sorghum may also contribute to the brewing microbiota. This may be because most of the processes, such as grain wetting, steaming, stacking, and cellaring, are conducted in the same undivided workshop during the production. Before grain wetting, workers spread several sorghum grains out of their packages and stack them into piles in the workshop as they wait for water to be added, allowing the microorganisms present on the sorghum surface to be dispersed into the surrounding environment. These microorganisms adhere to the drying halls, cellars, and tools during subsequent stacking and fermentation sessions, which initiates inoculation of the fermented grains.

The special flavor of Jiang-flavored baijiu is formed by the metabolites of different microorganisms that use fermented grains as the culture medium. Therefore, predicting the metabolic functions of microorganisms and analyzing the correlation between the microorganisms and the flavor substances of the baijiu play key roles in maintaining product quality (Tan et al., 2022). In our study, we found a correlation between ethyl acetate, ethyl caprylate, ethyl lactate, butyl caproate, and the microorganisms on the surface of raw materials in the first batch of baijiu. These results enrich the theory of the correlation between flavor substances and the microbiota of Jiang-flavored baijiu and provide a reference basis for further research.

Using functional gene prediction, we found that Proteobacteria, Bacteroidota, Firmicutes, Acidobacteriota, Actinobacteriota, Chloroflexi, Myxococcota, and Gemmatimonadota were strongly correlated with metabolic pathways for carbohydrates, amino acids, energy and cofactors, and vitamins. Acetobacter, Bacillus, and Lactobacillus species within the phyla Proteobacteria and Firmicutes were identified as key contributors to the production of ethyl lactate, butyric acid, hexanoic acid, lactic acid, and pyrazine compounds in baijiu. Bacillus species can break down proteins and starches into amino acids and monosaccharides via proteases and amylases, as well as generate organic acids via the tricarboxylic acid cycle. These microorganisms play crucial roles in the fermentation process of baijiu production (Wang et al., 2016; Pan et al., 2023). Although the abundance of Bacillus was not high at the genus level in this study, it does not exclude the possibility that microbial community succession may have made it the dominant species in the subsequent brewing process due to environmental changes. Finally, the number of functional genes in the bacterial communities of the samples varied slightly but not significantly, suggesting that there are other factors that determine the functional changes in the brewing microbial community besides the surface microorganisms of the raw materials, which have only a partial functional impact.

The quality of sorghum crops and the composition of surface microbiota are influenced by a variety of factors, including crop variety, cultivation techniques, ecological and climatic conditions, and soil characteristics (Bokulich et al., 2014; Ding et al., 2021; Chen et al., 2023). Guizhou is a karst farmland ecosystem characterized by intricate geomorphology and significant microclimate variations (Wang et al., 2019). Variations in soil composition can affect the development and spatial extent of sorghum root systems, affecting the ability of plants to absorb water and nutrients (Zhao and Hou, 2019). Climate variations affect sorghum photosynthesis and influence the rate of photosynthetic product accumulation (Zegada-Lizarazu et al., 2015). The interaction between the soil and climate influences nutrient transport and grain accumulation, leading to variations in sorghum quality. Additionally, alterations in topography, climate, and soil conditions modify the composition and structure of the sorghum microbiota by affecting the hydrothermal conditions in the field (Khalifa and Eltahir, 2023). In our study, we found that Pseudomonas, Pantoea, and Kosakonia were significantly correlated with soil total phosphorus, elevation and diurnal temperature difference, while the abundance of Bacteroides, unclassified Vicinamibacterales and Sphingomonas was mainly affected by the monthly mean temperature, precipitation, and effective accumulative temperature.

Although investigations into the surface microorganisms of sorghum are lacking, recent studies have explored the surface microorganisms of grapes as key components in wine production. Proteobacteria and Ascomycota were identified as the predominant bacterial and fungal taxa, respectively, in the microbial communities present on the surfaces of grapes in Xinjiang (Gao et al., 2019). The diversity of bacterial communities was associated with altitude, latitude, and longitude; whereas, the fungal community diversity was significantly influenced by the altitude, dryness, frost-free period, latitude, and longitude. By contrast, the microbial composition on grape surfaces in the Grenache and Carignan regions of Spain was characterized by the dominance of Ascomycetes, followed by Actinobacteria, Acidobacteria, and Bacillus, which was a significant factor in the distribution of bacteria (Portillo et al., 2016). Previous studies have successfully established correlations between the microbiota of raw brewing materials and ecological factors, albeit without consideration of varietal differences. To address this gap, we used a consistent variety of waxy sorghum to eliminate varietal influence. Our findings indicated that the microbiological composition of sorghum surfaces was influenced by a combination of the climate, altitude, and soil properties. However, this study only covers a relatively small area of the country and did not address how the microbial composition may also be influenced by land-use history or other bioenvironmental factors.

5 Conclusion

Variations in the physicochemical characteristics and surface microbial community diversity were observed in Hongliangfeng No. 1 sorghum cultivated in the RH, DY, and JS regions. The results showed that the difference of origin not only affected total starch, amylose, protein, and tannin content, but also formed a unique composition of surface microorganisms at different phylum and genus levels, with both the local soil and climate can have a key impact on the sorghum bacterial community. There was a correlation between the microbial diversity and baijiu flavor components, which provides a reference for further exploration of the brewing function of the raw sorghum materials. Our future research will further expand the range of sample collection, including after wetting, analyzing the brewing environment (e.g., tools, air, plant, and ground) during the same period and collecting data on additional environmental factors to help clearly establish the sources and metabolic pathways of microorganisms found on the surface of waxy sorghum and how they affect the flavor of Jiang-flavored baijiu.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found in the Genome Sequence Archive (Genomics, Proteomics & Bioinformatics 2021) in National Genomics Data Center (Nucleic Acids Res 2022), China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences (GSA: CRA CRA017410) that are publicly accessible at https://ngdc.cncb.ac.cn/gsa.

Author contributions

PX: Writing – review & editing, Writing – original draft, Investigation, Conceptualization. MS: Writing – original draft, Supervision, Methodology. XD: Writing – review & editing, Supervision. YR: Writing – review & editing, Validation. MC: Writing – review & editing, Software, Investigation. YJ: Writing – review & editing, Investigation. JS: Writing – review & editing, Writing – original draft, Conceptualization.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Tradition unveiled: a comprehensive review of microbiological studies on Portuguese traditional cheeses, merging conventional and OMICs analyses

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The microbial communities inhabiting Portuguese traditional cheeses play a fundamental role in shaping their unique flavor, texture, and safety characteristics. This comprehensive review synthesizes findings from both conventional microbiological studies and advanced OMICs analyses to provide a deeper understanding of the microbiota dynamics in these cheeses. We explore the microbial composition, diversity, and functional roles of bacteria, yeasts, and molds across various Protected Designation of Origin (PDO) cheeses, highlighting their contributions to cheese ripening, flavor development, and safety. Additionally, we discuss the potential of OMICs technologies, namely metagenomics, in unraveling the complex microbial ecosystems of Portuguese traditional cheeses. Through this integrative approach, we aim to shed light on the intricate interplay between microorganisms and cheese matrices, unveiling the secrets behind the rich heritage and distinctiveness of Portuguese traditional cheeses.

KEYWORDS

Portuguese traditional cheeses, microbiota, PDO cheeses, conventional microbiology, OMICs analyses, metagenomics, flavor development, cheese ripening

1 Traditional cheeses with Protected Designation of Origin: preserving heritage

Artisanal food products constitute an important cultural heritage. In Mediterranean countries, such as Portugal, a wide variety of traditional cheeses is manufactured, harboring distinct flavor, texture and overall composition (see Figure 1). These dairies hold significant importance, not only in social and cultural contexts, preserving history and tradition, but also in terms of economic impact, as they are regarded as value-added products (Guiné and Florença, 2020). The majority of Portuguese traditional cheeses, like those in many other countries, are manufactured in rural regions by small, multi-generational enterprises. Consequently, the sale of these products provides essential income to local communities, dependent on cattle farming and/or cheese production as their livelihood (Reis and Malcata, 2011). These products are manufactured using ancient traditions and expertise, which must be safeguarded to maintain their distinctiveness. In Portugal, the art of cheesemaking traces its origins to the Roman era, with raw milk and its byproducts, including cheese, being integral components of the European diet ever since (Araújo-Rodrigues et al., 2020). In those early periods, cheese production represented a pioneering approach to preserving milk products in a more stable form (Salque et al, 2013). Furthermore, during that era, cheese held such significant value that it was frequently utilized in commercial transactions (Freitas and Malcata, 2000). Today, PDO cheeses are manufactured in small-scale industrial dairies, preserving the rich tradition of their production.

Recognizing the significance of traditional products to their countries of origin, the European Union (EU) introduced geographical indication schemes for agricultural products and foodstuffs, including Protected Designation of Origin (PDO), Protected Geographical Indication (PGI), and Traditional Specialty Guaranteed (TSG) (EU, 2022). These designations serve to aid consumers in identifying traditional products, while also safeguarding and promoting their unique qualities, which are intricately tied to geographical origin and manufacturing expertise (Dias and Mendes, 2018).

According to Regulation (EU) No 1151/2012 (European Commission, 2012) and Commission Implementing Regulation

FIGURE 1
Geographical distribution of the Portuguese traditional PDO cheese production areas.

(EU) No 668/2014 (European Comission, 2015), implemented by EU, in PDO products "Every part of the production, processing and preparation process must take place in the specific region", while in PGI "at least one of the stages of production, processing or preparation takes place in the region" and TSG "highlights the traditional aspects, such as the way the product is made or its composition, without being linked to a specific geographical area" (EU, 2022). In this review manuscript, our focus will be exclusively on Portuguese PDO cheeses and their associated microbiota.

While manufacturing techniques may vary depending on the region, certain practices are consistent across all PDO cheese-making facilities. For example, these cheeses are exclusively made with raw milk, never heated above 40°C, and employ coagulating agents or rennet along with salt, limited to a maximum of 25 g/L. Additionally, no starter or non-starter microbiota is added during the manufacturing process (Freitas and Malcata, 2000). For each PDO cheese, a specification book outlines all mandatory production details, encompassing the type of milk utilized and any treatments applied, specified animal breeds (when applicable), coagulating agents, ripening temperatures, humidity levels, cheese dimensions, and labeling requirements.

In traditional cheese manufacturing, one of the pivotal steps is milk clotting, typically achieved using a coagulating agent such as animal, plant, or microbial rennet (Arbita et al., 2020). The choice of rennet not only impacts milk coagulation, but also influences the development of organoleptic characteristics, primarily attributed to various enzymatic activities (Andrén, 2021). For example, cheeses may acquire a bitter flavor if the rennet used exhibits high non-specific proteolysis activity, a trait often disfavored by consumers (Arbita et al., 2020; Faccia et al., 2020; Andrén, 2021).

Since the beginning of cheese production, calf rennet has been the primary animal coagulating agent utilized, specifically an extract derived from the abomasum of suckling calves. The abomasum of young calves produces caseinolytic enzymes, notably chymosin and pepsin (Arbita et al., 2020; Andrén, 2021). Additionally, chymosin, renowned for its high milk-clotting activity and low proteolysis, is one of the most frequently employed enzymes in cheese production (Mohanty et al., 1999; Andrén, 2021).

In Portuguese traditional cheeses bearing the PDO label, the use of thistle (*Cynara cardunculus L.*), as a coagulating agent, is prevalent. *C. cardunculus L.*, commonly known as cardoon, is an edible flower native to the Mediterranean region, characterized by its large heads and purple flowers (Gostin and Waisundara, 2019; Folgado et al., 2020). The significance of *C. cardunculus L.* in cheese manufacturing lies in its enzymes with proteolytic activity, which target milk proteins (Folgado et al., 2020). Among these enzymes, cardosins, particularly cardosin A, play a pivotal role in milk clotting. Cardosin A exhibits proteolytic activity similar to chymosin, specifically targeting κ-casein while also cleaving α and β-caseins, contributing to a softer texture and flavor in the cheese (Folgado et al., 2020; Barracosa et al., 2021).

Overall, the selection of coagulating agents in Portuguese traditional PDO cheeses can vary depending on geographical location and the type of milk utilized. Undoubtedly, these agents play a crucial role in shaping the microbial ecosystem and significantly impact the organoleptic characteristics of the final product. Besides playing an effective role in the coagulation process, coagulating agents also contribute to the products microbiota (Cruciata et al., 2014). Associated enzymes take part in the proteolytic process during cheese manufacture, contributing to organoleptic characteristics through volatile compound formation (Pereira et al., 2008). Moreover, a previous study on cheese produced from animal rennet, characterized the microbial load of the coagulating agent and found several genera, including LAB, *Enterococcus* and lower counts of *E.coli*, coliforms and *Staphylococcus aureus* (Voidarou et al., 2011).

In the rich landscape of Portuguese traditional PDO cheeses, beginning from the northern region of Portugal, two prominent PDO cheeses stand out: *Terrincho* and *Transmontano*. *Terrincho* cheese is manufactured using *Churra da Terra Quente* ewes milk and animal rennet. This cheese comes in two variants, one aged for a minimum of 30 days and another matured for 90 days (known as old *Terrincho cheese*), both marketed under the *Terrincho* PDO designation. The extended maturation period endows old *Terrincho* with a firmer texture and intensifies its flavor and aroma. In contrast, *Transmontano* goat cheese, produced from *Serrana* breed goats milk and animal rennet, features an exceptionally hard paste, along with a robust aroma and spicy flavor profile (Despacho 7822/2011, 2011).

In central Portugal, several PDO cheeses hold prominence: *Beira Baixa*, *Rabaçal*, *Serra da Estrela*, and *Azeitão* cheeses. The *Beira Baixa* PDO encompasses three variants: *Castelo Branco*, *Picante* from *Beira Baixa*, and *Queijo Amarelo* (Direção-Geral de Agricultura e Desenvolvimento Rural, 2022). *Castelo Branco* cheese is manufactures from ewes milk using *Cynara cardunculus* L. as a coagulant, resulting in a semi-hard or semi-soft paste with intense flavor and aroma. An aged variant, old *Castelo Branco* cheese, matures for a minimum of 90 days, yielding a harder paste and a spicy flavor profile (Despacho 9633/2016, 2016). *Picante* from *Beira Baixa*, made from a blend of ewe and goat milk with animal rennet, boasts a semi-hard or semi-soft paste with an intense aroma and spicy flavor. *Queijo Amarelo*, produced from a mixture of ewe and goats milk or solely ewes milk, also employs animal rennet, resulting in a semi-hard to hard paste with intense aroma and a slightly acidic and spicy flavor (Despacho 4185/2011, 2011). *Rabaçal* cheese, produced from a blend of ewe and goats milk with animal rennet, features a semi-soft or semi-hard paste with a distinctive flavor, imparted by the presence of *Santa Maria* thyme in the grazing pasture (Despacho 6400/2003, 2003). Moving to *Serra da Estrela* cheeses, they are made from ewes milk and *Cynara cardunculus* L., resulting in a semi-soft buttery paste with a mild aroma and slightly acidic flavor. *Serra da Estrela* cheese can undergo extended ripening, yielding *Serra da Estrela* old cheese, which presents a semi-hard to extra-hard paste, a robust aroma, and a slightly spicy and salty flavor. Lastly, in the Lisbon region, *Azeitão* PDO cheese is produced using ewes milk and *Cynara cardunculus L.*, resulting in a cheese with a semi-soft and buttery paste, a yellow hue, and a slightly spicy and acidic flavor (Despacho 6400/2003, 2003).

In the region of Alentejo, three PDO cheeses are produced: *Serpa*, *Évora*, and *Nisa*. These cheeses are all manufactured using ewes milk sourced from the *Merina branca* breed and employ plant-

based rennet *Cynara cardunculus L. Serpa* cheese boasts a cured, buttery, and semi-soft paste, characterized by a strong odor and a slightly spicy flavor (Despachon.5511/2020, 2020). *Évora* cheese features a semi-hard to hard paste with a robust aroma and a slightly spicy and acidic flavor profile (Despacho 8601-N/2005, 2005). Lastly, *Nisa* cheese presents a semi-hard paste with an acidic flavor and a strong odor (Direção-Geral de Agricultura e Desenvolvimento Rural, 2022).

Moving to the island of Azores, two PDO cheeses are manufactured: *Pico* and *São Jorge* cheeses. Both cheeses are made from cows milk without a defined breed, using animal rennet (Direção-Geral de Agricultura e Desenvolvimento Rural, 2022). *Pico* cheese is cured with a soft paste, offering a salty flavor and intense aroma (Despacho32/1996/SRAP, 1996). *São Jorge* cheese, on the other hand, features a semi-hard or hard paste with a slightly spicy flavor and intense aroma (DespachoSRAP/94/1, 1994).

All the aforementioned characteristics of the PDO cheeses are summarized in Table 1 for easier comprehension and comparison.

2 Microbial impact on cheeses' organoleptic characteristics

Cheese hosts diverse and intricate microbial communities, which evolve over time and differ based on the cheese type, especially traditional varieties that dont depend on starter cultures for fermentation, as well as regions. Microorganisms in cheese originate not only from milk but also from the production environment. The intricate interplay between microbes and growth substrates, such as milk proteins and fatty acids, significantly impacts the quality and safety of the end product (Afshari et al., 2020).

Due to the diversity of cheese-LAB, these microorganisms harbor both starter and non-starter features. Starter LAB (SLAB) arise in the first stages of manufacture, being crucial participants in the production of lactic acid inducing an acidic pH environment and consequentially curd formation. SLAB can originate from the autochthonous raw milk microbiota, or be added during production, although the latter is not the case in Portuguese PDO cheeses (Coelho et al., 2022). In contrast, non-starter LAB (NSLAB) have a more significant role in the subsequent stages of cheese manufacture (Settanni and Moschetti, 2010), being mostly associated with volatile production, and consequentially odor and flavor development, due to proteolytic activities, as further discussed below. NSLAB can also originate from the raw milk microbiota, or may be introduced from cheesemaking settings, equipment or operators (Irlinger et al., 2017; Coelho et al., 2022).

Considering that raw milk is nutrient rich matrix, the probability of microbial contaminations cannot be overlooked, and may occur by contact with the animals teat surface, milking machinery or collection and storage containers. Due to inadequate hygiene practices, contamination may also occur by staff handling and associated settings, such as bedding, feed or water. Additionally, at cheese production stage, contamination can be associated with manufacturing facilities (storage rooms and shelfs), as well as working staff (Quigley et al., 2013; OSullivan and Cotter, 2017).

In cheese manufacturing, a series of biochemical reactions occur, including glycolysis, proteolysis, and lipolysis, which are influenced by microorganisms, environmental conditions, and the type of coagulant used (Tavaria et al., 2002; Pereira et al., 2008). These reactions shape the microbial composition and organoleptic characteristics of the final cheese, with volatile compounds playing a significant role in flavor development. Lactic acid bacteria (LAB) are key players in these processes. LAB, including genera like *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, or *Enterococcus*, are non-motile, microaerophilic Gram-positive bacteria capable of tolerating environmental stresses such as high temperature, low pH, or high salt concentrations (Tavaria et al., 2002). Their ability to metabolize various carbon sources and produce antimicrobial compounds makes them indispensable in the food industry.

These microorganisms act as biopreservatives due to their fermentation and acidification capabilities. Milk acidification begins with LAB utilizing lactose as a carbon source, leading to lactate production. This decrease in pH causes the coagulation of casein, the main protein in milk, resulting in the formation of cheese curd, which contains protein, fat, and whey (the liquid portion containing serum proteins, lactose, minerals, and vitamins). Furthermore, the reduction in pH helps prevent the growth of undesirable microorganisms, including foodborne pathogens and spoilage microbes (Favaro et al., 2015). Additionally, the production of organic acids such as lactic, acetic, formic, or propionic acids contributes to extending the shelf life and enhancing the safety of the food product (Favaro et al., 2015). Apart from alterations in pH, another safety strategy employed by these bacteria is the production of bacteriocins. LAB are known to produce various types of bacteriocins such as nisin, reuterin, reutericyclin, pediocin, lacticin, enterocin, among others, as well as bacteriocin-like inhibitor substances (BLIS) (Favaro et al., 2015).

During cheese ripening, LAB also play a central role in the formation of aroma compounds due to their enzymatic activity, which involves the catabolism of aromatic compounds. Additionally, LAB contribute to the breakdown of peptides, which impacts the aroma and texture of the cheese (Cardinali et al., 2022). One group of proteins that is particularly important in this process is the cell-envelope proteinases. These proteins degrade caseins into oligopeptides, which are then transported across the bacterial membrane and further degraded into shorter peptides and amino acids that are essential for LABs survival (Ji et al., 2021). The action of these proteinases generates unique hydrolysates and peptides with distinctive sensory and bioactive properties, which contribute to the overall characteristics of the cheese. Moreover, the degree of proteolysis and the different catalytic properties of these proteases may vary among LAB strains, leading to further diversification of the organoleptic features appreciated by consumers (Ji et al., 2021).

In PDO Portuguese traditional cheeses, the main bacteria found in the final product are *Lactococcus* spp., *Leuconostoc* spp., *Lactobacillus* spp. and, in some cheeses, *Enterococcus* spp., as it will be discussed in the following sections. All these genera belong to LAB and contribute to cheese maturation in different stages and levels, either by acidification of the milk, proteolytic activity, or production of different bacteriocins active against foodborne

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TABLE 1 Distribution and characteristics of Portuguese PDO cheeses.

Cheeses	Region	Milk origin	Breed	Coagulating agent		Maturation periods	Organoleptic features
Terrincho	North	Ewe	*Churra da terra quente*	Animal-derived	Enzyme mix with chymosin, pepsin and other enzymes	Maturation at 5–12°C for 30–90 days, humidity between 80 and 85%	Semi-hard to hard paste and white to yellow color
Transmontano	North	Goat	*Serrana*	Animal-derived	Enzyme mix with chymosin, pepsin and other enzymes	Maturation at 5–18°C, humidity of 70–85% for 60 days	Extra-hard paste with white color
Beira Baixa – Castelo Branco	Center	Ewe	Ewe – *Assaf, Lacaune, Merina Branca* and *Merino da Beira Baixa*	Plant-derived	*Cynara cardunculus*	Maturation at 8–14°C, humidity of 80–90% for 45–90 days	Semi-hard or semi-soft paste and yellow color
Beira Baixa – Picante	Center	Ewe and goat	Ewe – *Assaf, Lacaune, Merina Branca* and *Merino da Beira Baixa*; Goat – *Charnequeira, Saanen* and *Granadina-Murciana*	Animal-derived	Enzyme mix with chymosin, pepsin and other enzymes	Maturation at 10–18°C, humidity of 70–80% for 120 days	Semi-hard or hard past with white color
***Beira Baixa* – Amarelo**	Center	Ewe and goat or only ewe	Ewe – Assaf, Lacaune, *Merina Branca* and *Merino da Beira Baixa*; Goat – *Charnequeira, Saanen* and *Granadina-Murciana*	Animal-derived	Enzyme mix with chymosin, pepsin and other enzymes	Maturation at 10–18°C, humidity of 70–80% for 45–90 days	Semi-hard or semi-soft paste and yellow color
Rabaçal	Center	Ewe and goat	Ewe – *Assaf* and *Lacaune*; Goat – *Saanen*	Animal-derived	Enzyme mix with chymosin, pepsin and other enzymes	Maturation at 10–15°C, humidity of 70–85% for 20 days	Semi-hard or hard paste with white color
Serra da Estrela	Center	Ewe	*Bordaleira Serra da Estrela* and *Churra Mondegueira*	Plant-derived	*Cynara cardunculus*	First phase of maturation – 6–12°C, humidity of 85–90% for 15–20 days; Second phase of maturation – 6–14°C, humidity of 90–95% until the 45th day	Semi-soft, buttery paste with a white-yellowish color
Old *Serra da Estrela*	Center	Ewe	*Bordaleira Serra da Estrela* and *Churra Mondegueira*	Plant-derived	*Cynara cardunculus*	First phase of maturation – 6–12°C, humidity of 85–90% for 15–20 days; Second phase of maturation – 6–14°C, humidity of 90–95% until the 120th day	Semi-hard to extra-hard paste with a brownish orange color
Azeitão	Lisbon	Ewe	No defined breed but in the area the most common breeds are *lâncome* and *israelita assaf*	Plant-derived	*Cynara cardunculus*	Maturation at 10–12°C, humidity of 85–90% for 20 days	Semi-soft cheese with butter paste and white or yellow color
Serpa	Alentejo	Ewe	*Merina Branca*	Plant-derived	*Cynara cardunculus*	Maturation at 6–12°C for 30 days	Semi-soft, buttery paste and whitish color

(Continued)

TABLE 1 Continued

Cheeses	Region	Milk origin	Breed	Coagulating agent		Maturation periods	Organoleptic features
Évora	Alentejo	Ewe	*Merina Branca*	Plant-derived	*Cynara cardunculus*	Maturation at 8–15°C, humidity of 80–95% for 30 days	Hard or semi-hard paste with yellow color
Nisa	Alentejo	Ewe	*Merina Branca*	Plant-derived	*Cynara cardunculus*	First phase of maturation – 8–10°C, humidity of 80–90% for 15–20 days; Second phase of maturation – 10–14°C, humidity of 85–90% for 30–40 days	Semi-hard paste with a yellowish white color
Pico	Autonomous region of Azores	Cow	No defined breed	Animal-derived	Enzyme mix with chymosin, pepsin and other enzymes	Coagulation at 26–37°C during 45–60 minutes; Maturation at 15–17°C humidity between 75 and 85% over 17–30 days	Semi-soft, buttery paste and white-yellowish color
São Jorge	Autonomous region of Azores	Cow	No defined breed	Animal-derived	Enzyme mix with chymosin, pepsin and other enzymes	Maturation at 12–14°C, humidity at 80–85% for 60 days	Hard or semi-hard past and yellow color

pathogens (i.e., nisin produced by *Lactococcus lactis* against *Listeria monocytogenes*) (Cotter and Beresford, 2017; Lee et al., 2020; Afrin et al., 2021).

The genus *Lactococcus* spp. has emerged as the predominant LAB group in all PDO Portuguese traditional cheeses studied to date (Allers et al., 2004; Ayrapetyan et al., 2015a, Ayrapetyan et al., 2015b; Baptista, 2018; Abbasi and Emtiazi, 2020; Câmara et al., 2020; Beltrán-Espinoza et al., 2021). Extensively researched for its influence on cheese manufacture and its applicability across various industries, *Lactococcus* spp. are starter LAB alongside certain species of *Lactobacillus* (*Lactobacillus delbrueckii and Lactobacillus helveticus*). During the initial stages of ripening, these bacteria produce lactic acid, leading to milk acidification (Ruggirello et al., 2014; Cotter and Beresford, 2017). *Lactococcus lactis*, one of the main species found in Portuguese PDO cheeses, plays a crucial role not only in ensuring the safety of the cheese but also in flavor development. In terms of safety, *Lactococcus lactis* is known to produce over 40 types of bacteriocins, ribosomally synthesized proteins with antimicrobial activity against other bacteria. These bacteriocins, belonging to class I and II, help control undesirable microorganisms, including potentially pathogenic bacteria (Takala et al., 2023).

Furthermore, apart from their role in acidification, *Lactococcus lactis* and related species can convert amino acids into aroma compounds through aminotransferase activity. They also contribute to cheese properties by producing exopolysaccharides (Van De Bunt et al., 2014; Cardinali et al., 2022). Given these characteristics, *Lactococcus lactis* is widely employed as a starter culture, either alone or in combination with other cultures, in the production of various dairy products (OSullivan and Cotter, 2017).

In addition to *Lactococcus* spp., other LAB genera such as *Leuconostoc*, *Lactobacillus*, and *Enterococcus* are also prominent in Portuguese PDO cheeses (Tavaria and Malcata, 1998; Dahl et al., 2000; Domingos-Lopes et al., 2017; Câmara et al., 2019; Rocha et al., 2021; Rampanti et al., 2023; Rocha et al., 2023). These microorganisms become more noticeable during the later stages of ripening and are often found at the core of the cheeses. They contribute significantly to the flavor, texture, and safety of the final product (Montel et al., 2014). *Leuconostoc*, much like certain species of *Lactobacillus*, possesses the capability to metabolize lactose into lactic acid. Thus, alongside *Lactococcus*, *Leuconostoc* plays a pivotal role in initiating the maturation process of cheese (OSullivan and Cotter, 2017).

Indeed, *Leuconostoc* spp. also exhibits several important technological aspects for cheese ripening. For instance, it contributes to the production of aromatic compounds such as diacetyl and acetoin through the degradation of citrate. Additionally, *Leuconostoc* spp. is also capable of producing gas and dextrans. Dextrans are homopolysaccharides of D-glucose known for their viscosifying, emulsifying, texturizing, and stabilizing attributes in food applications. Therefore, they have the potential to serve as substitutes for commercial hydrocolloids commonly used in the food industry for the same purposes. These glucans are synthesized by extracellular dextransucrase enzymes released by certain LAB, including *Leuconostoc* species like *Leuconostoc mesenteroides*, as well as other genera such as *Lactobacillus*, *Streptococcus*, *Weissella*, and *Pediococcus* (Morelli and von Wright, 2019).

Regarding *Lactobacillus* spp., this genus is highly diverse within LAB and is commonly found in dairy products, including *L. delbrueckii* subsp. *bulgaricus*, *L. helveticus*, and *Lacticaseibacillus casei*. Additionally, various *Lactobacillus* species are also found in the human gastrointestinal tract, such as *L. acidophilus*, *L. gasseri*, and *Lacticaseibacillus rhamnosus* (Morelli and von Wright, 2019).

As mentioned earlier, some species of *Lactobacillus* have starter activity. However, lactobacilli also play a crucial role in non-starter activities, significantly impacting flavor development and ensuring the quality and durability of cheeses. *Lactobacillus* (and related genera, according to the new taxonomy; Zheng et al., 2020) is one of the major contributors of non-starter LAB during cheese ripening (Morelli and von Wright, 2019). Some of the species commonly found at this stage of ripening include *L. casei*, *Lacticaseibacillus paracasei*, and *Lacticaseibacillus rhamnosus*. *L. casei* is a ubiquitous microorganism found in various niches, exhibiting great genetic versatility. In a study by Cai et al. (2009), it was observed that cheese-isolated *L. casei* displayed a significant number of genes related to carbohydrate metabolism, transcriptional regulation, and signal transduction compared to *L. casei* from other environments (Cai et al., 2009).

Moreover, besides its proteolytic activity and role in flavor development in cheeses, *L. casei* can also act as a protective microorganism by detoxifying biogenic amines (BA). These toxic compounds are formed through microbial degradation of amino acids and, if present in food products, may cause symptoms of intoxication such as headaches, itchy skin rashes, heart palpitations, or diarrhea (Linares et al., 2011). Cheese is a fermented food in which BA may be present, with tyramine, histamine and putrescine being the most commonly found (Linares et al., 2011; Herrero-Fresno et al., 2012; Renes et al., 2014). In a genomic study conducted with *L. casei*, genes for methyltransferases and oxidoreductases related to BA degradation were identified (Ladero et al., 2014). These adaptabilities of *L. casei* make it an appealing candidate for potential applications in food industry environments and products, as well as a possible source of probiotics (Morelli and von Wright, 2019).

As for *Enterococcus* spp., these bacteria are constituents of the native microbiota of several traditional products from the Mediterranean area and are used as sanitary indicators (Freitas and Malcata, 2000). However, due to safety concerns regarding specific species or strains with virulence factors that could pose a risk to consumers, the European Food Safety Authority (EFSA) has created a list of microorganisms with Qualified Presumption of Safety (QPS) for use in the food industry. For the *Enterococcus* genus specifically, since it is not included in this directory, safety assessments are performed on a case-by-case basis, including screenings for species or strain virulence factors (Câmara et al., 2020). Nevertheless, in Portuguese traditional cheeses, *Enterococcus* spp. are typically present in the raw milk microbiota used for cheese manufacture, resulting in their presence in the cheese itself. Their proteolytic and lipolytic activities have a significant impact on the development of the cheeses flavor characteristics, making this genus essential for cheese ripening (Dias et al., 2021). To date, no outbreaks related to the consumption of any Portuguese PDO cheese containing these microorganisms have been reported, further highlighting their importance in traditional Portuguese PDO cheese production (Rocha et al., 2023).

Regarding the fungal community in Portuguese PDO cheeses, the main genera encountered include *Candida* spp., *Debaryomyces* spp., *Yarrowia* spp., and *Kluyveromyces* spp. These microorganisms are typically found in the cheese rind, but they can also be present in the cheese matrix (OSullivan and Cotter, 2017). In the case of *Candida* and *Debaryomyces*, some studies suggest that these yeasts may enter the cheese through the salt used in traditional cheese manufacture (Cotter and Beresford, 2017). Yeasts influence all stages of cheese ripening, as they have the ability to ferment lactose, contribute to milk acidification, and perform lipolytic and proteolytic activities for texture and flavor development, as well as produce aromatic compounds for scent and flavor (Gonçalves Dos Santos et al., 2017). The influence of molds on cheese properties is less understood. While various molds are found in some types of surface-ripened cheeses like *Brie* or *Camembert* (Irlinger et al., 2017), in Portuguese cheeses, the presence of molds is mainly due to contamination.

Overall, apart from the diversity of the microbiota present in the milk, variations in manufacturing practices (e.g., ripening duration, temperature, and humidity conditions) also induce differences in the final product properties (Araújo-Rodrigues et al., 2022). Moreover, the dominance of LAB in the cheese microbiota plays a pivotal role in the organoleptic characteristics of cheeses regardless of the region or milk used for their production.

3 Integrating conventional microbiology and OMICs

To fully capture the intricate microbial ecosystem of traditional cheeses, it is crucial to employ both conventional microbiology and OMIC technologies. These complementary approaches provide a comprehensive understanding of the diverse microbial communities present in these cheeses.

3.1 Conventional microbiology

Conventional microbiological procedures encompass culture-dependent methods, which involve the growth and isolation of microbial populations utilizing selective media. Briefly, after collection the samples are processed by homogenization using an isotonic buffer on a stomacher blender, serial dilutions are prepared and plated in appropriate media. Media selection varies according to the targeted microbial groups, the most commonly used being Man, Rogosa and Sharpe (MRS) agar for total LAB, M17 for lactococci, Slanetz Bartley Agar (SBA) for enterococci, Mayeux, Sandine and Elliker Agar (MSE) for *Leuconostoc* spp., Rogosa Agar for lactobacilli, potato dextrose agar or Rose Bengal Chloramphenicol (RBC) for yeast and molds, Baird Parker agar for *Staphylococcus* spp. and Violet Red Bile Glucose Agar (VRBGA) for *Enterobacteriaceae* (Pinho et al., 2004; Câmara et al., 2017; Gonçalves et al., 2018; Dias, 2021; Rocha et al., 2023; Salamandane et al., 2024).

Subsequent identification relies on phenotypic characteristics, such as morphology and biochemical traits, as well as genotypic techniques such as species-specific PCR (Anastasiou et al., 2022) (Anastasiou et al., 2022). Over the years, numerous studies have been conducted to investigate the microbiota of PDO cheeses, as

microbial communities undergo changes during various stages of ripening. Previous research has identified several groups of microorganisms, including bacteria, filamentous fungi (molds), and yeasts. Among these, LAB were found to be the most prevalent group of microorganisms (Freitas and Malcata, 2000), namely in *Terrincho* (Pinho et al., 2004), *Picante* (Freitas et al., 1996; Freitas and Malcata, 2000), *Serra da Estrela* (Rocha et al., 2023), *Azeitão*, *Serpa* (Gonçalves et al., 2018; Araújo-Rodrigues et al., 2020), *Évora*, *Pico* and *São Jorge* cheeses (Kongo et al., 2008).

Terrincho cheese has not been extensively studied however, some authors have shown that LAB are the most representative group of microorganisms, based on counts of colony forming units (CFUs), it was verified that *Lactobacillus* spp. and *Lactococcus* spp. showed levels of 10^9 CFU/g followed by *Enterococcus* spp. with numbers ranging from 10^7 to 10^8 CFU/g (Pinho et al., 2004; Pintado et al., 2008). In the study performed by Pinho et al (2004), *Terrincho* cheese microbiota was studied during ripening time, and the presence of yeasts and molds, *Pseudomonas* spp., coliforms and *Staphylococcus* spp. was assessed, with the latest ranging up to 10^4 CFU/g at the end of the ripening process. Coliform abundance was also studied, with numbers reaching 10^6 CFU/g after 60 days of ripening (Pinho et al., 2004). Pintado et al (2008) also assessed *Terrincho* cheese microbiota, and, once again, LAB were the predominant group. *Lactococcus* spp. and *Lactobacillus* spp., presented counts of around 10^9 CFU/g, while *Enterococcus* counts were lower, with numbers of *ca* 10^7 CFU/g. The presence of yeast and molds (~10^4–10^6 CFU/g), *Pseudomonas* spp., and *Staphylococcus* spp., were equally studied, and the numbers of the latest were concordant with the previous study, ranging from 10^4 to 10^5 CFU/g. *Enterobacteriaceae* were also in the order of 10^6 CFU/g (Pintado et al., 2008).

In the case of *Beira Baixa* cheese, which encompasses *Amarelo*, *Picante*, and *Castelo Branco* PDO cheese types, there have been only a few studies on their microbiota to date, to the best of our knowledge. Cardinali et al (2022) investigated the microbiota of *Castelo Branco* cheese and identified *Lactococcus* spp. and *Lactobacillus* spp. as the predominant microorganisms with CFUs counts ranging from 10^6–10^9 to 10^8–10^9, respectively (Cardinali et al., 2022). In the same study, *Enterococcus* spp. presence was also determined (10^4–10^6 CFU/g), as well as *Enterobacteriaceae* (10–10^4 CFU/g) yeasts and molds (10–10^4 CFU/g). In the study by Freitas et al. (1996), *Picante* cheese was examined, and once again, LAB was identified as the predominant group. Additionally, the presence of *Enterobacteriaceae*, *Staphylococcus* spp., yeasts, and molds was evaluated, with no molds found in the studied cheeses (Freitas et al., 1996).

Due to the limited availability of published papers on some traditional Portuguese cheeses, masters theses have been utilized over the years to characterize their indigenous microbiota. For *Amarelo* cheese, a masters dissertation conducted by Rodrigues (2023) reported similar results to those found in other *Beira Baixa* cheeses, with a predominance of LAB (*ca* 10^8 CFU/g). In terms of different genera, as this was a preliminary study with some limitations, the authors did not provide detailed information on that matter. Yeasts and molds were also detected, albeit in smaller quantities (ranging from 10 to 10^4 CFU/g) (Rodrigues, 2023). Potentially pathogenic microorganisms were also investigated: *Salmonella* spp. or *Listeria monocytogenes* were not found, but *Pseudomonas* spp., on the other hand, were present at levels ranging from 10^5 to 10^6 CFU/g (Rodrigues, 2023).

Rabaçal cheese is one of the lesser-studied PDO cheeses, and similarly to *Amarelo* from *Beira Baixa*, our review was based on masters dissertations. The study conducted by Dias et al. (2021) assessed the microbiota of Portuguese PDO cheeses, including the examination of *Rabaçal* microbiota in both the cheeses crust/rind and interior. The results align with studies conducted on other cheeses, with LAB being the predominant group of bacteria, with counts of approximately 10^8 CFU/g. *Enterobacteriaceae*, coliforms, and yeasts were also isolated in both the cheeses rind and interior, with counts of around 10^4 CFU/g. *Staphylococcus* spp. and molds were also detected, although in lesser quantities (ranging from 10 to 10^2 CFU/g) (Dias et al., 2021).

Serra da Estrela cheese is often regarded as a hallmark of Portuguese traditional PDO cheeses and has been the subject of numerous studies, employing both conventional microbiology and metagenomic approaches. Tavaria and Malcata have contributed with various articles on *Serra da Estrela* cheese. In a study conducted in 1998, the cheeses microbiota was analyzed, revealing that LAB counts increased throughout ripening and remained predominant until the end of the ripening period (60 days), representing 55.1% of the total microbiota (Tavaria and Malcata, 1998). *Enterobacteriaceae* and yeasts were the second most abundant groups, each representing 20.4% of the cheeses microbiota, with *Staphylococcus* spp. also being present, even though in lower percentages (4.1%) (Tavaria and Malcata, 1998). Another study by Tavaria and Malcata (2000) focused on evaluating the microbiota of *Serra da Estrela* cheeses manufactured in different years and geographic areas. LAB were identified as the main group of bacteria, with other less abundant groups such as yeasts and *Staphylococcus* spp. also isolated. *Enterobacteriaceae* were present, and their numbers were observed to decrease throughout ripening. This study also highlighted the influence of geographical location on the cheese microbiota (Freitas and Malcata, 2000). In another study by Dahl et al. (2000), the dominance of LAB throughout ripening was once again confirmed, even in longer ripening periods (duration assessed ranged from 60 to 180 days). Conversely, *Enterobacteriaceae* and yeast species showed a significant decrease throughout ripening (Dahl et al., 2000). More recent studies on *Serra da Estrela* cheese attest the prevalence of LAB over other groups of microorganisms with yeasts and molds being the less represented groups (Rampanti et al., 2023; Rocha et al., 2023). A 2024 study performed by Salamandane et al (2024), including PDO and non-PDO *Serra da Estrela* cheeses, showed a richer microbiota in PDO cheeses, with count numbers ranging from 10^6 to 10^9 CFU/g for *Lactococcus* and *Enterococcus, ca* 10^9 for *Lactobacillus*. *E. coli* and *Staphylococcus* were also found in counts around 10^3–10^5 and 10^4–10^5 respectively. *Listeria monocytogenes* and *Salmonella* were not found in any of the samples analyzed. These results highlight the diversity of microorganisms in fermented foods, particularly those with no selection of initial microbiota, as Portuguese PDO cheeses (Salamandane et al., 2024).

Studies on *Azeitão* and *Nisa* cheeses microbiota have also been conducted, primarily through masters dissertations. Baptista (2018) characterized the microbiota of these cheeses by analyzing cheeses

manufactured in different dairies and different years. They found LAB counts ranging from 10^7 to 10^{10} CFU/g for *Azeitão* cheese and from 10^6 to 10^8 CFU/g for *Nisa* cheese. Specific counts of *Lactococcus* and *Enterococcus* were also assessed using selective media. In *Azeitão* cheeses, *Lactococcus* numbers ranged from approximately 10^7 to 10^{11} CFU/g, and *Enterococcus* counts ranged from 10^4 to 10^7 CFU/g. *Nisa* cheeses had *Lactococcus* counts of about 10^6–10^{12} CFU/g and *Enterococcus* counts of 10^4–10^5 CFU/g (Baptista, 2018). Another study conducted by MaChado (2020) assessed the presence of coagulase-positive *Staphylococcus* and *Escherichia coli* in *Azeitão* cheese. They found that unlike *Staphylococcus* counts, which decreased throughout the ripening period, *E. coli* counts increased (MaChado, 2020).

Regarding *Évora* cheese, a study by Dias et al. (2021) evaluated the cheese microbiota throughout ripening. Similarly to other cheeses, the majority of the microorganisms belong to the LAB group. At the end of ripening time (25 days), LAB counts were in the order of 10^7–10^8 CFU/g, while *Enterococcus* spp. showed levels of around 10^5 CFU/g and *Enterobacteriaceae* and yeasts were found in the same approximate range of counts 10^4–10^6 CFU/g (Dias et al., 2021).

Serpa cheese has undergone some studies regarding its microbial content (Roseiro et al., 2003; Gonçalves Dos Santos et al., 2017; Gonçalves et al., 2018). A study performed by Roseiro et al. (2003) identified LAB as the predominant group throughout ripening, with count numbers reaching 10^9 CFU/g. Molds, *Listeria monocytogenes* and coagulase positive *Staphylococcus* were not encountered, contrarily to *E. coli* and coliforms, that were found in numbers of around 10^5–10^6 CFU/g, having increased during ripening. The presence of yeasts was also acknowledged, reaching 10^3 CFU/g (Roseiro et al., 2003). Posterior studies, assessed the microbiological community, namely yeasts and bacteria, in different manufactures and seasons (winter and spring). Yeasts counts reached 10^6 CFU/g and were found to be higher in winter compared to spring (Gonçalves Dos Santos et al., 2017; Gonçalves et al., 2018). Regarding bacterial communities, concordantly to the study by Roseiro et al. (2003), LAB were the major constituents, with the following genus represented: *Lactobacillus* spp., *Lactococcus* spp., *Leuconostoc* spp. and *Enterococcus* spp. and found in numbers up to 10^{10} CFU/g. *Enterobacteriaceae* were shown to be in high numbers in winter (10^5–10^7CFU/g), but rather low in spring (10–10^4). *E. coli* was also present, as well as *Staphylococcus* spp. but in lower numbers (*Staphylococcus* spp. present only in spring) (Gonçalves Dos Santos et al., 2017; Gonçalves et al., 2018).

Moving to the Azores, Domingos-Lopes et al. (2017) performed the phenotypic identification of bacteria present in *Pico* cheese to the genus and species level: 56.1% of the total isolates were *Lactobacillus* spp., while 30.7% were identified as *Enterococcus* spp., 4.4% as *Lactococcus* spp., 3.5% as *Leuconostoc* spp. and 0.9% as *Streptococcus* spp (Domingos-Lopes et al., 2017). On the other hand, on a study performed by Câmara et al. (2017), *Lactococcus* spp. were the most abundant group at the end of the cheese ripening time (21 days), followed by *Leuconostoc* spp., *Lactobacillus* spp. and *Enterococcus* spp. *Staphylococcus* spp., and yeasts were also found, but no molds were detected (Câmara et al., 2017).

Kongo and colleagues performed some studies with *São Jorge* cheese throughout the years (Kongo et al., 2007, Kongo et al, 2008, Kongo et al, 2009). The main finding of these studies was that *Lactobacillus* is the predominant genera. The study performed in 2007, reveals that besides *Lactobacillus*, in the end of the cheese ripening time, *Enterococcus* spp. was the second most abundant group of microorganisms, followed by *Pediococcus* and *Leuconostoc* spp. *Lactococcus* species were also found in *São Jorge* cheese, but only in early stages of ripening (Kongo et al., 2007). A subsequent study in 2008 assessed the hygienic safety of this cheese by evaluating the presence of *Enterobacteriaceae* and *Micrococcaceae*. Some *Klebsiella* species were found, as well as *E. coli* and *Staphyloccocus*, while no *Salmonella* species were detected (Kongo et al., 2008). In another study performed in 2009, the second most prevalent group of microorganisms, after *Lactobacillus* spp. (10^7–10^8 CFU/g) found in this cheese were *Lactococcus* spp., with viable counts around 10^6–10^7 CFU/g, followed by *Enterococcus* spp. with 10^5–10^6 CFU/g. Yeasts and molds were also present in levels around 10^4 and 10^5 CFU/g, respectively. Moreover, the presence of *Enterobacteriaceae* was also assessed and observed in small quantities, around 10^2 CFU/g (Kongo et al., 2009).

In conclusion, lactic acid bacteria are undoubtedly the predominant group in all cheese types, although the most abundant genera may vary from cheese to cheese. Significant variations in cheese microbiota among dairies and seasons are also evident. All of the aforementioned information is summarized in Table 2.

3.2 OMIC technologies

While conventional microbiology remains fundamental and should be conducted concurrently, it presents several drawbacks, primarily due to its time-consuming nature, challenges in selecting suitable growth media and conditions, and the potential oversight of less abundant microorganisms that may be overshadowed by predominant ones. Additionally, the presence of viable but non-culturable (VBNC) microorganisms poses a significant challenge when employing culture-dependent techniques (Anastasiou et al., 2022; Araújo-Rodrigues et al., 2022). In contemporary times, the emergence of OMIC technologies has revolutionized the fields of food science and microbial ecology (Afshari et al., 2020). This new generation of culture independent techniques, like metagenomics, proteomics, metabolomics or transcriptomics, used individually or in integrative analysis, will undoubtedly shed light on the complex microbial ecology of traditional PDO cheeses.

Several reviews have described the importance and interest of studying the cheese microbiota, aiming to correlate microbial interactions with the quality and flavor of the final product (Sattin et al., 2016; Papademas et al., 2019; Afshari et al., 2020; Jiang et al., 2023). However, only a limited number of OMIC studies has been conducted on Portuguese traditional PDO cheeses, including lipidomics, volatilomics (Reis Lima et al., 2020; Inácio et al., 2023), and metagenomics (Riquelme et al., 2015; Rocha et al., 2021; Araújo-Rodrigues et al., 2022; Coelho et al., 2023).

TABLE 2 Microbial communities of Portuguese PDO cheeses assessed by conventional microbiology and metagenomic approach.

PDO Cheese	Microbial communities by conventional microbiology	Microbial communities by metagenomic	References
Transmontano	No studies available to date		
Terrincho	Predominant bacteria – LAB: *Enterococcus* spp., *Lactobacillus* spp. and *Lactococcus* spp. Other: yeasts and molds, *Staphylococcus* spp., *Enterobacteriaceae*	No studies available	(Pinho et al., 2004; Pintado et al. 2008)
Beira Baixa	Predominant bacteria – LAB: *Lactococcus* spp., *Lactobacillus* spp., *Enterococcus* spp. Other: *Enterobacteriaceae*, molds, yeasts, *Staphylococcus* spp.	Predominant: *Lactococcus lactis, Lactiplantibacillus plantarum* Other: *Lacticaseibacillus zeae, Streptococcus thermophilus, Loigolactobacillus coryniformis* Fungi – *Candida sakey*, *Ustilago*, *Starmerella*, *Cladosposium variabile* and *Pichia kluyveri*	(Cardinali et al., 2022; Freitas et al., 1996; Rodrigues, 2023)
Rabaçal	Predominant bacteria: LAB Other: *Enterobacteriaceae*, coliform bacteria, yeasts, molds, *Staphylococcus* spp.	No studies available to date	(Dias, 2021)
Serra da Estrela	Predominant bacteria: LAB Other: *Enterobacteriaceae*, yeasts, *Staphylococcus* spp., molds	Predominant bacteria: *Leuconostoc* spp., *Lactococcus* spp., *Lactobacillus* spp and *Enterococcus durans* Fungi – *Candida* spp., *Debaryomyces* spp., *Yarrowia* spp, *Starmerella*, *Vishniacozyma victoriae*, *Kurtzmaniella zeylanoide*, *Cladosporium variabile*, *Cutaneotrichosporon curvatus and Metschnikowia fructicola*	(Dahl et al., 2000; Rampanti et al., 2023; Rocha et al., 2021, 2023; Salamandane et al., 2024; Tavaria and Malcata, 1998)
Azeitão	Predominant bacteria: LAB	Predominant bacteria: LAB – *Lactococcus, Leuconostoc spp., Lactobacillus*	(Baptista, 2018; Machado, 2020)
Serpa	Predominant bacteria: LAB (*Lactobacillus* spp., *Lactococcus* spp.,	Predominant bacteria: *Lactococcus, Leuconostoc spp., Lactobacillus*	(Gonçalves et al., 2018; Gonçalves Dos Santos

(Continued)

TABLE 2 Continued

PDO Cheese	Microbial communities by conventional microbiology	Microbial communities by metagenomic	References
	Leuconostoc spp., *Enterococcus* spp.) Other: *Enterobacteriaceae* (*E.coli*) and coliforms, yeasts: *D. hansenni*, *Kluyveromyces* spp., *Staphylococcus* spp.	*Enterobacteria* Yeast: *Debaryomyces, Kluyveromyces, Galactomyces*	et al., 2017; Roseiro et al., 2003)
Évora	Predominant bacteria: LAB	No studies available to date	(Dias, 2021)
Nisa	Predominant bacteria: LAB	Predominant bacteria: LAB – *Lactococcus, Leuconostoc spp., Lactobacillus*	(Baptista, 2018)
Pico	Predominant bacteria: LAB (*Lactobacillus* spp. – *Lactiplantibacillus plantarum*, *Lacticaseibacillus. paracasei* subsp. *paracasei*, etc. *Enterococcus* spp., *Lactococcus* spp., *Leuconostoc* spp. – *Leuconostoc spp. lactis*, *Enterococcus* spp. – *E. faecalis*) Other: *Staphylococcus* spp., yeasts	Predominant bacteria: *Lactococcus (L. lactis ssp. lactis), Enterococcus, Lactobacillus (L. paracasei, L. casei), Leuconostoc spp. (L. pseudomesenteroides).* Other: *Acinetobacter, Staphylococcus, Panthoea, Rothia*	(Câmara et al., 2017, 2019; Domingos-Lopes et al., 2017; Riquelme et al., 2015)
São Jorge	Predominant bacteria: *Lactobacillus* spp. – *L. paracasei*, *Lacticaseibacillus rhamnosus*, *Lactococcus* spp. – *L. lactis*, *Enterococcus* spp. – *E. faecalis, E. faecium*, *Pediococcus* spp., *Leuconostoc* spp. Other: *Enterobacteriaceae*, yeasts and molds, *Micrococcaceae*, *Klebsiella*	Predominant bacteria: *Leuconostoc spp., Lactobacillus, Enterococcus*	(Coelho et al., 2023; Kongo et al., 2007, 2009; Kongo, Gomes, and Malcata, 2008)

Regarding the metagenomic analysis, to our knowledge, the following Portuguese PDO cheese have been studied using targeted metagenomics, directed to the 16S rDNA and/or 26S rDNA amplicons: *Beira Baixa, Serra da Estrela, Azeitão, Serpa, Nisa, Pico* and *São Jorge* cheeses. Moreover, a recently published report featuring *Serra da Estrela* cheese applied shotgun metagenomics (Salamandane et al., 2024).

In *Beira Baixa* cheese, specifically *Castelo Branco*, a survey of the microbial community was performed by Cardinali et al. (2022).

The authors sequenced the V3–V4 regions of the 16S rDNA for bacterial and the 26S rDNA for fugal analysis, respectively (Cardinali et al., 2022). In brief, the authors tested three producers and found no significant differences the in terms of microbial content being the most prevalent species *Lactococcus lactis* and *Lactiplantibacillus plantarum*. In lower prevalence there was also *Lacticaseibacillus zeae*, *Streptococcus thermophilus* and *Loigolactobacillus coryniformis* (Cardinali et al., 2022). As for the fungal community, there were some differences detected within the different producers where *Candida sake* and *Ustilago* were the most prevalent in producer 1, *Starmerella* and *Cladosposium variabile* in producer 2 and *C. variabile* and *Pichia kluyveri* in producer 3 (Cardinali et al., 2022).

For *Serra da Estrela* PDO cheese, Rocha et al (2021), conducted a similar study of the cheeses microbial communities. The sequencing strategy was similar to the one applied to *Beira Baixa* cheese, so for the 16S rDNA the regions V3–V4 were selected and for the fungi community the Internal Transcribed Spacer 2 (ITS-2). In summary, from the 500 taxa identified, the authors appointed 30 as core taxa, present in all samples tested, including genus like *Leuconostoc* spp. and *Lactococcus* spp. for bacteria, and *Candida* spp., *Debaryomyces* spp. and *Yarrowia* spp. for fungi (Rocha et al., 2021). Another study by Rampanti et al. (2023) has also resorted to 16S rDNA sequencing of the region V3–V4 to analyze the microbial content of four producers of PDO *Serra da Estrela* cheese. Their metataxonomic analysis showed, once again, *Lactococcus lactis* as one of the most prevalent species, being detected in all cheese samples analyzed. Moreover, some species of *Leuconcostoc* were also identified (i.e. *Leuconostoc mesenteroides* and *Leuconostoc sakei*) as well as *Enterococcus* spp (Rampanti et al., 2023). The authors also conducted a survey on the cheeses mycobiota, verifying that this community of microorganisms could be dependent on each producer. They observed a wide variation in the fungal content of cheeses from different producers, likely due to different manufacturing techniques. Nevertheless, the authors performed detection of major and minor taxa. Among the major taxa, *Debaryomyces hansenii, Starmerella*, *Vishniacozyma victoriae* and *Kurtzmaniella zeylanoides* were detected. As for minor taxa it was detected *Cladosporium variabile*, *Cutaneotrichosporon curvatus* and *Metschnikowia fructicola* (Rampanti et al., 2023).

Still in *Serra da Estrela* cheese, a shotgun metagenomic approach was used to evaluate the microbiome, resistome and virulome, of both PDO and non-PDO cheeses (Salamandane et al., 2024). Briefly, the authors explored four different producers, two PDO producers (QG1/QG2 and QI1/QI2) and two non-PDO producers (QL1/QL2 and QT1/QT2). Regarding the cheeses microbiota, the authors observed a clear predominance of *Leuconostoc mesenteroides* throughout the different samples. As for the PDO specific microbiota, samples from both producers harbored *Enterococcus durans*, *Kocuria salsicia*, *Glutamicibacter ardleyensis*, *Lactococcus lactis*, *Raoultella ornithinolytica*, *Lactobacillus coryniformis* and *Lactiplantibacillus plantarum*. Contrarily, non-PDO cheeses showed higher diversity of associated species, even within samples from the same producer. Briefly, in QL1 and QL2 there was a predominance of *Enterococcus durans* while in QL2 the predominant species was *Lactobacillus paraplantarum*. Moreover, increased disparities were found in QT producers, with three predominant species, namely *Lactococcus lactis*, *Lacticaseibacillus rhamnosus* and *Leuonostoc. Mesenteroides* being associated with QT1, while QT2 samples showed a set of five predominant species: *Lacticaseibacillus paracasei*, *Lacticaseibacillus rhamnosus*, *Lactiplantibacillus plantarum*, *Leuconostoc mesenteroides* and *Lactococcus lactis* (Salamandane et al., 2024). Overall, this study further highlights the importance of using metagenomic approaches for the in depth clarification of the complex microbial ecosystem associated with traditional cheeses, either PDO or non-PDO.

In the case of Azeitão and Nisa cheeses, owing to the scarcity of metagenomic studies on the microbiotas communities, we turned to a masters dissertation where a metagenomic study was conducted to characterize the bacterial community (Baptista, 2018).

In brief, the authors studied PDO cheeses from 2016 to 2018 produced in these two regions, by sequencing the regions V1–V3 of the 16S rDNA for bacterial identification. In the analyzed cheese samples, Baptista (2018) identified over 22 different genera, mostly belonging to the LAB group. Throughout the different producers in both regions, the most prevalent genus was *Lactococcus* followed by *Leuconostoc* spp. and *Lactobacillus*. Moreover, concerning the microbial diversity of each producer in different years, some differences were observed in the relative abundance of the most prevalent genera. Additionally, in the less abundant genera, there was higher diversity, with some genera not being present in different years (Baptista, 2018).

PDO cheeses produced in *Serpa* have also been studied. In a study by Gonçalves Dos Santos et al. (2017), an analysis of the yeast community of *Serpa* PDO and non-PDO cheeses was conducted. In brief, sequencing of the 26S rDNA was performed for the ITS region and D1/D2 to enable identification at the species level. The authors assessed the diversity of the fungal community during different seasons (winter and spring) and verified that the most common fungal genera were *Debaryomyces* and *Kluyveromyces*. Moreover, the genus *Galactomyces* was also found, however its abundance varied within the different cheese factories (Gonçalves Dos Santos et al., 2017). Another study, also from the same research group, focused on the 16S rRNA sequencing of the V3–V4 regions, verifying a prevalence of the *Lactococcus* followed by *Leuconostoc* spp., *Lactobacillus* genus and *Enterobacteriaceae* family (Gonçalves et al., 2018).

To our knowledge, only one study has focused on the analysis of microbial communities in *Pico* cheese using metagenomics, specifically targeting the 16S rRNA sequencing of the V3–V4 regions. Riquelme et al. (2015), verified that the most prevalent microorganisms, (included in the dominant category defined by the authors), as core bacteria of *Pico* cheese were: *Lactococcus*, *Streptococcus*, *Acinetobacter*, *Enterococcus, Lactobacillus, Leuconostoc* spp., *Staphylococcus, Panthoea* and *Rothia* (Riquelme et al., 2015). Another study, also from the same research group used 16S rRNA gene sequencing for identification of autochthonous LAB from the *Pico* cheese, aiming to assess their technological potential. The most interesting species in terms of technological potential

were *Lactococcus lactis* ssp. *lactis*, *Lacticaseibacillus paracasei*, *Leuconostoc pseudomesenteroides* and *L. casei* (Câmara et al., 2019).

Regarding the *São Jorge* PDO cheese a metagenomic study was performed by Coelho et al. (2023) focusing on next-generation sequencing (NGS) as a tool to distinguish PDO from non-PDO cheeses. The authors observed that certified cheeses were richer in *Leuconostoc*, *Lactobacillus*, and *Enterococcus*, whereas in the non-certified cheeses, there was a prevalence of *Streptococcus* followed by *Lactococcus* (Coelho et al., 2023).

Regarding the PDO cheeses *Rabaçal*, *Terrincho*, *Évora*, and *Transmontano*, to our knowledge, there is still no metagenomic data available.

Overall, the most commonly used strategy for metagenomic studies involves sequencing the 16S rDNA of the V3–V4 regions for bacterial characterization and the 26S rDNA of the ITS or D1/D2 regions for fungal characterization. From the information gathered in this section and Section 3.1, it is evident that an OMIC approach, combined with culture-dependent techniques, provides several beneficial aspects for studying PDO cheese microbiota. This approach not only helps assess the quality of the cheeses but also aids in distinguishing certified cheeses. However, to our knowledge, research in this field, particularly regarding Portuguese cheeses, is still limited. All of the above information is summarized in Table 2.

4 Conclusions

In conclusion, our review underscores the pivotal role of microbial communities in shaping the distinct characteristics of Portuguese traditional cheeses, including their flavor, texture, and safety profiles. By synthesizing findings from conventional microbiological studies and cutting-edge OMICs analyses, we have gained valuable insights into the complex dynamics of cheese microbiota. Our exploration of microbial composition, diversity, and functional roles across various PDO cheeses has revealed the significant contributions of the microbiota to cheese ripening, flavor development, and safety assurance. Moreover, our discussion highlights the potential of OMICs technologies, particularly metagenomics, in elucidating the intricate microbial ecosystems of these cheeses.

Through this integrative approach, we have attempted to unveil the secrets behind the rich heritage and distinctiveness of Portuguese traditional cheeses. By furthering our understanding of the interplay between microorganisms and cheese matrices, we pave the way for continued advancements in cheese production, quality assurance, and preservation of cultural heritage.

Author contributions

TS-L: Conceptualization, Funding acquisition, Supervision, Writing – review & editing. SS: Writing – original draft, Writing – review & editing. SM: Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Deciphering the microbial succession and color formation mechanism of "green-covering and red-heart" *Guanyin Tuqu*

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"Green-covering and red-heart" *Guanyin Tuqu* (GRTQ), as a type of special fermentation starter, is characterized by the "green-covering" formed on the surface of *Guanyin Tuqu* (SQ) and the "red-heart" in the center of *Guanyin Tuqu* (CQ). However, the mechanisms that promote temporal succession in the GRTQ microbial ecology and the formation of "green-covering and red-heart" characteristics remain unclear. Herein, we correlated the temporal profiles of microbial community succession with the main environmental variables (temperature, moisture, and acidity) and spatial position (center and surface) in GRTQ throughout fermentation. According to the results of high-throughput sequencing and culture-dependent methods, the microbial communities in the CQ and SQ demonstrated functional complementarity. For instance, the bacterial richness index of the CQ was greater than that of SQ, and the fungal richness index of the SQ was greater than that of CQ at the later stage of fermentation. Furthermore, *Saccharomycopsis*, *Saccharomyces*, *Aspergillus*, *Monascus*, *Lactobacillus*, *Bacillus*, *Rhodanobacter*, and *Chitinophaga* were identified as the dominant microorganisms in the center, while the surface was represented by *Saccharomycopsis*, *Aspergillus*, *Monascus*, *Lactobacillus*, *Acetobacter*, and *Weissella*. By revealing the physiological characteristics of core microorganisms at different spatial positions of GRTQ, such as *Aspergillus clavatus* and *Monascus purpureus*, as well as their interactions with environmental factors, we elucidated the color formation mechanism behind the phenomenon of "green" outside and "red" inside. This study provides fundamental information support for optimizing the production process of GRTQ.

KEYWORDS

"green-covering and red-heart" *Guanyin Tuqu*, microbial community, temporal succession, environmental variables, spatial position, color formation

Introduction

"Green-covering and red-heart" *Guanyin Tuqu* (GRTQ), as a kind of special fermentation starter in the southern provinces of China, plays a vital role in initiating the fermentation of the light-flavor *Baijiu* (Zheng and Han, 2016; Jiang et al., 2018; Zhu et al., 2022). The raw material of GRTQ mainly consisted of "*Guanyin*" clay, rice bran, *Zhongqu* (mother *Tuqu*), and

Monascus wheat bran, which were manually kneaded into a spherical billet with a diameter of approximately 8 cm. The open manufacturing and fermentation environment of GRTQ provided conditions for the enrichment of microorganisms (Du et al., 2019). During the fermentation process of GRTQ, the green part formed on the surface was called "green-covering," and the red part formed in the center was called "red-heart." According to practical production experiences, GRTQ with an appropriate amount of "red-heart" and "green covering" was usually considered to be a reflection of high quality (Li et al., 2010; Hu et al., 2016). Previous study has reported that *Neurospora crassa*, *Aspergillus nidulans*, *Bacillus subtilis*, and *Oceanobacillus iheyensis* were chameleon-like microbes that regulated the metabolic differences of five-member heterocyclic amino acids in three-color sauce-flavor *Daqu*, resulting in microecological differentiation (Yang et al., 2023). Dong et al. (2024) found that *Kroppenstedia*, *Virgibacillus*, and *Bacillus* as dominant bacteria in black *Daqu*, yellow *Daqu*, and white *Daqu* of sauce-flavor *Jiuqu*, severally. However, no in-depth research has been conducted on how the "green-covering and red-heart" coloration arises and how they affect the quality of GRTQ.

To date, the production of GRTQ mainly depends on traditional crafts, which are characterized by low mechanization and rely on the experience of craftsmen to adjust the process parameters, which easily leads to dissimilar communities and qualities among different batches of GRTQ (Han et al., 2023). The quality of *Jiuqu* (a fermentative agent) depends largely on the composition of the microbial community and metabolic characteristics. Microbes can secrete lots of hydrolases, flavor compounds, and functional substances during the fermentation of *Jiuqu* (Wang and Xu, 2015). Thus, it is necessary to obtain a comprehensive understanding of the microbial diversity and function of GRTQ and brewing microbial resources, which is the premise and foundation for fully analyzing the fermentation mechanism of light-flavor *Baijiu* and improving its the product quality. Therefore, a comprehensive understanding of the spatial distribution of microbial composition and microbial succession patterns during *Tuqu* fermentation is required (Jin et al., 2019). Previous studies have reported that environmental factors such as moisture (Lei et al., 2020), temperature (Xiao et al., 2017; Fu et al., 2021; Zhao et al., 2022), pH (Narendranath and Power, 2005), and acidity (Zheng et al., 2018; Hao et al., 2021) were the driving factors of microbial community succession in *Jiuqu*. For example, Ma et al. (2022) reported that temperature, humidity, and acidity were the main driving factors of microbial succession during *Daqu* fermentation. Deng et al. (2020) reported that variations in temperature and moisture led to microbial diversity and various metabolites during spontaneous solid-state fermentation of high-temperature *Daqu*. To our knowledge, the major driving factors for microbial community in *Tuqu* have not been reported. Therefore, it is crucial to study the microbial community succession mechanism and environmental factors affecting microbial growth during the cultivation process of GRTQ to stabilize the quality and establish a modern production workshop for GRTQ.

In this study, samples of GRTQ from different fermentation stages were collected. By high-throughput sequencing combined with a classical culture-dependent approach, the microbial community succession in the samples were statistically analyzed to determine the dynamic changes in microorganisms throughout the production process at the center and on the surface. Furthermore, the correlations between the core microbiota and environmental variables (temperature, moisture, and acidity), as well as the growth characteristics of *Aspergillus clavatus* and *Monascus purpureus* were investigated, revealing the mechanism underlying the "green-covering" and "red-heart" phenomenon. These results could provide reference support for optimizing the production process of GRTQ and establishing modern production lines.

Materials and methods

Sample collection

"Green-covering and red-heart" *Guanyin Tuqu* (GRTQ) samples were collected from the *Tuqu*-making workshop in Daye, Hubei province, China. GRTQ was made from the *Guanyin* clay [73.8%, weight (w)], rice bran (24.6%, w), *Zhongqu* (1.6%, w), and *Monascus* wheat bran (1.2%, w), and the water content was kept at 32%. Next, the mixture was manually kneaded into a spherical billet with a diameter of approximately 8 cm. These spherical billets were placed in the box bed for the preculture at 27°C for 23 h termed culture microorganisms. Then, the obtained billets were transferred to the steel shelf in another room for the second incubation, and the temperature of the culture room was controlled at 30°C. After cultivation for 7 days, the culture room was maintained at 35°C to reduce the water content of GRTQ to <10%, which was called the dry period. The selected samples included: GRTQ cultured microorganisms, GRTQ fermented for days 1–7, and drying stage, which were labeled as 0, 1, 2, 3, 4, 5, 6, 7, and D, respectively. Each GRTQ sample was divided into two parts, included the surface layer of 1.5 cm thick was named surface part of *Tuqu* labeled as SQ, and the remaining central part was named central part of *Tuqu* labeled as CQ (Figure 1). Each sample had three biological replicates. A total of 54 samples were obtained. These samples were stored at 4°C until further analysis, and −20°C for DNA extraction.

Physicochemical properties

The moisture content, total acids, and temperature of GRTQ samples were determined. After drying at 150°C for 90 min, the moisture content of the GRTQ powder was calculated using the gravimetric method. Total acids was determined by titration with 0.1 M NaOH and 100 μL of phenolphthalein to obtain a titration endpoint of pH 8.2. The temperature was measured by inserting the thermometer directly into the center of the *Tuqu*.

Isolation and enumeration of different culturable microbes

Ten grams of GRTQ powder were homogenized in 90 mL of sterile water, and incubated for 25 min continuously stirred at 160 rpm under 20°C. This suspension was defined as a 1/10 dilution and was serially diluted 10-fold with sterile water. A 100 μL aliquot of each dilution was spread and incubated on the agar media (showed in Table 1; Rosfarizan and Ariff, 2000; Shi et al., 2006; Zhou et al., 2021). The media for culturing fungi and bacteria were supplemented with final concentrations of 0.5 g/L chloramphenicol and 0.5 g/L nystatin,

FIGURE 1
Schematic of the production process of "green-covering and red heart" *Guanyin Tuqu* and the appearance of the center and the surface.

respectively. The enumerations of each group of microbes for each sample were performed in triplicate. The above reagents were purchased from Solarbio Science & Technology Co., Ltd., Beijing, China.

The growth characteristics of *A. clavatus* and *M. purpureus*

Preculture conditions: strain was inoculated on a PDA plate at 30°C, pH 6.0 and cultured for 5 days on the aerobic incubator. Regulated batch cultures: the effects of PDA medium at pH (3.07, 4.02, 5.00, 6.09, and 6.97), temperature (25, 30, 35, 40, and 45°C), and oxygen (aerobic incubator and anaeropack box) on the growth of *M. purpureus* and *A. clavatus* were investigated, respectively. Anaerobic box was used to generate anaerobic conditions.

DNA extraction, amplification, and sequencing

Total genomic DNA of samples was extracted with the TGuide S96 Magnetic Soil/Stool DNA Kit [Tiangen Biotech (Beijing) Co., Ltd.] according to manufacturer's instructions. The DNA concentration of the samples was measured with the Qubit dsDNA HS Assay Kit and Qubit 4.0 Fluorometer (Invitrogen, Thermo Fisher Scientific, Oregon, United States). The 27F: AGRGTTTGATYNTGGCTCAG and 1492R: TASGGHTACCTT GTTASGACTT universal primer set was used to amplify the full-length 16S rDNA gene, and the 1F: 5′-CTTGGTCATTTAGAGG AAGTAA-3′ and 4R: 5′-TCCTCCGCTTATTGATATGC-3′ universal primer set was used to amplify the ITS rDNA gene from the genomic DNA, extracted from each sample. All PCR reactions were carried out in 20 μL reaction system containing 0.5 mM of each primer, 10 ng of template DNA, and the KOO One PCR Master Mix (TOYOBOLife Science) was used to perform 25 cycles of PCR amplification, the PCR conditions of 16S rDNA region amplifcation were as follows: pre-denaturation at 95°C for 2 min, followed by 25 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min 30 s, and a final extension at 72°C for 2 min. The PCR conditions of ITS rDNA region amplifcation were as follows: pre-denaturation at 95°C for 5 min, followed by 25 cycles of denaturation at 98°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s, and a final extension at 72°C for 10 min. The total of PCR amplicons were purified with Agencourt AMPure XP Beads (Beckman Coulter, Indianapolis, IN) and quantified using the Qubit dsDNA HS Assay Kit and Qubit 4.0 Fluorometer (Invitrogen, Thermo Fisher Scientific, Oregon, United States). After the individual quantification step, amplicons were pooled in equal amounts. SMRTbell libraries were prepared from the amplified DNA by SMRTbell Express Template Prep Kit 2.0 according to the manufacturer's instructions (Pacific Biosciences). Purified SMRTbell libraries from the pooled and barcoded samples were sequenced on a single PacBio Sequel II 8 M cell using the Sequel II Sequencing kit 2.0. Raw data FASTQ files were imported into the format which could be operated by QIIME2 system using QIIME tools import program. Demultiplexed sequences from each sample were quality filtered and trimmed, de-noised, merged, and then the chimeric sequences were identified and removed using the QIIME2 dada2 plugin to obtain the feature table of amplicon sequence variant (ASV). The QIIME2 feature-classifier plugin was then used to align ASV sequences to a pre-trained GREENGENES 13_8 99% database to generate the taxonomy table (Bokulich et al., 2018). Any contaminating mitochondrial and chloroplast sequences were filtered using the QIIME2 feature-table plugin. Sequencing service and data analysis service were provided by Wekemo Tech Group Co., Ltd. Shenzhen China. For the sequence data, they were deposited in the national center of biotechnology information (NCBI) database with accession number: PRJNA1080012.

TABLE 1 Isolation materials and methods for different culturable microorganisms.

Microbial species	Media component	Separation principle	Culture conditions
Yeasts	Yeast extract peptone dextrose agar (YPD): 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, and 20 g/L agar.	Peptone and yeast extract powder provide carbon and nitrogen sources, and glucose provides fermentable sugars for yeasts.	30°C for 72 h
Aerobic bacteria	Wallerstein nutrient agar (WL): 5 g/L casein peptone, 4 g/L yeast extract, 50 g/L glucose, 0.425 g/L KCl, 0.125 g/L $CaCl_2$, 0.125 g/L $MgSO_4$, 0.0025 g/L $FeCl_3$, 0.0025 g/L $MnSO_4$, 0.55 g/L KH_2PO_4, 0.022 g/L bromocresol green, 20 g/L agar, and pH 5.5.	KCl, $CaCl_2$, and $FeCl_3$ are critical ions and help maintain osmotic balance.	37°C for 48 h
Molds and *Aspergillus clavatus*	Potato dextrose agar (PDA): 6 g/L potato infusion powder, 20 g/L glucose, 20 g/L agar, and pH 5.6.	Potato extract is beneficial to the growth of all kinds of molds, and glucose can provide carbon source.	30°C for 72 h
Lactobacillus	De Man, Rogosa, and Sharpe agar (MRS): 10 g/L peptone, 8 g/L beef extract, 4 g/L yeast extract, 20 g/L glucose, 2 g/L KH_2PO_4, 2 g/L ammonium citrate dibasic, 5 g/L CH_3COONa, 0.2 g/L $MgSO_4$, 0.04 g/L $MnSO_4$, 1 g/L Tween-80, 20 g/L agar, and pH 6.5.	Ammonium citrate dibasic, CH_3COONa, $MgSO_4$, $MnSO_4$, and tween-80 provide growth factors for the culture of various *Lactobacillus*.	37°C for 48 h
Monascus	PDA medium, and 0.2 mL of sterilized lactic acid solution (50%, V:V) was added to cover the sample.	*Monascus* likes lactic acid, and high concentration of lactic acid can inhibit the growth of miscellaneous bacteria.	30°C for 72 h

Statistical analysis

Origin Pro 2018 was used to draw the histogram and line chart. Spearman's correlation heat map analysis was performed to explore the correlations between environmental variables and microorganisms using the "pheatmap" packages of R (version 4.0.3). Significant differences were tested using one-way ANOVA in SPSS. The microbial communities in surface and center of GRTQ were further compared using the linear discriminant analysis effect size (LEfSe) analysis, Venn diagram and PCoA analysis with the online interface using the Wekemo bioincloud: https://www.bioincloud.tech/standalone-task-ui/lefse.

Results

Diversity of microbial community in the fermentation process of GRTQ

After filtering the raw sequencing data by QIIME 2, 152,683 bacterial reads and 138,708 fungal reads from the surface parts of *Tuqu* (SQ) and 164,628 bacterial reads and 161,582 fungal reads from the central parts of *Tuqu* (CQ) were obtained. These bacterial and fungal reads were clustered into 3,808 and 525 OTUs, respectively. The total sequences belonged to 16 phyla and 153 genera of bacteria and 7 phyla and 63 genera of fungi. Among them, 5 phyla and 43 genera of fungi were detected in the SQ. On the other hand, the fungi in the CQ were identified as belonging to 6 phyla and 51 genera (Figure 2A). In addition, 13 phyla and 107 genera of bacteria were detected in the SQ, while the bacteria in the CQ were identified as 12 phyla and 112 genera (Figure 2B). The results indicated that both the SQ and the CQ were inhabited by a quantity of microbial populations, which might indicate that they are highly important for the production of *Baijiu* (Chen et al., 2021).

For alpha diversity, a greater richness index indicated greater community diversity. The fungal richness index of the CQ first increase from 38.5 (Day 0) to 45.5 (Day 2), and then decreased to 8.5 during the drying process (Figure 2C). However, the bacterial richness of the CQ presented an opposite trend to that of fungi, which first decreased from 219.5 (Day 0) to 104 (Day 4), and then raised to 157.5 during the drying process (Figure 2D). The richness index of fungi changed from 61 (Day 0) to 17 (Day 3), then gradually raised to 41 (Day 5), and finally decreased to 19.5 during the drying process in the SQ (Figure 2C). However, there was no obvious change in the richness index of bacteria in the SQ in a narrow range, fluctuating between 130 and 170 (Figure 2D). The richness index of bacteria was far greater than that of fungi both in the SQ and CQ, which suggested that the diversity of bacteria was greater than that of fungi. After Day 4, the fungal richness index of the SQ was greater than that of the CQ, indicating the SQ had more fungal diversity, which might be related to the oxygen content of the SQ (Mo et al., 2022). At the later stage of fermentation (After Day 7), the bacterial richness index of the CQ was greater than that of the SQ, indicating that the CQ had greater bacterial diversity.

We then investigated the number of common and unique genera of the CQ and the SQ during the succession process via a Venn diagram. There were 20 and 12 unique fungal genera in the CQ and SQ, respectively, while there were 46 and 41 unique bacterial genera, respectively (Figures 2E,F), suggesting that the CQ possessed slightly higher uniqueness than the SQ.

Microbial composition and community succession

PCoA analysis based on Bray–Curtis dissimilarity distances revealed that both bacterial and fungal communities between the CQ and the SQ on different days were clearly separated. The succession process of fungi could be divided into phase 1 (C0-C3 and S0-S4) and phase 2 (C4-CD and S5-SD) throughout the production of GRTQ (Figure 3A). In addition, the bacteria were classified as phase 1 (C0 and S0), phase 2 (C1-C4 and S1-SD), and the subsequent fermentation of the CQ (C5-CD) formed an independent phase 3 (Figure 3B). These

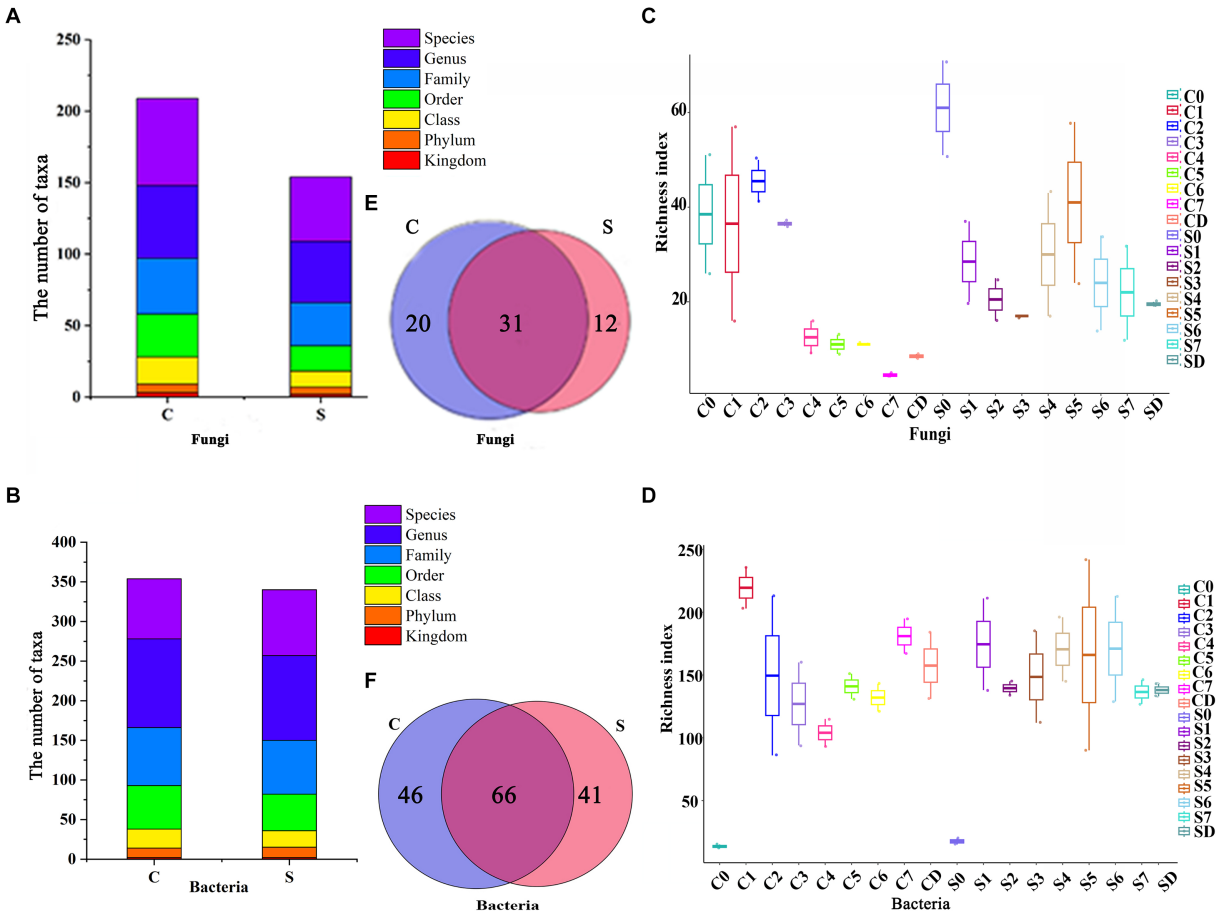

FIGURE 2
Alpha diversity and the microbial community composition of center and surface. Diagram for the **(A)** fungi and **(B)** bacteria from phylum to genus level. **(C)** Fungal and **(D)** bacterial diversity in terms of richness index at different fermentation times. The lines inside the boxes denote the median at 95% confidence. Venn diagram of **(E)** fungi and **(F)** bacteria according to the genus level. C, Center; S, Surface, 0–7, Days 0–7; D, drying.

results indicated that the successional rhythms of the fungal communities in the CQ and SQ were basically similar, while there were significant differences in the bacterial communities. Specifically, the bacterial communities in the CQ exhibited more intense succession during the later fermentation stage (After Day 4).

The relative abundances and genus compositions of dominant microorganisms were investigated between the CQ and SQ on different days during the fermentation process (Figures 3C,D). Among the top 20 genera with relative abundances were greater than 0.1%, *Saccharomycopsis*, *Saccharomyces*, *Aspergillus*, *Monascus*, *Lactobacillus*, *Bacillus*, *Rhodanobacter*, and *Chitinophaga* were identified as the dominant microorganisms in the CQ, while those in the SQ were represented by *Saccharomycopsis*, *Aspergillus*, *Monascus*, *Lactobacillus*, *Acetobacter*, and *Weissella*. *Mortierella* only appeared in the CQ, while *Beauveria* only existed in the SQ. During the C0 and S0 periods, the relative abundances of dominant microorganisms were relatively high, and the species and quantities of microorganisms were largely similar, mainly due to the straw curtains covered the GRTQ samples at the beginning of fermentation, providing heat insulation and moisture preservation. Thus, there were few differences in the environments of the SQ and CQ. As fermentation progresses, fungal community succession began to occur. The abundance of *Saccharomycopsis* first increased and then decreased in CQ and SQ. The abundance of *Saccharomycopsis* in the SQ was higher than that in the CQ. At the S2 stage, the maximum proportion of *Saccharomycopsis* was 61.66%, after which the percentage decreased slowly until the end of fermentation. The highest percentage of *Saccharomycopsis* was 32.41% at the C1 stage but distinctly decreased to 1.19% at the C5 stage. Notably, *Saccharomyces*, as dominant yeast in the CQ, was much greater at the early stage of fermentation than at the end of fermentation. Additionally, *Saccharomyces* in the CQ reduced gradually from 44.03% in the C0 period to 0.50% in the C5 period. However, *Saccharomyces* was 38.96% at the initial S0 stage in the SQ, and its abundance was relatively low (< 4%) at the other stages. In the preliminary stage of the SQ (S1-S4), *Aspergillus* was more dominant (> 30%), then gradually decreased with the fermentation process, but it was still higher than 10%. However, *Aspergillus* predominated at the C3 stage (58.58%) and was relatively rare at other stages (< 1%). *Monascus* accounted for a small proportion in the CQ and the SQ during the initial stage of fermentation, but quickly increased from 63.38 to 99.67% in C4-CD, and rising from 58.32 to 75.43% in S5-SD.

We further investigated the distribution and succession of the core bacteria during the GRTQ fermentation process (Figure 3D). The bacterial genus *Lactobacillus* was significantly prevalent at both the CQ (42.87–84.92%) and the SQ (47.05–88.18%). *Bacillus* was present mainly in the late stage of fermentation in the CQ, and multiplied

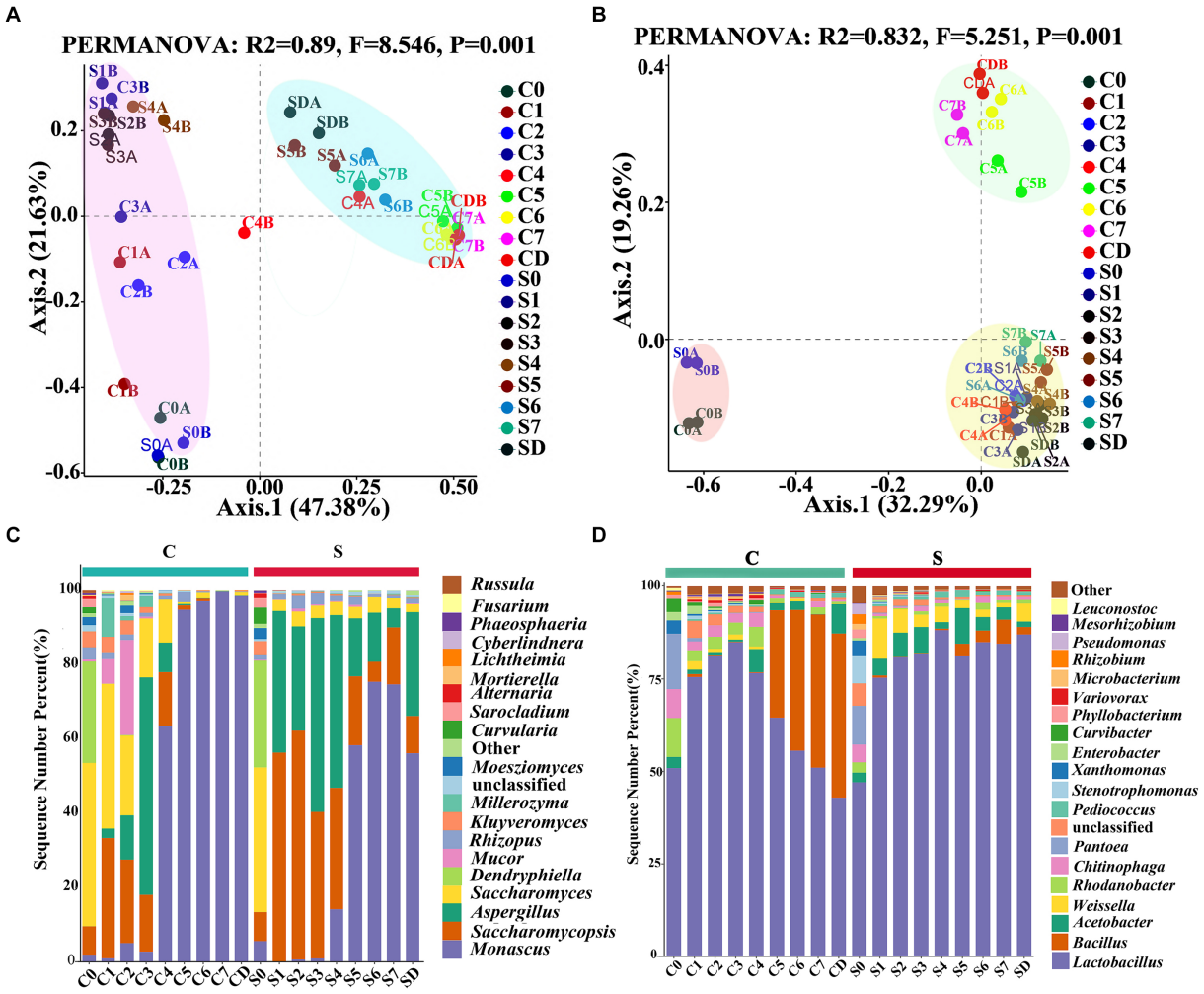

FIGURE 3
PCoA analysis of the microbial communities associated with the *Tuqu* fermentation process. **(A)** Fungi and **(B)** bacteria based on Bray-Curtis distance. **(C)** Fungal and **(D)** bacterial community compositions and succession at the genus level. Among the top 20 genera with relative abundance were greater than 0.1%. C, Center; S, Surface; 0–7, Days 0–7; D, drying.

quickly from the C5 to CD stage (29.12–44.43%), which might be due to its ability to withstand high temperature and low water activity in the late stage of fermentation (Zhang et al., 2021). *Acetobacter* mainly existed in the SQ, accounting for 9.67–1.48% (S5-SD). Similarly, *Weissella* also mainly presented in the SQ, occupying for 10.89–1.21% (S1–S7). This is mainly because their growth requires more oxygen. *Rhodanobacter* and *Chitinophaga* grew in the early stage of fermentation (C0-C4), accounting for 2.68–10.52% and 2.67–7.83%, respectively.

Because there was little difference in microbial abundance between the CQ and SQ on Day 0 (Figures 3C,D), the abundances of the detected fungal and bacterial taxa in the CQ and SQ samples (excluding Day 0) were further analyzed using the linear discriminant analysis (LDA) effect size (LEfSe) method. Notably, *Saccharomycopsis*, *Aspergillus*, *Rhizopus*, *Beauveria*, and *Neocosmospora* had statistical differences and were distinctive fungal genera in the SQ, while *Monascus*, *Mortierella*, *Lichtheimia*, *Fusarium*, and *Moesziomyces* were statistically different and distinctive fungal genera in the CQ (Figure 4A). Additionally, *Lactobacillus*, *Weissella*, *Acetobacter,* and *Gluconobacter* were discriminant bacterial taxa in the SQ, while *Mesorhizobium* was a discriminant bacterial taxon in the CQ (Figure 4C).

Enumeration of representative bacteria and fungi

LEfSe analysis revealed that *Aspergillus*, *Lactobacillus*, and *Monascus* were significant discriminant taxa (Figure 4). Furthermore, through high-throughput sequencing, it was found that *Lactobacillus*, *Monascus,* and *Aspergillus* accounted for a large proportion and played an important role in the fermentation of GRTQ (Figures 3C,D). Therefore, the dominant microorganisms (viable yeasts, aerobic bacteria, *Lactobacillus*, *Aspergillus*, and *Monascus*) were quantitatively investigated using the plate-counting method throughout the fermentation process of the SQ and the CQ. As shown in Figure 5, yeasts proliferated extensively in the later stage of fermentation in GRTQ, with a higher number of yeasts in the SQ than in the CQ. *Aspergillus* was mainly distributed in the SQ, and its biomass peaked on Day 3, followed by a continuous decline. *Monascus* was

mainly detected in the CQ and reached its maximum quantity at the end of fermentation (Day 6). Aerobic bacteria and *Lactobacillus* increased rapidly in the first 2 days and followed by a decrease in biomass.

Temporal changes in the environmental variables

The dynamics of the environmental variables throughout the fermentation process were shown in Figure 6. Temperature is an important indicator of GRTQ fermentation and a valuable factor for screening functional microorganisms of GRTQ (Wang and Xu, 2019). When the prepared GRTQ entered the culture room, its temperature gradually increased and then plateaued for a few days (Day 1–5). After the 5th day, with the rapid reproduction of microorganisms, the temperature of GRTQ rapidly increased and reached its highest value (approximately 45°C) on the 6th day. At the end of cultivation, the microorganisms tended to stabilize, and the temperature of the GRTQ decreased to around 40°C. In the early stage of cultivation, the temperatures of CQ and SQ were similar. However, in the rapid heating phase (Day 5–6), the temperature of the CQ was significantly higher than that of the SQ until the end of cultivation (Figure 6A). The overall moisture content of the GRTQ exhibited a continuous downward trend, ranging from 32 to 3%. Before the 4th day, the moisture of the CQ was lower than that of the SQ, while after the 4th day, the opposite trend was observed. In the early stage, due to the high ambient humidity, the moisture increased slightly. In the later stage, owing to the increase in temperature, the internal water retention of GRTQ was better and its water evaporation was less. Therefore, the moisture content of the CQ was higher than the SQ (Figure 6B). The acidity of the GRTQ first increased, then fluctuated, and finally decreased. The acidity of the CQ was greater than that of the SQ on Days 1–6, and the situation reversed in the later period (Day 7-drying process) (Figure 6C).

Correlations between environmental variables and microbial community

There was a correlation between environmental variables (temperature, moisture, and acidity) and microorganisms at the genus level (Figure 7). Specifically, *Monascus* was positively correlated with temperature ($p < 0.001$) and negatively correlated with moisture ($p < 0.05$) and acidity ($p < 0.01$). Moreover, *Saccharomycopsis* was negatively correlated with temperature ($p < 0.05$) and positively

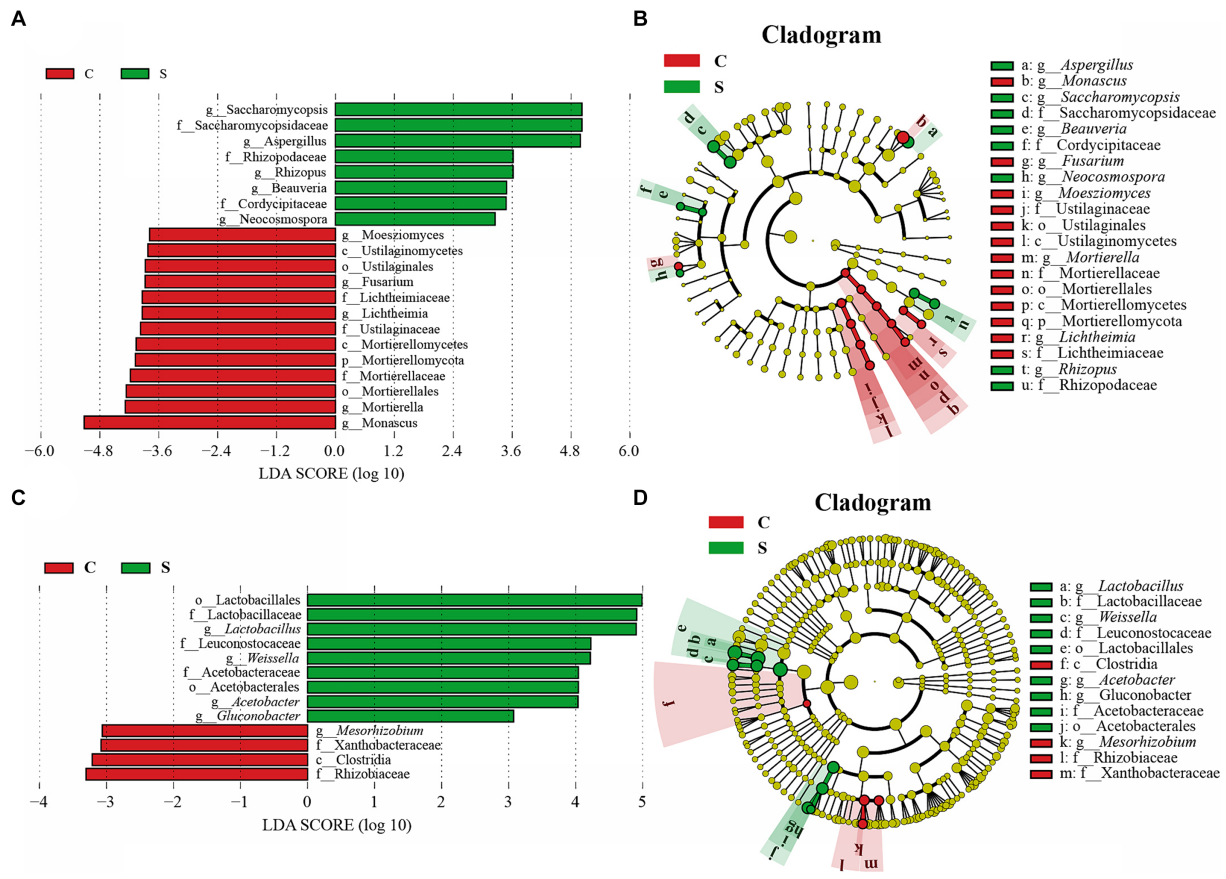

FIGURE 4
Linear discriminant analysis (LDA) effect size (LEfSe) of the central of *Tuqu* (CQ) and the surface of *Tuqu* (SQ) based on genus level (LDA > 3, $p < 0.05$). The LDA score indicates the level of differentiation between CQ and SQ, and the horizontal bar chart showing discriminant taxa of the fungi **(A)** and bacteria **(C)**. Cladogram of the fungi **(B)** and bacteria **(D)**. Significant discriminant taxon nodes of CQ and SQ are represented by green and red, respectively, while no discriminant taxon nodes are represented by yellow. C, Center; S, Surface.

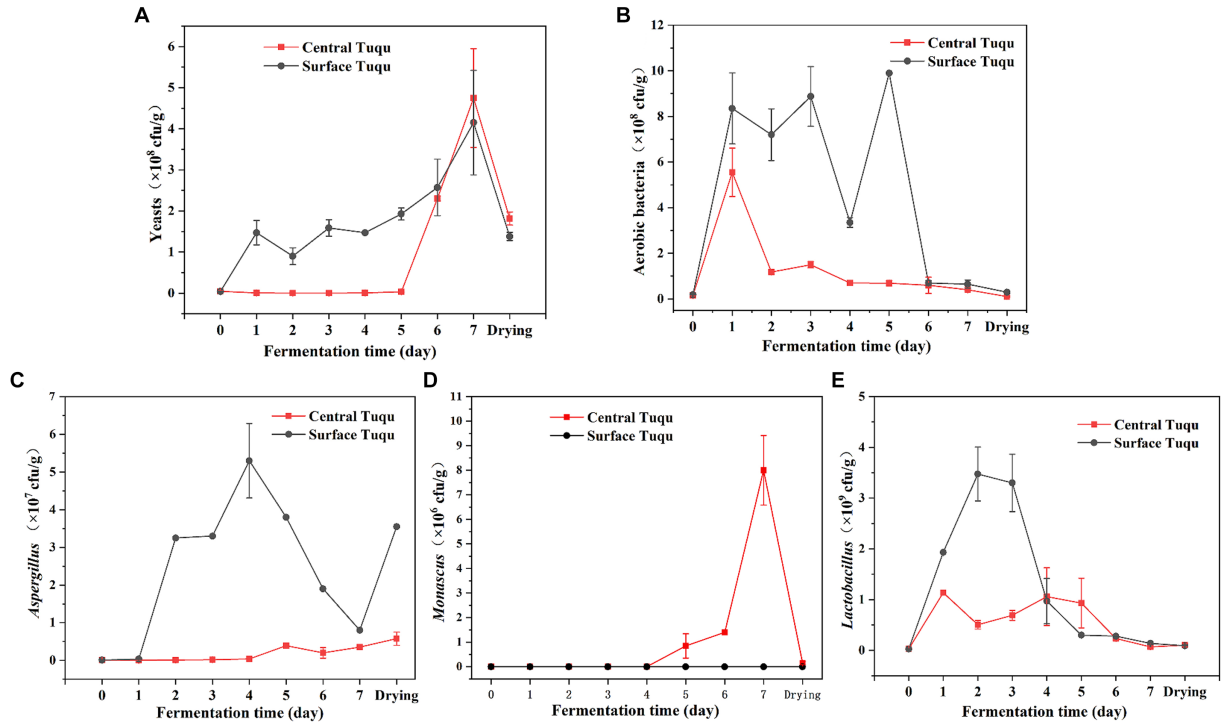

FIGURE 5
Dynamics of the biomass of different groups of microbes **(A)** yeasts, **(B)** aerobic bacteria, **(C)** *Aspergillus*, **(D)** *Monascus*, and **(E)** *Lactobacillus*, during the *Tuqu* fermentation process. Values are presented as the mean ± SEM.

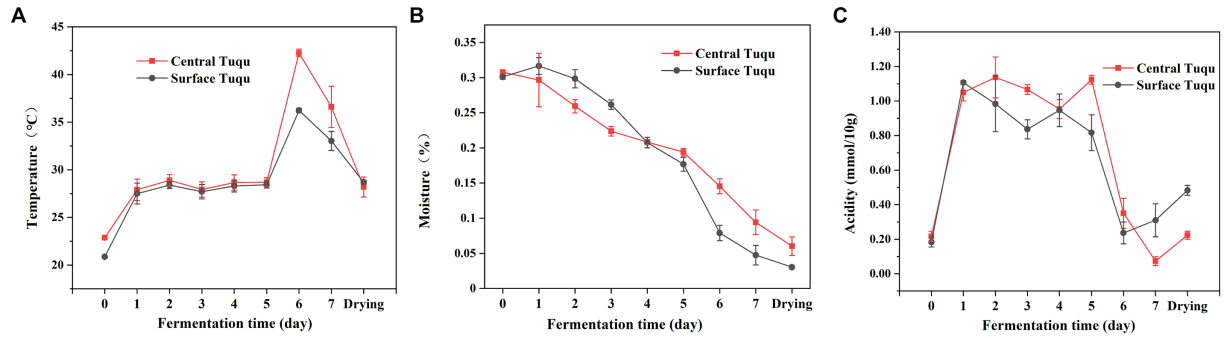

FIGURE 6
Dynamics of physicochemical characteristics **(A)** temperature, **(B)** moisture, **(C)** acidity during the *Tuqu* fermentation process. Values are presented as the mean ± SEM.

correlated with moisture ($p < 0.001$) and acidity ($p < 0.001$). These results demonstrated that the changes in temperature, moisture, and acidity had opposite effects on the growth of *Saccharomycopsis* and *Monascus*. *Saccharomyces* was negatively correlated with temperature ($p < 0.01$) and positively correlated with moisture ($p < 0.001$). *Bacillus* was positively correlated with temperature ($p < 0.001$) and negatively correlated with moisture ($p < 0.001$). *Aspergillus* was positively correlated with acidity ($p < 0.001$). Additionally, *Phaeosphaeria*, *Alternaria*, *Clonostachys*, *Ophiosphaerella*, *Nigrospora*, and *Bipolaris* were negatively related to temperature and acidity, illustrating that the fermentation of GRTQ could inhibit undesirable microbes (Licandro et al., 2020; Zhou et al., 2021; Dong et al., 2022).

Study on the growth characteristics of *A. clavatus* and *M. purpureus*

The apparent characteristics of GRTQ during the fermentation process were shown in Figure 1. The "green-covering" was found to be produced by the growth of *A. clavatus* through separation, screening, and identification experiments (Figure 8A), however, we did not isolate any other microbes that produced green colonies on the green surface of GRTQ, except for *A. clavatus*. On the other hand, according to the previous research in our laboratory, the "red-heart" was generated by the metabolism of *Monascus* sp. (*M. purpureus* and *Monascus ruber*) (Zhu et al., 2022).

These results were consistent with the identification of *A. clavatus* and *M. purpureus* as significant discriminant taxa in the SQ and CQ samples, respectively, based on LEfSe analysis at species level (Supplementary Figure S1).

The physicochemical properties of GRTQ play an important role in the growth of microorganisms, providing indispensable support for microbial physiological activity. Therefore, the physiological characteristics of *Monascus* sp. (*M. purpureus* was selected as the experimental object) and *A. clavatus* under the different environmental conditions were investigated to determine the reasons for the "green-covering" and "red-heart" phenomena.

Acidity is one of the essential factors affecting microbial growth, and the results were shown in Figure 8B. When the pH was <3.07, the growth of *M. purpureus* was inhibited (Zhou et al., 2021). However, the growth of *A. clavatus* was inhibited when the pH was <4.02, which indicated that the acid resistance of *M. purpureus* was greater than that of *A. clavatus*. In addition, the optimum growth temperatures for *M. purpureus* and *A. clavatus* were 35 and 30°C, respectively (Figure 8C). The growth of *A. clavatus* was inhibited at temperatures >40°C. Similarly, it also had an inhibitory effect on *M. purpureus* when the temperature was above 45°C, and *M. purpureus* could still grow slowly. This result might indicated that *M. purpureus* was more resistant to high temperature than was *A. clavatus*. The oxygen contents in the SQ and the CQ were obviously different, which might affect the growth of microorganisms (Cui, 2007). Therefore, the tolerances of *M. purpureus* and *A. clavatus* to microaerobic or even anaerobic conditions were analyzed. As shown in Figure 8D, in a microaerobic environment, *A. clavatus* grew slowly without green spores, while *M. purpureus* grew normally. In an oxygen-free environment, *A. clavatus* stopped growing, while *M. purpureus* grew slowly but did not produce pigments. *M. purpureus* had better tolerance to hypoxia and better adapt to the hypoxic environment of the CQ, while *A. clavatus* had a stronger dependence on oxygen and was more suitable for the environment of the SQ.

Discussion

"Green-covering and red-heart" *Guanyin Tuqu* (GRTQ), is a special fermentative starter (also known as *Jiuqu* in Chinese) that originated in southern China and is characterized by a layer of green covering the surface and with a red heart. It plays a vital role in the production of light-aroma-type *Baijiu*. The GRTQ with a green covering and red heart is often considered to be of high quality. However, the mechanisms that promote temporal succession in the GRTQ microbial ecology and the formation of a "green covering and red heart" remain unclear. Therefore, we correlated the temporal profiles of microbial community succession with the main environmental variables (temperature, moisture, and pH) and spatial position (center and surface) in GRTQ throughout fermentation to reveal the underlying mechanisms involved.

For the microbial community of the whole GRTQ, *Saccharomycopsis*, *Saccharomyces*, *Aspergillus*, *Monascus*, *Lactobacillus*, *Bacillus*, *Rhodanobacter*, and *Chitinophaga* were identified as the dominant microorganisms in the CQ, while the SQ was represented by *Saccharomycopsis*, *Aspergillus*, *Monascus*, *Lactobacillus*, *Acetobacter*, and *Weissella*. *Saccharomycopsis*, *Saccharomyces*, *Aspergillus*, *Monascus*, and *Lactobacillus* were

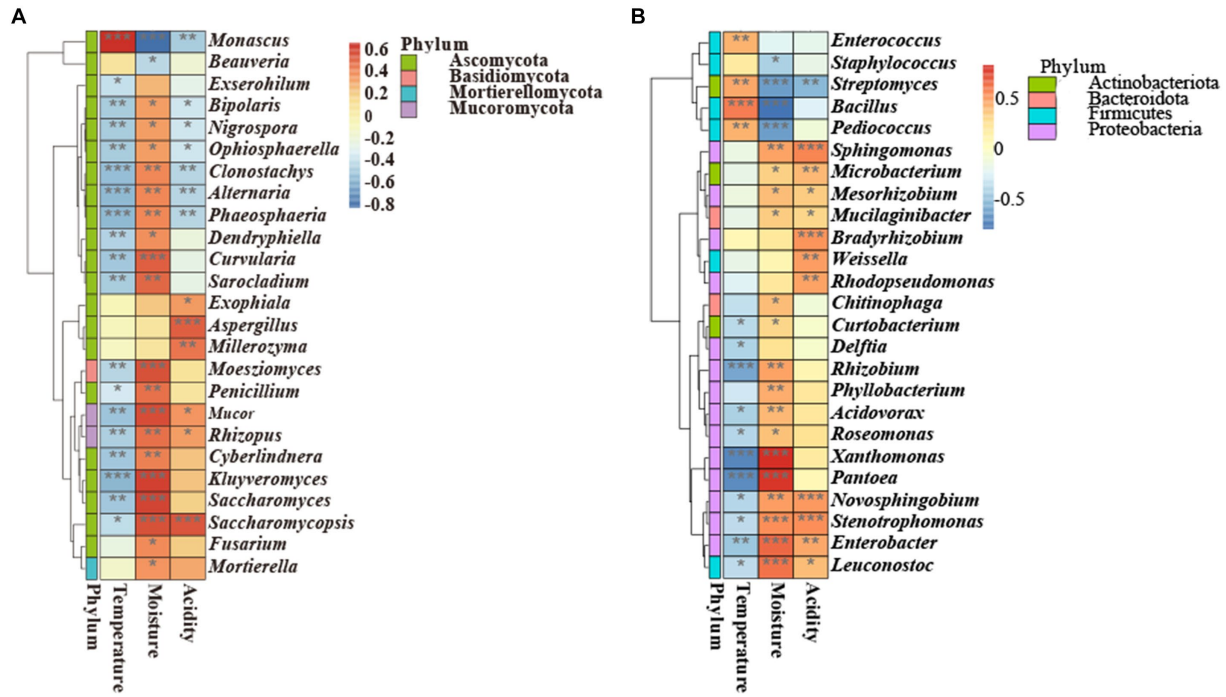

FIGURE 7
Correlation heatmap analysis of the **(A)** fungi and **(B)** bacteria with environmental factors. With red standing for a perfect positive relationship and blue for a perfect negative correlation. *$0.01 \leq p < 0.05$, **$0.001 \leq p < 0.01$, ***$p < 0.001$.

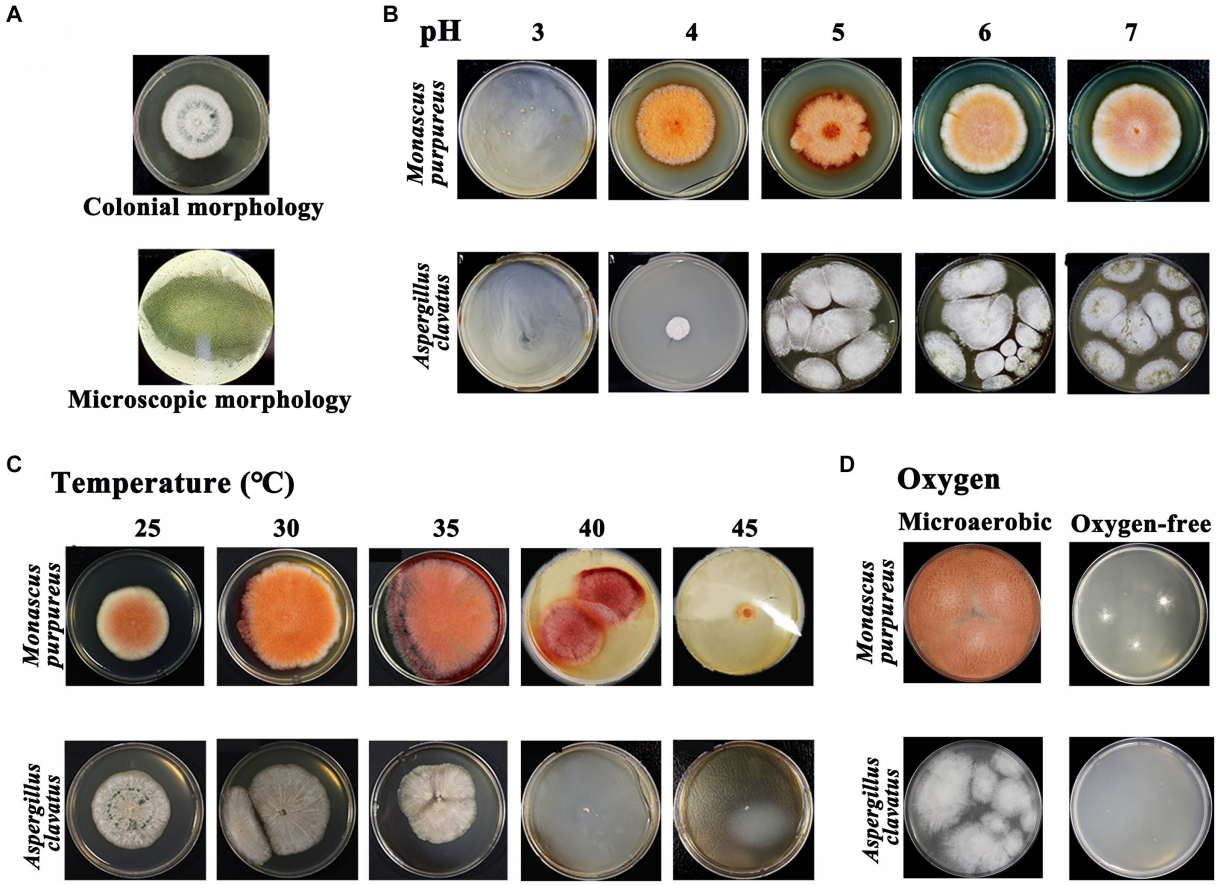

FIGURE 8
(A) Colonial morphology and microscopic morphology (×100) of *Aspergillus clavatus*. Morphology of *Monascus* sp. and *Aspergillus clavatus* at different levels under various environmental factors: **(B)** pH, **(C)** temperature, and **(D)** oxygen. *Monascus* sp.: *Monascus purpureus* was selected as the experimental object.

common dominant microorganisms in the SQ and CQ and were the main microorganisms because of their unique functions during the brewing and fermentation process. For instance, *Saccharomyces* served as the main strain for the production of alcohol and other aromatic substances (Chi et al., 2009). *Saccharomycopsis* could produce α-amylase and glucoamylase, contributing to the strong glycosylation ability of the mixture (Chi et al., 2009). *Aspergillus* could produce a large amount of saccharifying hydrolases, which degraded and converted starch into sugars that could be used by bacteria and yeasts and produced proteolytic enzymes that contributed to protein hydrolysis and flavonoid formation (Machida et al., 2008; Gou et al., 2015). *Monascus* not only excreted a variety of hydrolytic enzymes (saccharification enzymes, proteases, esterases, etc.) to improve the brewing quality of Baijiu (Chen et al., 2015), but also produced abundant beneficial metabolites, such as *Monascus* pigments (red, yellow, and orange pigments) (Chen and Li, 2023), gamma-aminobutyric acid (Song et al., 2021), and monacolin K (Zhang et al., 2020), which have antimicrobial functions, lowering blood pressure and blood lipid levels, respectively. *Lactobacillus* could use fermentable sugars to produce lactic acid, acetic acid, and other organic acids to provide *Baijiu* with a unique flavor and regulate the microbial abundance (Xue et al., 2022). Wang and Xu (2019) reported that *Lactobacillus* regulated the composition of other bacteria and yeasts and synthesized flavor compounds to affect the organoleptic properties of liquor. The characteristics of special aromas might be related to unique microorganisms (by Venn diagram) and discriminant microorganisms (by the linear discriminant analysis (LDA) effect size (LEfSe) method), which might also affect the spatial composition and function of microbial communities. For example, *Aspergillus* was identified as a significant discriminant taxon in the SQ, while *Monascus* was identified as a significant discriminant taxon in the CQ based on LEfes analysis (Figure 4). In addition, at the later stage of fermentation, the bacterial richness index of the CQ was greater than that of the SQ and the fungal richness index of the SQ was greater than that of the CQ. These results indicated that the microbial communities in the CQ and SQ demonstrated functional complementarity (Hou et al., 2024).

High-throughput sequencing technology can accurately reveal the species and genetic diversity of microbial communities (Caporaso et al., 2012). However, this the method cannot effectively exclude the impact of dead microorganisms on live microorganisms. Additionally, real microbial strains could not be obtained (Song et al., 2017). Traditional culture-dependent method can obtain a large number of live microorganisms, but it might be affected by the type of culture medium, incubation time, temperature, interactions between species, operation error and so on, making it difficult to

accurately reconstruct a large amount of microbial information in the samples (Zheng et al., 2012). The principles of these two methods lead to certain differences in the detection results. These differences need to be analyzed in conjunction with actual situations in order to truly reflect the changes in microbial growth in the GRTQ. For instance, the dynamic changes in the quantity of *Aspergillus*, *Monascus*, and *Lactobacillus* detected by high-throughput sequencing methods (Figures 3C,D) were similar to the results detected via traditional culture-dependent methods in the early stage of fermentation, but there were notable differences in the later stage of fermentation (Figures 5C,E). Due to the increase in temperature and decrease in moisture during the late stage of the fermentation and drying process, some microorganisms died in large numbers. The actual microbial community might be consistent with the results of traditional culture-dependent methods. In other words, traditional cultivation methods with selective culture media could better reflect the actual information. For example, Luo et al. (2023) added 3.3 mL/L acetic acid to YPD medium to achieve effective separation and counting of yeast. Therefore, to better reflect the microbial community information in complex environmental samples, it is necessary to combine two methods for analysis to obtain the most authentic microbial information.

The "green covering" started to appear on Day 2 and peaked on Day 4 from the SQ appearance (Figure 1). These results were basically consistent with the dynamic variation in *Aspergillus* abundance in the SQ from the high-throughput sequencing results (Figure 3C). Through microscopic morphological observation, it was found that the formation of "green-covering" was due to the production of green spores by *Aspergillus* (Figure 8A). The "red heart" began to appear on Day 6, and after the drying process, the red-heart area reached its maximum. Interestingly, this phenomenon was hysteretic compared to the high-throughput sequencing results, which showed that *Monascus* was present in large quantities on Day 3 (Figure 3C). Analyzing the reason, the appearance of *Monascus* did not immediately produce pigments. However, in the later stage of growth, it produced large *Monascus* pigment, which caused the appearance of red color in the core (CQ). However, it is unclear why *A. clavatus* grows externally and *Monascus* prefers to propagate internally. The amounts of "green-covering" and "red-heart" coloring were crucial parameters for evaluating the quality of GTRQ. Excess or low numbers of "green-covering" and "red-heart" led to insufficient microbial diversity and the metabolism of hydrolases. Exploring their growth patterns will enable better guidance for workshop production.

The mechanism of the external growth of *A. clavatus* and the internal reproduction of *Monascus* were analyzed based on the relevant microbial physiology and environmental factors data. The correlation analysis between *Monascus* and environmental indicators was consistent with the research results on the physiology characteristics of *Monascus* (Figure 7A). *Monascus* was positively correlated with temperature ($p < 0.001$). Previous studies demonstrated that *Monascus* required a large amount of water in the early stages of growth (Chen et al., 2015), which was consistent with our finding that as the *Monascus* increased, the water content ($p < 0.05$) decreased (Figure 7A). According to the above results, we speculated that *Monascus* consumed a large amount of water in the CQ at relatively high temperature, resulting in the mass growth of *Monascus* and the production of *Monascus* pigments to further form the red heart. In the early stage of fermentation in the SQ, the moisture and oxygen contents were relatively higher, while the temperature and acidity were lower, which was consistent with the physiological characteristics of *A. clavatus* (Figures 8B,D). Besides, *Aspergillus* grew rapidly and had an advantage in niche competition, thus shaping a "green covering" on the SQ.

Conclusion

Thus, this study investigated the changes in microbial quantity and community succession during different stages of the fermentation process in the SQ and the CQ. The results showed that there were more bacteria than the fungi in both the SQ and CQ. Furthermore, at the later stage of fermentation, the bacterial richness index of the CQ was greater than that of the SQ, and the fungal richness index of the SQ was greater than that of the CQ. In addition, there were significant differences in the types and quantities of the dominant microorganisms in the CQ and SQ. The dominant microorganisms in the CQ included *Saccharomycopsis*, *Saccharomyces*, *Aspergillus*, *Monascus*, *Lactobacillus*, and *Bacillus*, while those in the SQ were represented by *Saccharomycopsis*, *Aspergillus*, *Monascus*, *Lactobacillus*, *Acetobacter*, and *Weissella*. The study on the correlation between environmental factors (temperature, moisture, and acidity) and dominant microorganisms at the genus level revealed that environmental factors were closely correlated with the reproductive succession of microorganisms. By revealing the physiological characteristics of core microorganisms at different spatial positions of GRTQ, such as *A. clavatus* and *M. purpureus*, as well as their interactions with environmental factors, we elucidated the color formation mechanism behind the phenomenon of "green" outside and "red" inside. This study provides data support for optimizing the production process of GRTQ and offers potential guidance for establishing modern production lines.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Author contributions

LiZ: Writing – original draft, Writing – review & editing, Conceptualization, Data curation, Formal analysis, Methodology, Software, Supervision, Validation. LC: Data curation, Investigation, Validation, Writing – original draft. BL: Software, Writing – review & editing. YX: Formal analysis, Funding acquisition, Investigation, Writing – original draft, Writing – review & editing. WD: Conceptualization, Data curation, Methodology, Writing – review & editing, Formal analysis. YL: Methodology, Software, Validation, Writing – original draft. JT: Formal analysis, Investigation, Writing – original draft. GZ: Investigation, Methodology, Validation, Writing – original draft. LeZ: Funding acquisition, Resources, Writing – original draft. SY: Supervision, Validation, Writing – original draft. QY: Formal

analysis, Funding acquisition, Investigation, Supervision, Writing – original draft. SC: Funding acquisition, Project administration, Supervision, Validation, Writing – original draft, Writing – review & editing.

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Conflict of interest

LiZ, LC, BL, YX, YL, JT, GZ, LeZ, SY, QY, and SC were employed by the Jing Brand Co., Ltd.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1412503/full#supplementary-material

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Effects of sorghum varieties on microbial communities and volatile compounds in the fermentation of light-flavor Baijiu

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Light-flavor Baijiu (LFB) fermentation is a representative spontaneous mixed-culture solid-state fermentation process in which sorghum is used as the raw material. Raw materials and microorganisms are crucial to the flavor formation and quality of LFB. However, the microbial and physicochemical dynamics of different sorghum varieties during LFB fermentation, as well as their impact on flavor compounds are still largely unknown. Herein, PacBio single-molecule real-time (SMRT) sequencing and headspace solid-phase microextraction coupled with gas chromatography–mass spectrometry (HS-SPME-GC–MS) were applied to investigate microbial community succession and volatile flavor formation in glutinous/non-glutinous sorghum-based fermented grains during LFB fermentation. Fermented grains made of glutinous sorghum Liangnuo No. 1 (GLN) had higher bacterial α-diversity and lower fungal α-diversity than those with fermented grains prepared with non-glutinous red sorghum (NRS) ($p < 0.05$). The dominant microbial species were *Saccharomyces cerevisiae*, *Acetobacter pasteurinus*, and *Lactobacillus helveticus*, the latter two of which were the predominant bacteria observed at the end of fermentation in GLN and NRS, respectively. Moisture content and reducing sugar had a more significant impact on the microorganisms in GLN, while amino acid nitrogen, total free amino acids, and residual starch were the main driving factors driving the microbial community in NRS. The correlation network and discriminant analysis indicated that a relatively high content of 4-vinylguaiacol showed a significant positive association with significant differential microbial species in GLN. These results provided valuable insights for improving the quality of LFB.

KEYWORDS

light-flavor Baijiu, sorghum varieties, fermented grains, microbial community, volatile compounds

Introduction

Baijiu (Chinese liquor), which is one of the six well-known distilled spirits throughout the world, plays an indispensable role in Chinese culture, economy, and dietary profiles (Jin et al., 2017; Wang, 2022). Based on its unique taste and characteristic flavor profile, Baijiu can be divided into four basic categories: sauce-flavor Baijiu, strong-flavor Baijiu, light-flavor

Baijiu, and rice-flavor Baijiu (Ye et al., 2021). Light-favor Baijiu (LFB) is a type of Chinese liquor with a pure and mild flavor produced by traditional spontaneous mixed-culture solid-state fermentation. Unlike other types of Baijiu, traditional LFB is fermented in ceramic jars and mainly includes Jiuqu (starter) preparation, material pretreatment, alcoholic fermentation, distillation, and aging (Pang et al., 2020). The flavor components of Baijiu are subjected to complicated interactions among several factors during the whole process, involving the properties and processing of the raw material, substances produced during fermentation, microbial metabolites from starter and fermented containers, characteristics of the environmental microbiota, and distillation of fermented grains (Pang et al., 2020; Wu et al., 2021). Thus, raw materials and microbial metabolites are of great importance to the formation of Baijiu flavor.

As the principal raw material in the manufacturing of LFB, sorghum is rich in starch and protein and contains small amounts of tannin and fiber, which influence microbial communities and the flavor profile of fermented grains (Xu et al., 2018; Chen et al., 2019; Liu C. et al., 2021; Wang et al., 2021). According to the ratio of amylopectin to amylose in the grain, sorghum varieties can be classified as glutinous or non-glutinous, and the amylopectin/amylose ratio for common sorghum is approximately about 80/20, whereas in glutinous sorghum, there is little or no amylose (Chen et al., 2019). Compared to that in non-glutinous sorghum, a high ratio of amylopectin to amylose in glutinous sorghum can more effectively convert starch into ethanol (Wang et al., 2008). However, the yield of glutinous sorghum is relatively low, and the cost of using these grains for Baijiu production is greater. The selection of raw materials may affect the quality and value of Baijiu. To clarify the effects of sorghum varieties on the quality of Baijiu, different types of sorghum and microbial community association in Chinese strong-flavor and Xifeng Baijiu fermentation systems have been examined *in situ* (Liu C. et al., 2021; Liu et al., 2023). However, to our knowledge, the relationships between sorghum varieties, microbial communities and volatile compounds during LFB fermentation have not yet been reported.

The flavor of Baijiu is the foremost factor in determining its quality and is formed by microbial community under the driving force of various environmental factors, such as moisture, temperature, and acidity (Zhang et al., 2020; Ji et al., 2023; Wang et al., 2023). Hence, the exploration of the associations among the microbial community, environmental factors, and flavor compounds has become the key to clarify the mechanism underlying the formation of Baijiu flavor. Recent studies on LFB have mainly focused on the elucidation of microbial community and their correlation with flavor compound formation (Luo et al., 2023; Pan et al., 2023), as well as correlational analyses of physicochemical properties, microbial communities, and volatile components in Jiuqu (Hu et al., 2023; Yu et al., 2023) and comparative analyses of the microbial community structure and screening of functional microbial strains (Tang et al., 2022a; Xiang et al., 2023). However, the dynamics of the environmental factors, microbial community, and flavor compounds in fermented grains are still not fully understood. In particular, the impact of sorghum varieties on microbial communities and flavor compounds has rarely been considered.

The fermented grain samples that were used in this study were collected from Hubei Province and fermented by glutinous sorghum and non-glutinous sorghum with Jiuqu, respectively. Headspace solid-phase microextraction coupled with gas chromatography–mass spectrometry (HS-SPME-GC–MS) and PacBio single-molecule real-time (SMRT) sequencing were applied to determine the volatile compounds and microbial community structure during different stages of LFB fermentation, respectively. Moreover, the driving effect of environmental factors on microbial communities and the correlation between microbiota and the volatile flavor profiles were investigated. The results can provide a theoretical basis for elucidating the brewing mechanism and improving the quality and fermentation efficiency of LFB.

Materials and methods

Sample collection and treatment

Fermented grain samples were collected in July 2022 from Jing Brand Co., Ltd., a LFB producer in Huangshi, Hubei Province, China, which has an annual Baijiu production of 70,000 tons. The mechanized technology for the brewing process of LFB was showed in detail in our previous study (Tang et al., 2022b). Two sorghum varieties were selected: glutinous sorghum Liangnuo No. 1 (GLN) and non-glutinous red sorghum (NRS). Fermented grain samples (250 g) were collected on days 0, 1, 2, 3, 4, 5, 7, 9, 11, and 14 during fermentation from fermentation tanks in the middle layer, and labeled as GD0, GD1, GD2, GD3, GD4, GD5, GD7, GD9, GD11, GD14 (fermenting with GLN), and JD0, JD1, JD2, JD3, JD4, JD5, JD7, JD9, JD11, JD14 (fermenting with NRS), respectively. These samples were stored at 4°C for physicochemical properties analysis, and −80°C for DNA extraction and volatile compounds analysis. Three independent batches were sampled for adequate representation. A total of 60 samples were collected for analysis.

Physicochemical analysis

Moisture was measured by estimating the weight loss using drying fermented grain samples (10 g) to a constant weight at 110°C for 3 h. Acidity, amino acid nitrogen, and reducing sugar were detected as described by Lin et al. (2022). Direct titration was used to determine the acidity and amino acid nitrogen of fermented grains. Reducing sugar was detected by the DNS method. Hydrochloric acid dissolution was used to determine residual starch content (Tang et al., 2022b). The determination of free amino acids content referred to"GB5009.124–2016 Determination of Free amino acids in Foods." All physicochemical analyses were conducted in triplicate.

HS-SPME-GC–MS analysis of volatile compounds

The volatile compounds in fermented grains were determined by HS-SPME-GC–MS according to the protocol described previously (Sun et al., 2022). Concretely, 10 g of each fermented grain sample was added to 25 mL of sterilized ultrapure water and ultrasonicated for 30 min, and then soaked overnight at 4°C. The suspension was centrifuged at 8000 × g at 4°C for 10 min. 8 mL of the supernatant was transferred to a 20 mL headspace vial containing 20 μL of internal standard mixer (ethyl caproate-d11, hexanal-d12, n-hexanol-d13, and

ethyl octanoate-d15) and 7 g of sodium chloride. The headspace vial was placed in the automatic microextraction device and extracted at 50°C for 45 min. A total of 60 fermented grain samples were collected, followed by GC–MS analysis. Volatile compounds were identified by matching with the National Institute of Standards and Technology (NIST) library (Gaithersburg, MD, United States), and the matching masses were more than 80%. The semi-quantification of the volatiles was determined with the internal standard method. The relative concentration of volatile compounds was calculated based on the ratio of volatiles' peak area and internal standard peak area.

DNA extraction, amplification, and sequencing

Total genomic DNA from the fermented grain samples was extracted by using the TGuide S96 Magnetic Soil/Stool DNA Kit (Tiangen Biotech (Beijing) Co., Ltd.) according to the manufacturer's instructions. The quality and quantity of the extracted DNA were examined via electrophoresis on a 1.8% agarose gel, and the DNA concentration and purity were determined with a NanoDrop 2000 UV–Vis spectrophotometer (Thermo Scientific, Wilmington, United States). The full-length 16S rRNA gene in bacteria was amplified with the primer pairs 16S-F (5′-AGRGTTTGATYN TGGCTCAG-3′) and 16S-R (5′-TASGGHTACCTTGTTASGACTT-3′) (Johnson et al., 2019). The primers for fungal analysis were designed on the basis of the full-length internal transcribed spacer (ITS) regions of the rRNA (ITS1F: 5′-CTTGGTCATTTAGAGGAAGTAA-3′; ITS4: 5′-TCCTCCGCTTATTGATATGC-3′) (Banerjee et al., 2019). All PCR reactions were performed in a 30-μL reaction system. The KOD One PCR Master Mix containing KOD DNA polymerase (TOYOBOLife Science) was used to perform PCR amplification. The PCR conditions for 16S rRNA region amplification were as follows: initial denaturation at 95°C for 2 min, followed by 25 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min 30 s, and a final step at 72°C for 2 min. The PCR conditions for fungal full-length ITS amplification were as follows: pre-denaturation at 95°C for 2 min, 32 cycles of denaturation at 98°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s, as well as a final extension at 72°C for 5 min. The amplicons were quantified, after which the normalized equimolar concentrations of amplicons were pooled and sequenced on the PacBio Sequel II platform (Allwegene Tech., Beijing, China).

Bioinformatics and statistical analysis

Raw circular consensus sequencing (CCS) was performed by identifying CCS reads through barcodes via the Lima v1.7.0 software. Cutadapt v2.7 was applied to identify and remove primer sequences and acquire clean CCS sequences by filtering sequence length. Subsequently, effective CCS sequences were obtained by identifying and removing chimeric sequences via UCHIME v4.2 (Lai et al., 2023). The qualified sequences with more than 97% similarity thresholds were clustered into operational taxonomic units (OTUs) by using USEARCH v10.0 (Robert, 2013). The Naive Bayes classifier in QIIME2 using the SILVA database (release 138.1) with a confidence threshold of 70% was used to annotate the prokaryotic OTUs (Bolyen et al., 2019). Fungal OTUs were annotated with the fungal ITS database, UNITE (Release 8.0) as the reference sequence database. Microbial α-diversity indices (Chao 1 richness estimator and Shannon's diversity index) were calculated to determine the complexity of the species diversity of each sample utilizing QIIME2 software. Additionally, we employed linear discriminant analysis (LDA) effect size (LEfSe) to evaluate the differentially abundant taxa among the groups, and the threshold for discriminative features was a logarithmic LDA score of 4.0 (Segata et al., 2011).

The dynamic changes in physicochemical indicators, microbial α-diversity indices, and relative abundance of bacteria and fungi in the samples were plotted using OriginPro 2018 software (Origin Lab Corporation, United States). Advanced Circos barplot, heatmap plots, PLS-DA (partial least squares-discriminant analysis), STAMP (statistical analysis of metagenomic profiles), heatmap barplot, Procrustes analysis graph, and linear regression plot were performed using the OmicStudio tools at https://www.omicstudio.cn/tool. Redundancy analysis (RDA) performed using the vegan packages in R was chose to determine the correlations between physicochemical properties and microbial community and the Monte Carlo permutation test was used to check the significance (Pan et al., 2022). To explore the correlations between the dominant microbiota and important aroma components, Spearman's correlation coefficient (ρ) was explored between microorganisms and metabolites via IBM SPSS Statistics (version 19.0), and visualized as a correlation network with $|\rho| > 0.6$ and $p < 0.05$ in Cytoscape (v3.9.1) (Lin et al., 2022). The correlations between physicochemical properties and dominant microbial species were calculated by Spearman's correlations (Huang et al., 2018; Luo et al., 2023).

All the experiments for physiochemical properties determination were conducted in triplicate. Data were presented as mean values ± standard deviation. One-way ANOVA analysis and the multiple comparisons were performed using Duncan's test by SPSS software (version19.0, Chicago, IL, United States).

Results

Physicochemical properties of fermented grains

The physicochemical properties of fermented grains, including the moisture content, acidity, amino acid nitrogen content, reducing sugar, residual starch, and total free amino acids, were determined. Figure 1 illustrated that the dynamics of the physicochemical properties throughout the fermentation process of the two types of sorghum exhibited similar trends. The moisture content was approximately 63.0% at the beginning of fermentation, quickly increased to 68.4–70.0% on day 3, and then moderately escalated to approximately 72.0% thereafter (Figure 1A). The moisture content in GLN were generally higher than that in NRS (Supplementary Figure S1A). The titratable acidity rapidly decreased from 0.43 to 0.38 mmol/10 g from day 0 to day 2 in GLN, while the acidity in NRS witnessed a slight drop in the first 3 days and then gradually increased in the later stage (Figure 1B). Throughout fermentation, the acidity in GLN was often lower than that in NRS (Supplementary Figure S1B). Figures 1B,C showed that the dynamics of amino acid nitrogen and acidity in GLN and NRS were similar, and amino acid nitrogen content in GLN were markedly lower than that in NRS ($p < 0.001$)

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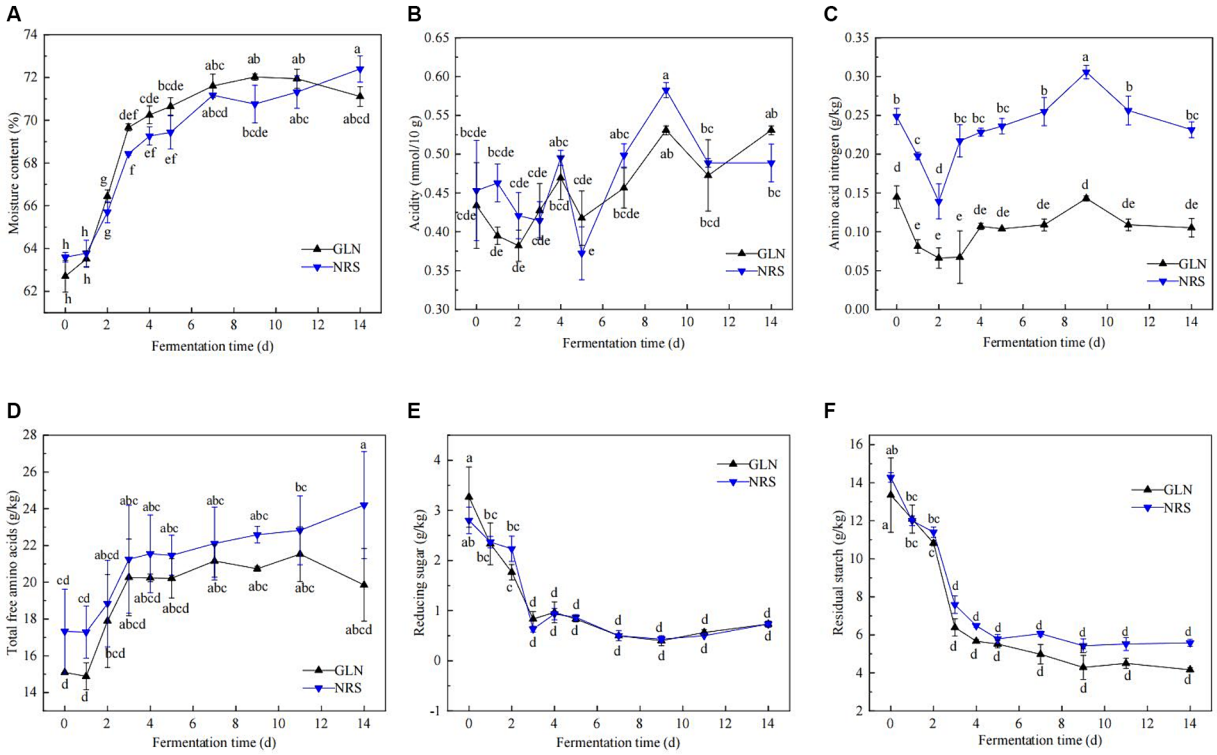

FIGURE 1
Dynamic changes of physicochemical properties during the fermentation process using two types of sorghum. (**A**) Moisture content. (**B**) Acidity. (**C**) Amino acid nitrogen. (**D**) Total free amino acids. **(E)** Reducing sugar. **(F)** Residual starch. GLN: Representing the samples fermented using glutinous Liangnuo No.1. NRS: Representing the samples fermented using non-glutinous red sorghum. Different lowercase letters indicate significant differences at the 0.05 level. The same below.

(Supplementary Figure S1C). During fermentation, the trend of the total free amino acids content curve was similar to that of the moisture content curve (Figure 1D). And the total free amino acids content in GLN was significantly lower than that in NRS ($p < 0.05$) (Supplementary Figure S1D). As shown in Figures 1D,F, the variations in reducing sugar were consistent with those in residual starch. Specifically, both of them rapidly decreased from day 0 to day 3, and then remained largely stable in the later stage, while GLN had high reducing sugar and low residual starch contents compared to NRS (Supplementary Figures S1E,F).

Microbial diversity and structure of microbial communities

Following quality control, 758,105 and 2,502,927 effective CCS sequences for bacteria and fungi, respectively, were found in all samples. All of the rarefaction curves of the tested species became saturated, thus demonstrating the effectiveness of the sequencing data (Supplementary Figure S2). Changes in microbial diversity were investigated according to PacBio SMRT sequencing data. Throughout the fermentation using two types of sorghum, the α-diversity of bacteria exhibited a clear decreasing trend, especially in the later stage (Figures 2A,B). Overall, the bacterial α-diversity in GLN was higher than that in NRS, particularly for bacteria, which had a higher Shannon index in GLN ($p < 0.05$) (Supplementary Figure S3B). For fungi, the Chao 1 index showed no significant change during fermentation, while the Shannon index reached a maximum value at the beginning of fermentation and then decreased and remained stable (Figures 2C,D). Overall, NRS had a higher fungal Chao 1 index than that in GLN ($p < 0.05$) (Supplementary Figure S3C).

The top 10 microorganisms in the rankings dominated the microbial community during the fermentation (most of them with average relative abundances >1%). The top 10 bacteria included *Levilactobacillus brevis* (1.75–58.01% in GLN, 0.92–90.71% in NRS), *Lactobacillus helveticus* (0.04–54.04% in GLN, 0.01–58.17% in NRS), *Acetobacter pasteurinus* (0.87–69.68% in GLN, 0.07–2.18% in NRS), *Limosilicobacillus pontis* (0.02–13.17% in GLN, 0.002–23.51% in NRS), *Klebsiella pneumoniae* (0.02–19.22% in GLN, 0.04–8.94% in NRS), *Acetobacter tropicalis* (0.05–10.00% in GLN, 0.30–11.32% in NRS), *Gluconobacter oxydans* (0.00–15.13% in GLN, 0.12–11.72% in NRS), *Weissella confusa* (0.00–12.11% in GLN, 0.003–28.35% in NRS), *Lentilactobacillus buchneri* (0.02–5.00% in GLN, 0.02–14.44% in NRS), and *Acinetobacter baumannii* (0.00–3.62% in GLN, 0.05–21.45% in NRS) during fermentation. On day 0 and 1, the most common bacteria were *A. baumannii*, *W. confusa*, *G. oxydans*, *A. tropicalis*, and *K. pneumoniae*. As fermentation progressed, acetic acid bacteria and lactic acid bacteria dominated in the middle and later phases of fermentation. The relative abundance of *La. brevis* first increased and then decreased, and that for GLN was lower than that for NRS. *La. helveticus* became the dominant species after 5 days and rapidly increased in the later stage, and it predominated (58.17%) at

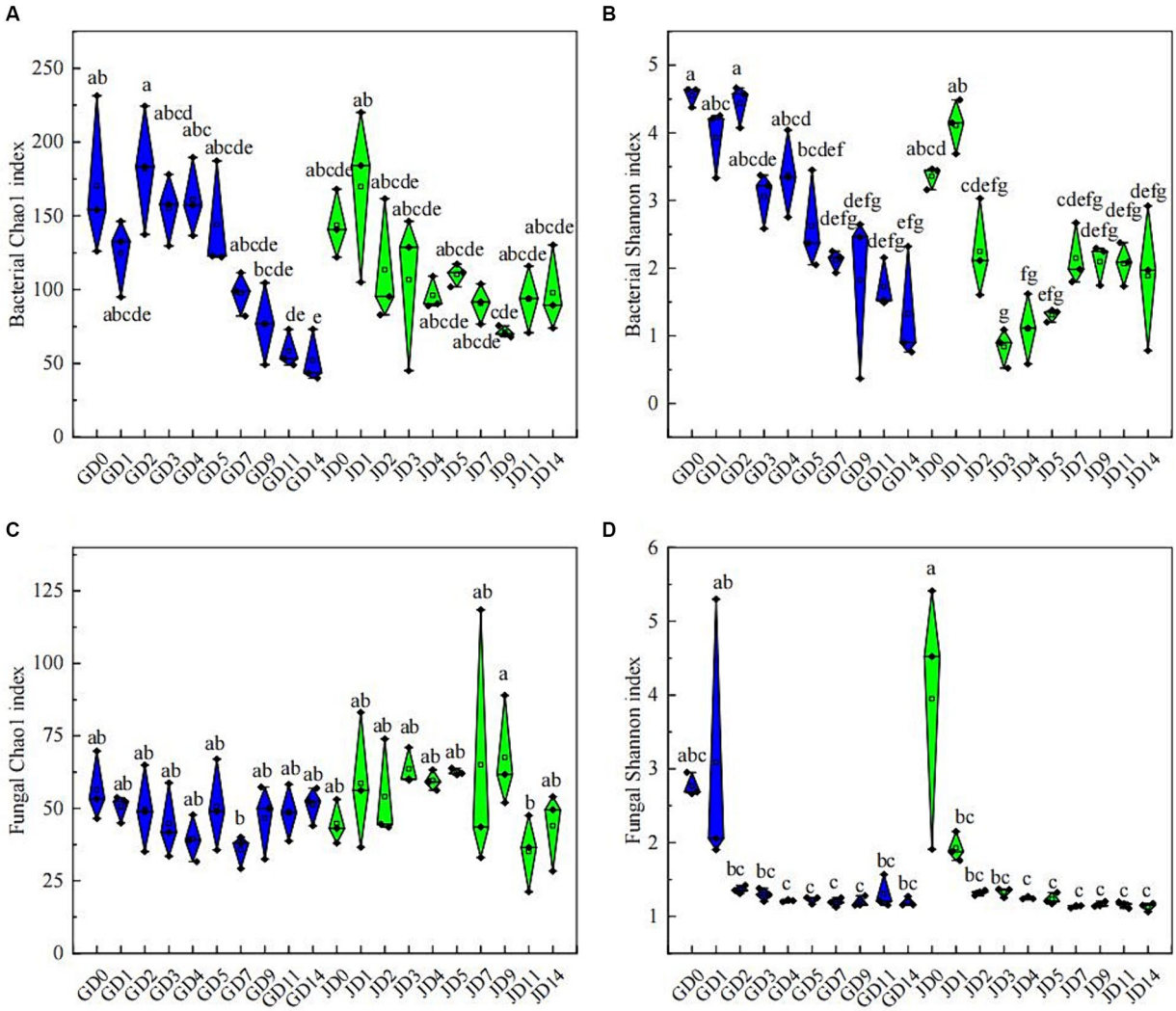

FIGURE 2
Microbial community α-diversity indices in fermented grains. **(A)** Bacterial Chao 1 indices, **(B)** bacterial Shannon indices, **(C)** fungal Chao 1 indices and **(D)** fungal Shannon indices at different fermentation times.

the end of fermentation in NRS, which was higher than that in GLN. However, *A. pasteurinus* became the dominant specie (69.68%) at the end of fermentation in GLN, and its relative abundance was higher than that in NRS (Figure 3A).

The most common fungal species were *Saccharomyces cerevisiae*, *Wickerhamomyces anomalus*, and *Rhizopus oryzae*. Throughout the entire fermentation, *S. cerevisiae* was the dominant yeast, and its relative abundance reached over 95% after day 1. The relative abundance of *S. cerevisiae* on day 0 in GLN was higher than that in NRS; however, the proliferation rate of *S. cerevisiae* in NRS (51.73–96.31%) was greater than that in GLN (60.94–95.70%) from day 0 to day 2 (Figure 3B).

The linear discriminant analysis (LDA) effect size (LEfSe) method was also used to analyze the differences in the microbial taxa detected in all of the samples. For bacteria, LDA highlighted that a total of 64 biomarkers were identified via the statistically significant LDA threshold of >4 (Figure 4A). A phylogenetic tree of the total bacterial community from the phylum to the species level was constructed for these differentiating taxa by using LEfSe (Figure 4B). A total of 2 phyla, 3 classes, 5 orders, 7 families, 20 genera, and 27 species displayed significant differences in abundance among all of the samples. The 27 bacterial species with significant differences included *K. pneumoniae* (6.06% in GLN, 1.48% in NRS), *Limosilactobacillus fermentum* (3.40% in GLN, 0.01% in NRS), *Lactiplantibacillus plantarum* (2.82% in GLN, 0.01% in NRS), *G. oxydans* (3.50% in GLN, 2.70% in NRS), *Gluconobacter japonicus* (2.45% in GLN, 1.13% in NRS), *A. pasteurianus* (14.39% in GLN, 0.74% in NRS), *A. tropicalis* (4.02% in GLN, 2.58% in NRS), *W. confusa* (2.31% in GLN, 3.73% in NRS), *Bacillus velezensis* (0.59% in GLN, 0.92% in NRS), *A. baumannii* (0.93% in GLN, 4.82% in NRS), *La. buchneri* (1.57% in GLN, 5.01% in NRS), *La. pontis* (4.99% in GLN, 6.88% in NRS), *Lentilactobacillus hilgardii* (0.12% in GLN, 0.83% in NRS), *La. acetotolerans* (0.30% in GLN, 1.22% in NRS), *La. helveticus* (12.90% in GLN, 14.83% in NRS), and *La. brevis* (22.62% in GLN, 43.12% in NRS), etc. Among them, 15 and 12 species were distributed in the fermented grains with GLN and NRS, respectively.

For fungi, 29 biomarkers, which included 2 phyla, 6 classes, 4 orders, 5 families, 6 genera, and 6 species, were detected in all of the

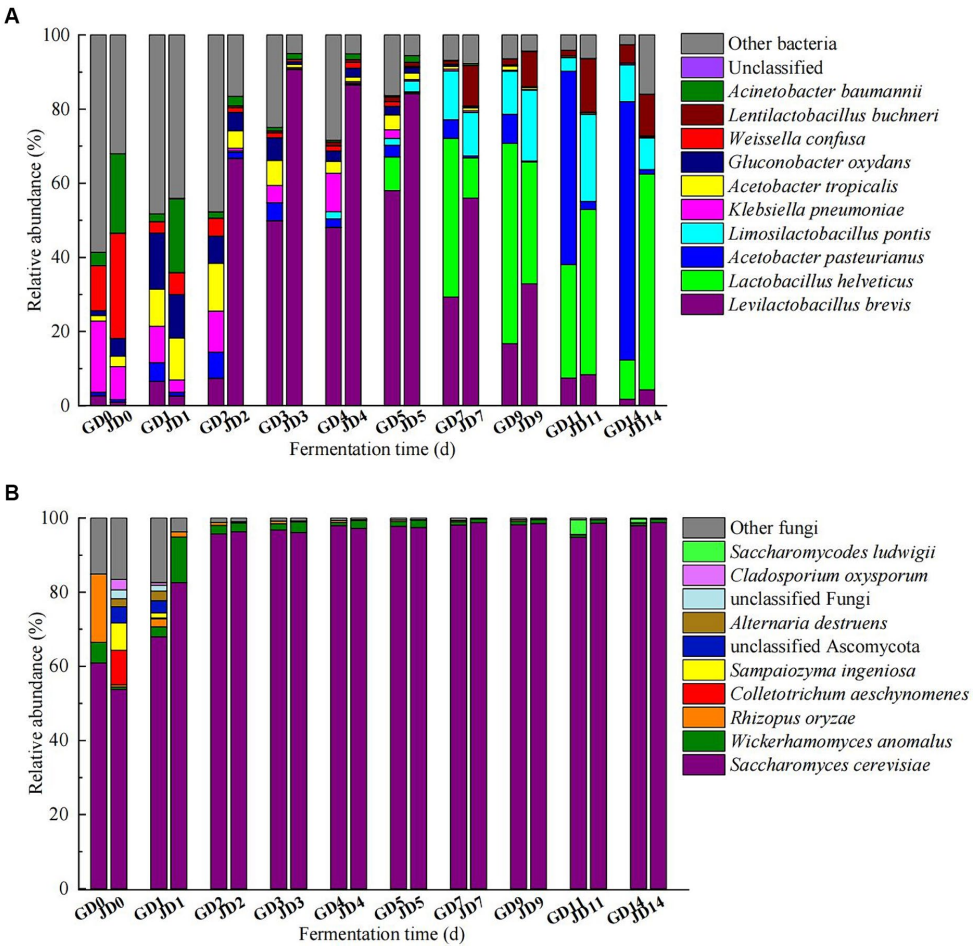

FIGURE 3
Dynamic changes of bacterial **(A)** and fungal **(B)** communities at the species level in fermented grains.

samples (Figure 4D). The six fungal species were *R. oryzae*, *S. cerevisiae*, *W. anomalus*, *C. aeschynomenes*, *Stagonosporopsis inoxydabilis*, and unclassified Ascomycota. More differential fungal species were detected in the NRS-treated fermented grains.

Characteristics of volatile flavor compounds

Approximately 59 and 65 major volatile compounds in the fermented grains prepared with GLN and NRS, respectively, were identified by HS-SPME-GC–MS. All of the volatiles were composed of nine categories, including esters, alcohols (excluding ethanol), acids, phenols, aldehydes and ketones, aromatics, lactones, terpenoids, and others. Figure 5A showed that esters, which were the most abundant volatiles in the early stage, followed by alcohols and acids, had the highest content in both GLN and NRS. The relative content of esters reached its maximum on day 1, and rapidly decreased from day 1 to day 3, and then remained relatively stable. The contents of esters (average content 0.64 mg/kg) in GLN were lower than those (average content 0.82 mg/kg) in NRS. Alcohols rapidly increased from day 0 (0.12 mg/kg-0.14 mg/kg) to day 1 (0.28 mg/kg-0.45 mg/kg) and then remained relatively stable (0.30 mg/kg-0.40 mg/kg). The contents of alcohols (average content 0.33 mg/kg) in GLN were higher than those (average content 0.28 mg/kg) in NRS. The change in acids in GLN showed a clear trend with respect to the ester content, whereas the acid content in NRS was greatest (0.075 mg/kg) on day 3 and then decreased.

Heatmap analysis of 49 flavor components in the fermented grains, including 18 esters, 9 alcohols, 6 acids, 6 phenols, 6 aldehydes and ketones, 2 aromatic compounds, 1 lactone, and 1 terpenoid, which suggested that the relative content of volatiles in NRS was relatively higher than that in GLN (Figure 5B). The 15 main flavor components with relative contents greater than 1% included ethyl acetate, phenethyl acetate, ethyl palmitate, isoamyl acetate, ethyl oleate, ethyl linoleate, ethyl decanoate, ethyl caprylate, diethyl succinate, phenylethyl alcohol, isobutanol, isoamyl alcohol, and acetic acid, etc. Ethyl acetate was the most abundant ester in the early stage and then decreased and remained relatively stable in the later stage. In addition to ethanol, phenylethyl alcohol was the most abundant alcohol, which showed a rapid increase from day 0 to day 3 and then remained relatively stable. The change in isoamyl alcohol content exhibited a similar trend to that of phenylethyl alcohol. The contents of phenylethyl alcohol and isoamyl alcohol in GLN were higher than those in NRS. Acetic acid was the most abundant acid and its content in GLN was lower than that in NRS.

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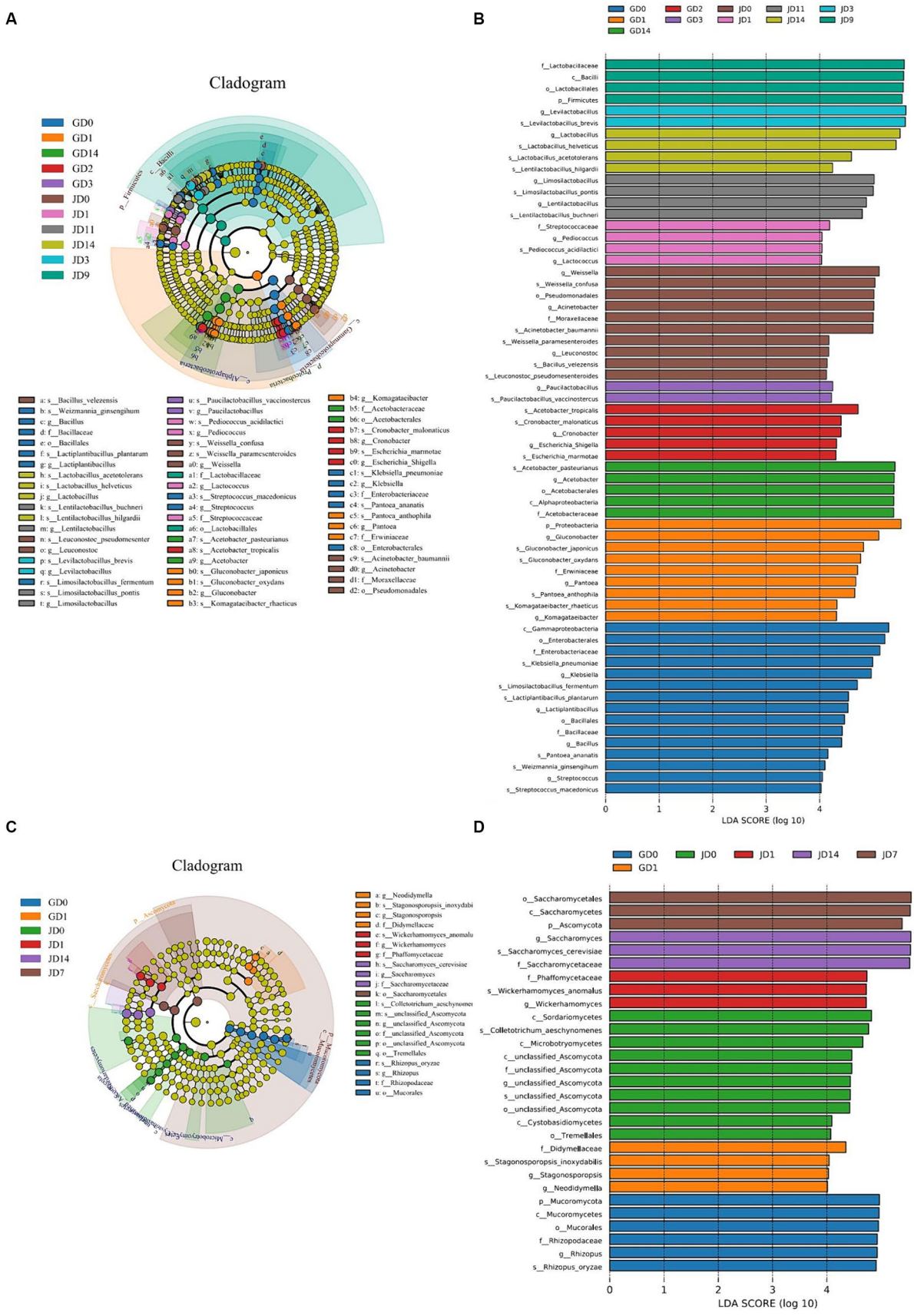

FIGURE 4
LEfSe analysis of inter group samples during fermentation process. **(A)** Bacterial and **(C)** fungal taxonomic cladogram of the discriminant analyzed by LEfSe. Taxonomic ranks from phylum to genus were represented by rings from the inner to the outer portion of the graph. The nodes in each

(Continued)

FIGURE 4 (Continued)
classification were defined at the same level of taxonomic rank and the sizes of the nodes were proportional to their relative abundances. Nodes with lime color indicated no significant variation in the abundances of the taxa. Highlighted areas with the additional different colors implied different groups, and the nodes in the extra different colors distinguished the different samples. Nodes with the same color in the branches were defined as significantly different taxonomic biomarkers representing the different taxa in the same color group. (**B**) Bacterial and **(D)** fungal taxa that showed significantly different abundances for samples.

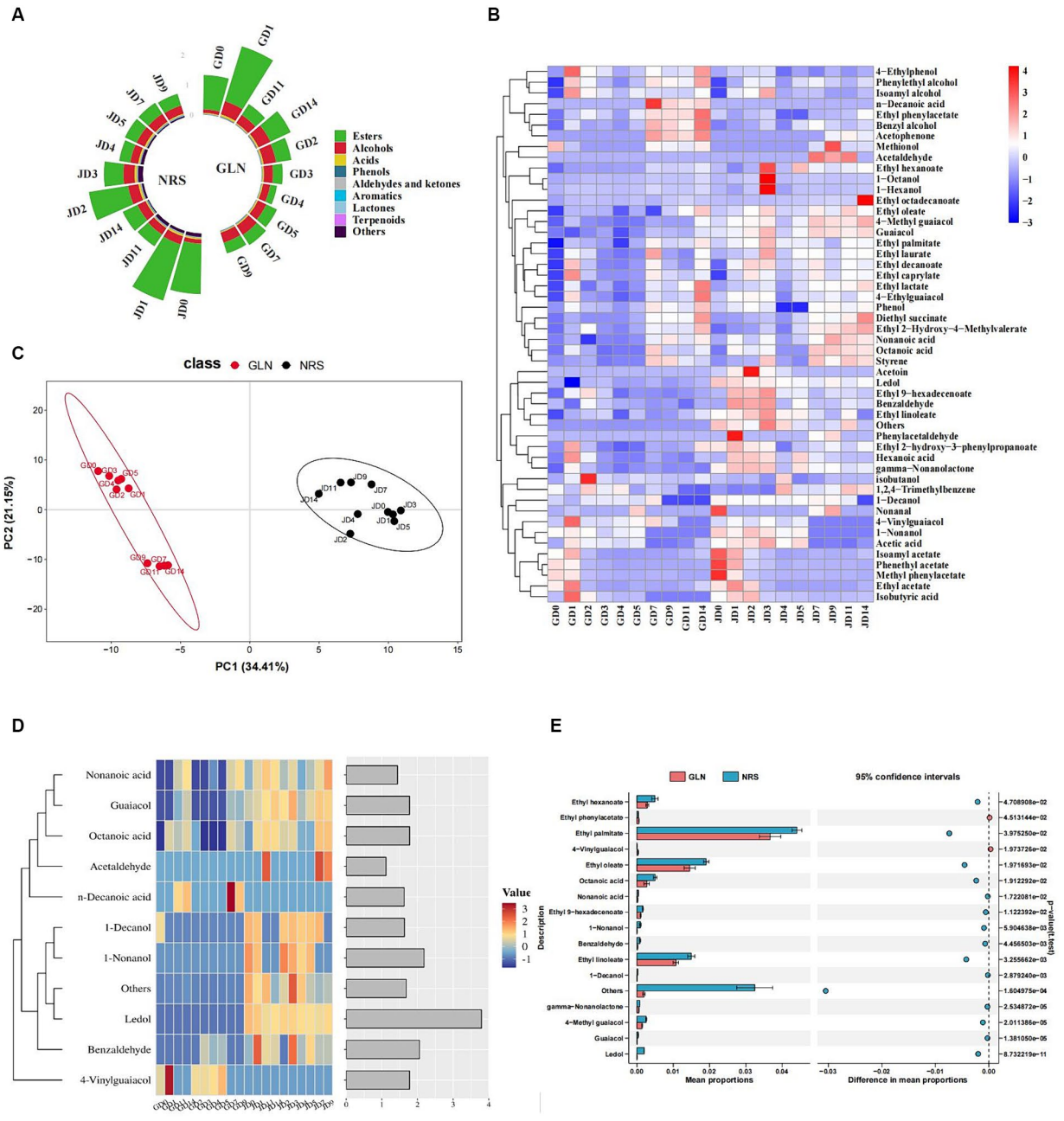

FIGURE 5
The key volatile substances composition in GLN and NRS. **(A)** The content of volatile flavor compounds in GLN and NRS. **(B)** Heatmap of flavor components in GLN and NRS. **(C)** PLS-DA between volatile compounds in 20 types of samples in GLN and NRS. **(D)** A heatmap and classification of 11 volatile compounds with the variable importance (VIP) value >1.0 based on the PLS-DA of GLN and NRS. **(E)** STAMP analysis of significant differential flavor compounds in GLN and NRS.

All of the volatiles were split into 20 groups for PLS-DA to illustrate the grouping of GLN and NRS, flavor compounds, and notable distinctive substances. The R^2 (0.98) and Q^2 (0.95) values implied that the established model was valid. PLS-DA demonstrated that sorghum variety had a significant effect on the flavor profiles of the fermented grains. In addition, the flavor structures of the samples

from day 0 to day 5 were relatively similar, and those of the samples from day 7 to day 14 were clustered together (Figure 5C). The fermentation could be divided into two stages (stage 1: day 0 to day 5; stage 2: day 7 to day 14) based on the composition of flavor substances in GLN, whereas the flavor substances varied slightly in NRS, thus resulting in no significant difference during fermentation. Flavor substances with variable importance in the projection (VIP) > 1.0 are usually used to explain the clustering of diverse groups in discriminant analysis. Eleven distinctive compounds with VIP > 1.0 were identified by using PLS-DA, including 2 alcohols, 3 acids, 2 aldehydes, 2 phenols, 1 terpenoid, and 1 other (Figure 5D). STAMP was applied to compare the differences in flavor substances between GLN and NRS to obtain significantly different flavor compounds. Thirteen distinctive compounds were identified via STAMP, including 6 esters, 1 aldehyde, 3 phenolics, 1 lactone, 1 terpenoid, and 1 other (Figure 5E). Among them, 4-vinylguaiacol and n-decanoic acid were more abundant in GLN (Figure 5D).

Abiotic factors driving microbial community succession

The RDA results showed that the two axes explained 98.16% of the total variation in the microbial community (Figure 6A). The Monte Carlo permutation test results showed that moisture and reducing sugar both had a more significant impact on microorganisms in GLN than those in NRS. However, amino acid nitrogen, total free amino acids, and residual starch were more important for microorganisms in NRS. As shown in Figure 6B, on the basis of the NMDS analysis of both microbial abundance and environmental factors, the results of Procrustes analysis indicated that there was a significant correlation between the microbial community and environmental factors in different samples ($M^2 = 0.3631$, $p < 0.001$).

Additionally, to better understand the impact of abiotic factors on microorganisms, Spearman correlation coefficients were used to examine the correlations between physicochemical properties and dominant microbial species (Figure 6C). There was a significant correlation between moisture content, reducing sugar, residual starch and a considerable fraction of fungi and bacteria. *S. cerevisiae*, *La. buchneri,* and *La. pontis* were positively correlated with moisture content and negatively correlated with reducing sugar and residual starch, while *R. oryzae*, *K. pneumoniae*, *A. tropicalis*, *G. oxydans*, *W. confusa*, and *A.baumannii* were negatively related to moisture content and positively related to reducing sugar and residual starch (Supplementary Tables S2, S3). Compared to those in NRS, more microbial species in GLN were driven by physicochemical properties. For instance, acidity was only significantly correlated with microbial species in GLN, thus indicating the importance of acidity to the microbial community in GLN.

Relationships between major flavor components and microbial species

Flavor components are usually produced during fermentation by the microorganisms involved in the process. To determine the microbes that produce the main flavor components, co-occurrence network analysis was used to analyze the positive and negative correlations between the microbial taxa and major flavor components with relative contents over 0.1% (Figure 7). The results showed that *A. pasteurianus* exhibited a positive correlation with 17 flavor substances (10 ethyl ester compounds, 2 alcohols, 1 acid, 3 phenols, and 1 aromatic), thus indicating that this bacterium may have an important contribution to the formation of flavor substances in GLN. However, *La. buchneri* was significantly correlated with 11 flavor compounds, thus implying that it may play an important role in the formation of flavor compounds in NRS. Acetic acid was strongly positively associated with *A. tropicalis* ($\rho > 0.8$ and $p < 0.01$) and exhibited a significant negative association with *La. helveticus* and *La. pontis* in GLN, while it had an extremely significant negative correlation with *La. buchneri* and *La. pontis* ($\rho > 0.8$ and $p < 0.001$) in NRS. Ethyl acetate showed a strong positive association with *W. anomalus*, *R. oryzae*, *G. oxydans*, *A. baumannii*, and *A. tropicalis* ($\rho > 0.8$) and was negatively correlated with *S. cerevisiae* in NRS, while it only had a negative correlation with *S. cerevisiae* and *La. brevis* in GLN. Phenylethyl alcohol was positively correlated with *S. cerevisiae* and negatively correlated with 7 microbial species, including *W. confusa*, *K. pneumoniae*, and *A. baumannii*, etc., in NRS, while it only had a positive association with *A. pasteurianus* in GLN. In summary, 14 microorganisms and 29 flavor substances were found to form 117 co-occurrence networks in GLN, while 100 co-occurrence networks of 15 microorganisms with 21 flavor compounds were detected in NRS.

Furthermore, the correlation between the microbial community and the content of volatile compounds was calculated to evaluate the importance of biotic factors to volatile metabolites. The results showed that both the bacterial and fungal communities were significantly correlated with the volatile compounds ($p < 0.05$) in GLN and NRS (Figures 7C,D).

Discussion

In traditional fermentation, the raw material plays a key role in the assembly of the core microbiota and the formation of volatile compounds. However, the effects of raw materials on the microbial composition and microbial function during Baijiu fermentation are still unclear. LFB is a valuable and convenient model for exploring the mechanism of microbial fermentation due to its short fermentation period and simple production process. Thus, the microbial composition and diversity and physicochemical dynamics during the fermentation of LFB produced from two types of sorghum varieties were investigated in this study.

We showed that the sorghum variety affected not only microbial succession but also microbial metabolism in Baijiu fermentation, based on both full-length amplicon sequencing and flavoromics. Previous studies have indicated that different sorghum varieties had significant discrepancy in their physicochemical constituents, such as amylopectin, tannins, and crude fat, which led to the different substrates available to microorganisms during the Baijiu fermentation (Wu et al., 2017). In this study, the glutinous sorghum had significantly higher amylopectin and tannin contents than non-glutinous sorghums ($p < 0.05$) (Supplementary Table S1), which was consistent with previous studies (Wu et al., 2017; Liu et al., 2023). Amylopectin, which has more short-chain branches, is easily decomposed into glucose by amylase; thus, the glutinous sorghum with a high content of

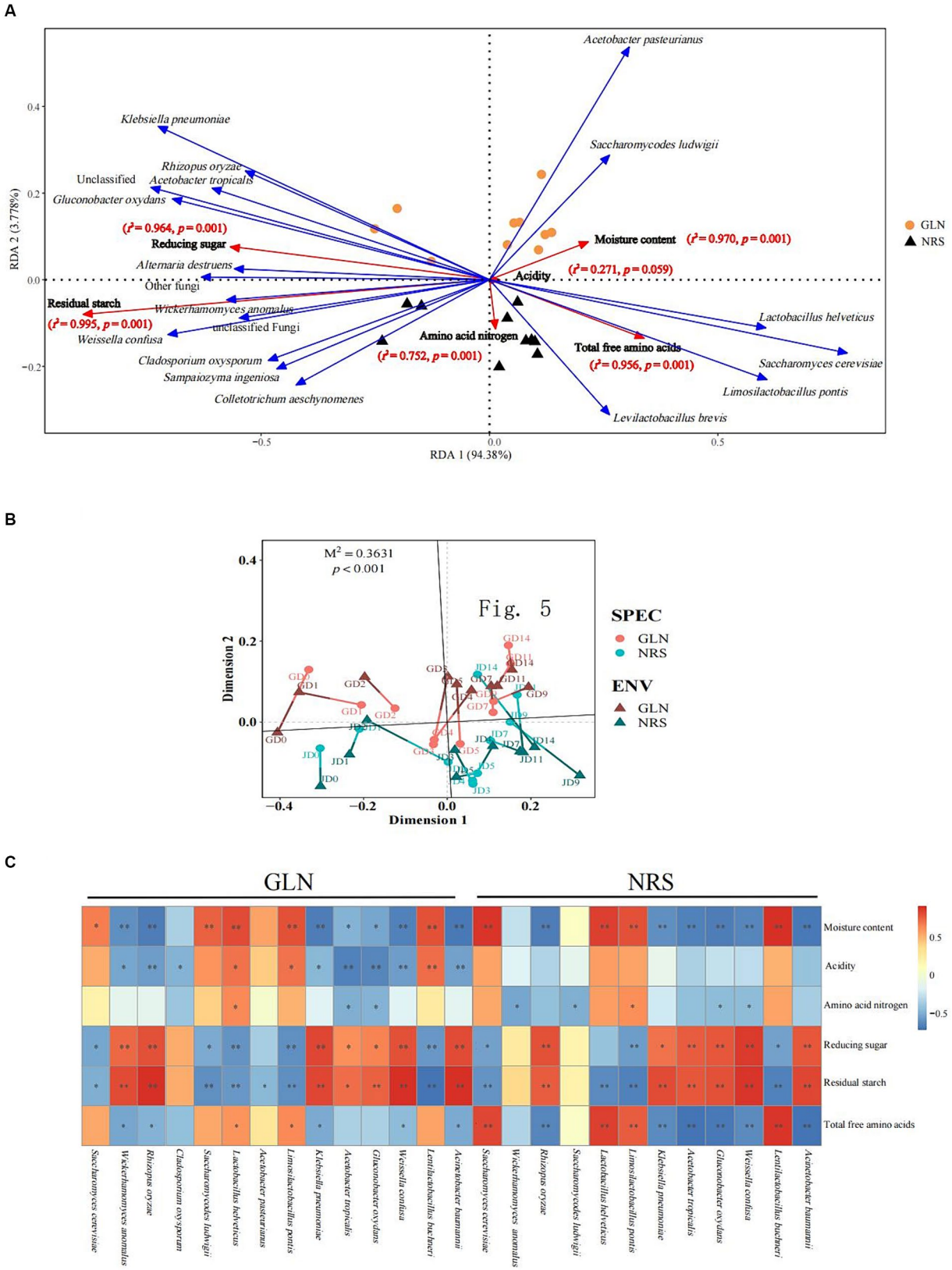

FIGURE 6
The driving force of microbial community succession. **(A)** Redundancy analysis of microbial and abiotic factors in fermented grains. **(B)** Procrustes analysis of the correlation between microbial community and abiotic factors in fermented grains ($M^2 = 0.3631$, $p < 0.001$, 999 permutations). **(C)** Correlation of microorganisms and physicochemical properties (Spearman's $|\rho| > 0.6$ and $p < 0.05$).

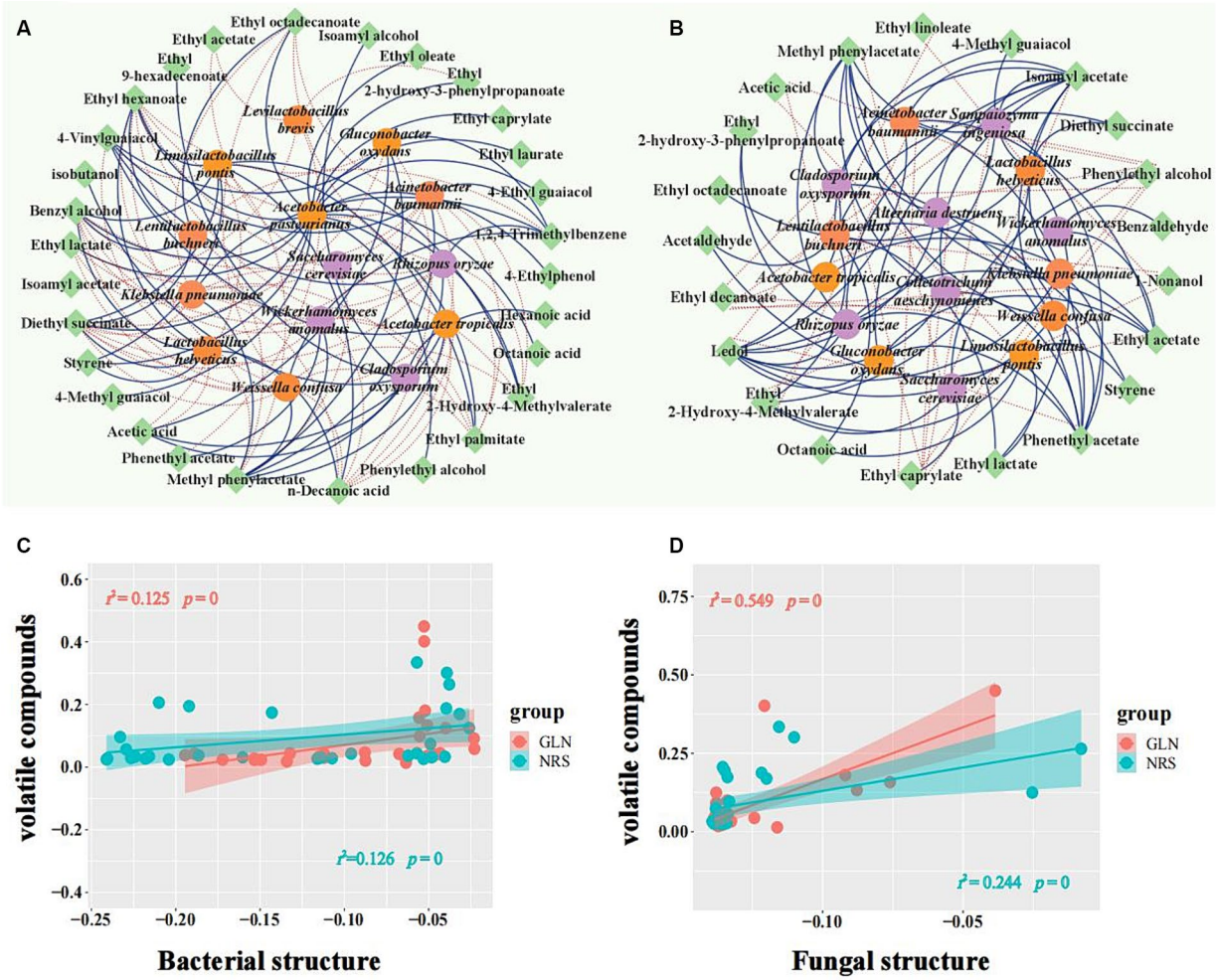

FIGURE 7
Relationship between microbial composition and volatile metabolites. A network of microorganisms related to major volatile compounds in GLN **(A)** and NRS **(B)** with a strong (Spearman's $|\rho| > 0.6$) and significant ($p < 0.05$) correlation. Fungi, bacteria, and volatiles are represented by purple, orange, and green circle modules, respectively. Positive and negative correlations among microorganisms and volatiles are represented by solid and dotted edges, respectively. The relationship between volatile compounds and bacteria **(C)**, and fungi **(D)** was estimated with Pearson correlation. The microbial structure and content of volatile metabolites are indicated by the first axis of the PCA. The shaded area denotes 95% confidence intervals. Significance is represented by $p < 0.05$.

amylopectin could provide more reducing sugar for microbial growth and metabolism at the beginning of fermentation (Wang et al., 2008; Xu et al., 2021). This may explain why the content of reducing sugar in GLN was higher than that in NRS in the early stages, while the residual starch content in GLN was lower during fermentation (Figures 1E,F). Similarly, previous studies have shown that the fermented grains of glutinous sorghum presented higher reducing sugar contents and lower starch contents than their non-glutinous counterparts during fermentation (Liu C. et al., 2021). In addition, tannins are also present at relatively high levels in glutinous sorghum and can affect the formation of flavor substances and microbial composition during Baijiu fermentation (Salami et al., 2018; Xu et al., 2018).

In the closed fermentation cellar, microenvironment plays a crucial role in regulating microbial succession and metabolism during fermentation. For instance, a variety of physicochemical factors, including acidity, amino nitrogen, residual starch, reducing sugar, and moisture contents, can regulate microbial dynamics during LFB brewing (Lin et al., 2022; Tang et al., 2022b). Our results indicated that moisture content, amino acid nitrogen, reducing sugar, residual starch, and total free amino acids were identified as the driving factors of the fungal and bacterial variation (Figures 6A,B). RDA further demonstrated that the microbial changes in GLN were positively related to moisture content, while amino acid nitrogen, residual starch, and total free amino acids were positively associated with the microbial community in NRS. These results were in agreement with the previous studies showing that differences in the composition of environmental factors produced by sorghum varieties had different effects on the microbial community (Liu et al., 2019; Wang et al., 2021). Moreover, compared with those in NRS, the microbial species in GLN responded more rapidly to environmental changes. Thus, sorghum varieties indirectly affected the microbial community by regulating the physicochemical properties of fermented grains.

Previous studies have suggested that the LFB fermentation process can be divided into two stages according to microbial changes in fermented grains (Shen et al., 2021; Lin et al., 2022). As illustrated in

Figure 3, our current research also indicated that fungal and bacterial succession included two stages, and the microbial community changed the most after 2 days of fermentation, regardless of the variety of sorghum that was used. As fermentation proceeded, *Lactobacillus*, *Acetobacter*, and *Saccharomyces* became the dominant genera in the fermented grains in the middle and later stages. Further analysis revealed that the fungal community was more sensitive to changes in the fermentation environment due to rapid community structure changes after day 1 (Figure 3B), which was consistent with the findings of a previous study (Lin et al., 2022). Compared with the bacterial community, the fungal species composition showed less discrepancy between two varieties of sorghum in later fermentation, and anoxic and highly acidic fermentation environments promoted the formation of a fungal community that was mainly composed of *S. cerevisiae*. Besides, more differential species belonging to lactic acid bacteria were found in GLN, which correspondingly inhibited other bacteria by secreting lactic acid. In contrast, the differentially abundant strains in NRS were mainly non lactic acid bacteria (Figures 4A,B). *Lactobacillus* has been reported to be abundant in the final stage of the fermentation of different flavors of Baijiu and to greatly contribute to the production of flavor substances, such as lactic acid (Dong et al., 2020; Du et al., 2020; Ji et al., 2023; Li et al., 2023). Moreover, the rapid increase in *Lactobacillus* abundance during LFB fermentation can also inhibit unnecessary microbes by forming a highly acidic fermentation environment, thus leading to similar fungal communities in different sorghum varieties during the later stage of fermentation.

The microbial species involved in Baijiu fermentation determine the flavor composition and textures of the raw liquor. Microbial changes caused by the application of different varieties of sorghum eventually led to changes in the composition of flavor substances. In this study, the content of esters was higher in NRS than that in GLN (Figures 5A,B), which represented the majority of volatiles found in LFB fermentation samples, consistent with prior studies (Huang et al., 2020; Zhu et al., 2022). *W. anomalus* was reported to be conducive to the synthesis of ester compounds during Chinese Baijiu brewing, and the content of esters can be increased by adding *W. anomalus* strains (Fan et al., 2019; Wang et al., 2020). Figures 4C,D, 7B indicated that *W. anomalus* was a differential fungal specie and showed a strong positive association with ethyl acetate in NRS; thus, the higher biomass of *W. anomalus* possibly improved the production of esters in the fermented grains with NRS. Additionally, the content of ethyl acetate, which was the main aromatic substance in LFB, first increased, then decreased and remained relatively stable in the later stage of fermentation in the two grain varieties (Figure 5B). This may be the result of high oxygen content in the early stages of fermentation and low oxygen content in the middle and later stages of fermentation (Shen et al., 2021).

Furthermore, 8 volatiles were identified as being important differential substances in fermentation by using two sorghum varieties based on the PLS-DA and STAMP (Figures 5D,E); however, these compounds were not the major flavor substances in LFB. This indicated that sorghum variety not only caused changes in the content of the main aroma substances (such as ethyl acetate) but also regulated the composition of several micro-flavor components (such as 4-vinylguaiacol, ledol, and benzaldehyde). Among them, 4-vinylguaiacol is an important fragrance component in Baijiu, wine, and beer (Xu et al., 2020b; Wang et al., 2022) and has medicinal value because of its potential anticancer and antioxidant activities (Bortolomeazzi et al., 2007; Luo et al., 2021). In addition, 4-vinylguaiacol was more abundant in GLN (Figure 5D) and had a significant positive association with *R. oryzae*, *A. tropicalis*, and *G. oxydans* and a negative correlation with *La. helveticus* and *La. pontis* (Figure 7A). Multiple microorganisms, such as *Lactobacillus* spp., *Bacillus* spp., *Candida*, *Brettanomyces*, and *Aspergillus* spp., have been reported to degrade ferulic acid into 4-vinylguaiacol (Suezawa and Suzuki, 2007; Xu et al., 2020a). Notably, *R. oryzae*, *A. tropicalis*, and *G. oxydans* were significantly differential and more abundant microbial species in GLN (Figure 4), likely resulting in higher 4-vinylguaiacol content in GLN samples. Thus, different sorghum varieties played important roles in the flavor component formation of LFB.

Some studies have demonstrated the key effect of microbial structure on volatile metabolites (Jia et al., 2020; Liu D. et al., 2021). Herein, we found that both fungi and bacteria were the main drivers of volatile metabolites, regardless of the sorghum varieties (Figures 7C,D). This study highlighted the idea that the structure of both fungi and bacteria played an important role in maintaining volatile metabolites during the LFB fermentation. Overall, different sorghum varieties led to differences in the succession of the microbial community and changes in environmental factors, thus ultimately resulting in differences in flavor compounds.

Conclusion

To our knowledge, this is the first study to reveal the effect of sorghum varieties on microbial community and volatile compounds in the fermentation of LFB. In this study, the microbial composition and diversity, physicochemical dynamics, and flavor components during the fermentation of LFB produced from two types of sorghum varieties were investigated. Based on the microbial structure, aromatic compounds, and physicochemical factors, our research successfully elucidated why GLN and NRS exhibited distinct characteristics. NRS exhibited higher amino acid nitrogen, residual starch, and total free amino acids contents, which were the main driving factors for the microbial community succession of the fermented grains in NRS. Higher bacteria α-diversity and more differential bacterial species were detected in GLN, while a higher fungal α-diversity and more differential fungal species were detected in NRS. In addition, the relative contents of volatiles in NRS were relatively higher than those in GLN, and 8 flavor compounds were identified as being important differential substances in the fermented grains of two sorghum varieties. The findings can provide guidance for selecting brewing materials and optimizing fermentation parameters to improve the quality of Baijiu.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Author contributions

JT: Conceptualization, Funding acquisition, Investigation, Methodology, Writing – original draft, Writing – review & editing.

BL: Investigation, Methodology, Writing – original draft. YS: Data curation, Methodology, Writing – original draft. SR: Data curation, Methodology, Writing – original draft. WJ: Data curation, Visualization, Writing – original draft. QL: Investigation, Methodology, Writing – original draft. LZ: Investigation, Methodology, Writing – original draft. RL: Investigation, Methodology, Writing – original draft. QY: Funding acquisition, Resources, Supervision, Writing – review & editing. HD: Investigation, Methodology, Resources, Writing – review & editing. SY: Resources, Supervision, Writing – review & editing. QS: Resources, Supervision, Writing – review & editing. SC: Conceptualization, Funding acquisition, Supervision, Validation, Writing – review & editing.

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Conflict of interest

JT, BL, YS, SR, WJ, QL, LZ, RL, QY, SY, QS, and SC were employed by the Jing Brand Co., Ltd.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1421928/full#supplementary-material

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